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Temperature and pH requirements for viral haemorrhagic septicemia virus induced cell fusion

A. Estepa, J. M. Coll*

INIA, Sanidad Animal, CISA Valdeolmos, E-28130 Madrid, Spain

ABSTRACT: The rhabdovirus causing viral haemorrhagic septicemia (VHS), an important disease of salmonids, induced formation of large syncytia in fish cell monolayers by cell fusion. For maximal percentage of fusion, not only was a pH of 5.6 needed but also a temperature of at least 14°C. The fusion was dependent on the G protein of VHSV as shown by inhibition of fusion with specific anti-G polyclonal and monoclonal antibodies. Part of the region of the G protein involved in fusion was mapped to amino acids 82 to 109 because anti-peptide antibodies binding to that region also inhibited fusion. The fusion assay described here can be useful for studying the functionality of the G protein of fish rhabdoviruses in the early steps of infection and this information can, in turn, help to designing effective vaccination and/or therapeutic strategies against fish rhabdoviruses. The described fusion assay could also be applied to other fish rhabdoviruses such as infectious haematopoietic necrosis virus (IHNV) and spring viraemia of carp virus (SVCV).

KEY WORDS: Viral haemorrhagic septicaemia · Rhabdovirus · Neutralizing-enhancing antibody · Monoclonal antibodies · Fusion

INTRODUCTION

The homotrimeric glycoprotein (G protein) of mammalian rhabdoviruses (Gaudin et al. 1993) is responsible for the binding of these viruses to the receptor of the host cellular membranes and for the subsequent virus and cell membrane fusion activity (Schlegel et al. 1982, Superti et al. 1984, Rigaut et al. 1991). The kinetics of membrane fusion for mammalian rhabdoviruses shows a typical lag time, the duration of which depends upon both pH and temperature. For rabies (Gaudin et al. 1993) and vesicular stomatitis (Puri et al. 1992, Gaudin et al. 1995) viruses, the lag time decreases at low pH (~5.5) and at physiological temperatures (37°C). For the fish rhabdovirus causing viral haemorrhagic septicemia (VHS), low-pH dependent fusion has been shown in insect cell lines (Lecocq-Xhonneux et al. 1994, Lorenzen & Olesen 1995) but not in fish cell lines.

Fish rhabdoviruses cause highly damaging diseases in wild fish and fish reared by the international salmon culture industry. A vaccine for fish rhabdoviruses is not yet available (Leong & Fryer 1993, Lorenzen et al. 1993, Estepa et al. 1994, Leong et al. 1995). The study of fish rhabdovirus-fusion could be of importance in the design of alternative methods to combat these diseases.

In the experiments reported here, we describe an *in vitro* assay for VHSV-induced fusion of fish cells and show that cell fusion induced by this fish rhabdovirus depends not only on low pH but also on relatively high temperatures. In contrast to mammalian rhabdoviruses which require 37°C, only 14°C was required for VHSV-induced fusion. The use of antibodies to inhibit VHSV fusion confirmed that the observed fusion reaction was mediated by the G protein of VHSV and provided a preliminary map of some of the regions involved.

MATERIALS AND METHODS

The VHSV 07.71, isolated in France from rainbow trout *Oncorynchus mykiss* (LeBerre et al. 1977), was grown and/or assayed for infectivity in *epithelioma papillosum cyprini* (EPC) cells (DeKinkelin 1972) and

*Addressee for correspondence. E-mail: coll@inia.es

purified as described previously (Basurco et al. 1991). Anti-p2 and anti-Gs polyclonal antibodies (PAbs) were obtained as described previously (Estepa & Coll 1996). The p2 peptide, corresponding to amino acids 82 to 109 of the G protein of VHSV, was synthesized by Clontech (Palo Alto, CA, USA). It was selected for this study because of its possible participation in cell fusion (Estepa & Coll 1996). The soluble protein G (Gs) was purified from VHSV-infected EPC cell supernatants as previously described (Ruiz-Gonzalvo & Coll 1993). PAbs were prepared by injecting BALB/c mice with 20 µg of p2 or Gs in Freund's complete adjuvant. This was followed with 4 monthly injections of each respective protein in Freund's incomplete adjuvant. To obtain ~40 ml of pooled diluted ascites containing PAbs, 3 immunized mice were each infected intraperitoneally with 0.5 to 2×10^6 viable myeloma X63/Ag8653 cells (Coll 1989). Ascites fluid was harvested after 7 to 10 d. The anti-p2 PAb was purified by chromatography over a p2-activated CH-Sepharose-4B column with an 8-atom spacer (Sigma, St. Louis, MO, USA). The PAbs were further purified from ascites by passage through a 5 × 1.5 cm column containing rabbit serum bound to CNBr-activated Sepharose and followed by passage through a 3 × 1 cm column of Protein A-Sepharose (Pharmacia). The antibodies were eluted with ethylenediamine pH 12.5, neutralized with Tris, dialysed, lyophilized and reconstituted in phosphate buffered saline pH 7.4. The protein concentration was adjusted to about 1 mg ml^{-1}.

Anti-G 1H10 monoclonal antibody (MAb) was obtained, produced in ascites and purified by ammonium sulphate precipitation from the ascites as described previously (Sanz & Coll 1992b).

To assay for VHSV-induced cell to cell fusion or fusion from within, EPC cells in 96-well plates (about 300 000 cells $well^{-1}$) were incubated with 100 µl of cell culture medium infected with VHSV-07.71 at a multiplicity of infection (MOI) of 3 by agitation and then incubated overnight at 14°C (Estepa & Coll 1996). A temperature of 14°C for the fusion experiments was used because it was the optimal temperature for VHSV infection of EPC cells. Further details are explained in Fig. 2.

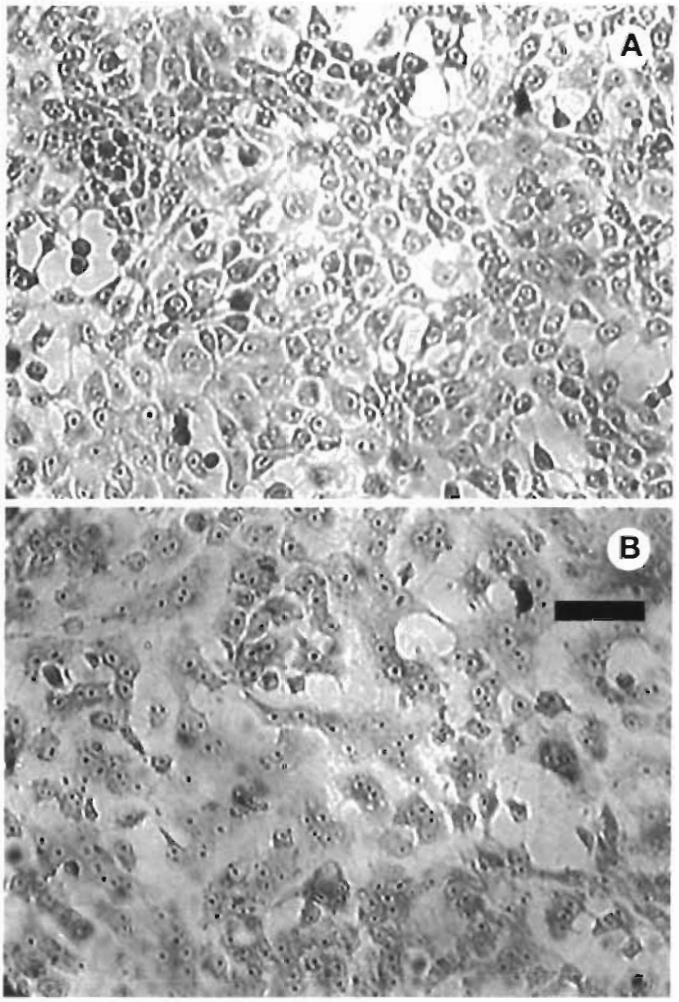

Fig. 1. Micrographs of fixed/stained VHSV-infected EPC cultures 16 h post-infection and after 30 min at (A) pH 7.6 or (B) at pH 5.6. Scale bar = 100 µm

RESULTS

Fig. 1 shows micrographs of overnight VHSV-infected EPC cells after 30 min incubation at pH 7.6 (Fig. 1A) and at pH 5.6 (Fig. 1B) after fixation and staining. Large syncytia were visible only at pH 5.6, either after fixing and staining or by phase contrast microscopy. MAb 2C9 detected the presence of the N-protein (the major and the earliest viral protein being made in VHSV-infected cells) in >95% of the overnight VHSV-infected EPC cells (estimated as described in Sanz & Coll 1992a). The percentage of fused EPC cells varied from 33 to 100%. For a given experiment, the maximal percentage of fusion was obtained after 30 min of incubation at 14°C. At 20°C fusion was also observed with the same kinetics. The maximal percentage of fusion was obtained at pH <6.5 whereas at pH >6.5 the formation of syncytia was not detectable, a result that has been previously reported. Control cultures of non-infected EPC cells did not show syncytia formation at any pH tested (Fig. 2).

Inhibition of fusion was obtained when the VHSV-infected EPC cells were incubated at pH 5.6 with anti-G PAbs (Fig. 3). The percentage of inhibition obtained was dependent both on the amount of anti-Gs PAbs

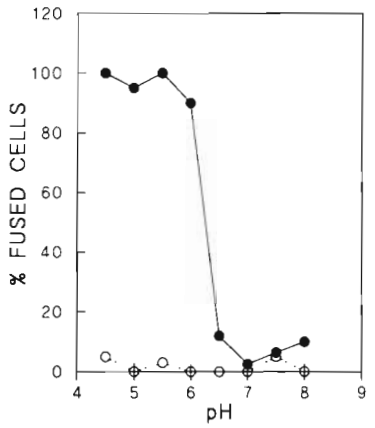

Fig. 2. pH-dependence of cell to cell fusion induced by VHSV infection. Fusion was induced after 16 h infection with 3 plaque-forming units of VHSV per cell. The next day, the EPC plates were washed with RPMI-1640 medium at different pHs (obtained without bicarbonate and buffered with 20 mM HEPES and 20 mM MES and adjusted to the desired pH). After 30 min incubation at 14°C with manual agitation, the cultures were fixed for 10 min with cold methanol, stained with Giemsa, washed and dried. About 200 nuclei per well were counted. Percentage of fusion was calculated as the (number of nuclei in syncytia/total number of nuclei) × 100. (●——●) VHSV-infected EPC cells (fusion was observed from pH 4 to pH 6.5 but not at higher pH values). (O·····O) control EPC cells (no fusion was observed at any of the assayed pH values)

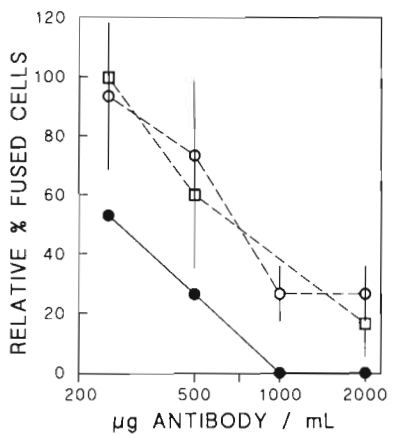

Fig. 3. Inhibition of cell to cell fusion with anti-Gs polyclonal antibodies. Relative percentage of VHSV-induced cell fusion was estimated by incubating EPC cell monolayers overnight at 14°C with a VHSV multiplicity of infection (MOI) of 3 (●——●), 1.5 (O– –O) or 0.75 (□– –□) in 3 different experiments. After overnight incubation, serial dilutions of anti-Gs PAbs in pH 5.6 buffer were added. Control anti-N MAb 2C9 (Sanz & Coll 1992b) did not inhibit fusion. Fusion was estimated at 30 min at pH 5.6 and 14°C after fixing and staining. The percentage of fused cells in the controls at 3, 1.5 and 0.75 MOI in the absence of Abs was 93, 50 and 33%, respectively. The relative percentage of fusion was obtained by the formula (% of fusion in the presence of antibodies/% of fusion in the controls) × 100. Means and standard deviations from 3 to 4 wells are represented

and on the amount of VHSV used (MOI) (Fig. 3). However, maximal percentage of inhibition was obtained only at relatively high concentrations of PAbs. Because anti-p2 antibodies only bound to G at pH 5.6 but not at pH 7.6, suggesting a pH-dependent conformation shift (Estepa & Coll 1996), one possible explanation for the result mentioned above was that fusion was so rapid that PAbs had no time to react with the hypothetical exposed regions of G to inhibit the fusion. To allow the PAbs to react with the hypothetical pH-exposed G regions, infected cells were first incubated with anti-Gs PAbs at 4°C. During the 4 h at pH 5.6 and 4°C in the absence or in the presence of anti-Gs PAbs, no fusion was detected. Upon returning the cultures to 14°C, fusion began immediately in the cultures incubated in the absence of anti-Gs PAbs and inhibition of fusion was observed in the cultures treated with the anti-Gs PAbs. Under these conditions inhibition of fusion was observed at anti-Gs PAb concentrations that were approximately 5-fold lower than the concentration required for inhibition at 14°C (not shown). Fig. 4 shows that at pH 5.6 both PAb anti-p2 and MAb 1H10 also inhibited fusion at lower concentrations after a first step of incubation at 4°C and then at 14°C than when incubated at 14°C only.

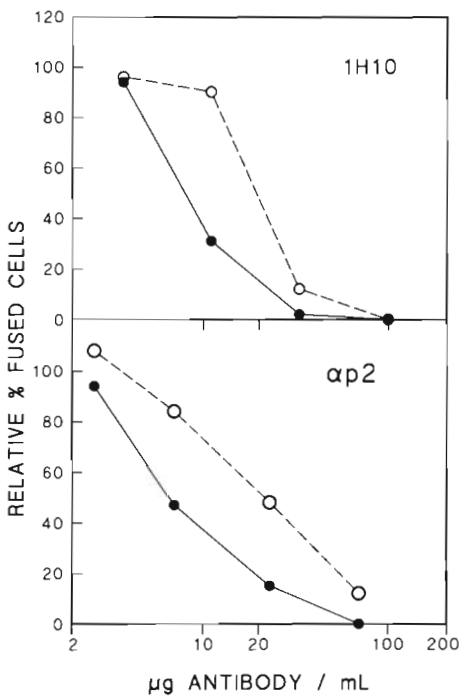

Fig. 4. Inhibition of cell to cell fusion with MAb anti-1H10 and with anti-p2 at 4 and 14°C. EPC monolayers were infected overnight with VHSV at a multiplicity of infection of 3. Anti-G MAb 1H10 and anti-p2 PAb were incubated with the infected cells at either 14°C for 30 min (O– –O) or first at 4°C for 4 h and then at 14°C for 30 min (●——●)

DISCUSSION

We have shown that cell to cell fusion mediated by VHSV infection in EPC cells was dependent on both pH and temperature. The optimal pH of fusion was approximately 6 (Fig 2). Similarly, infection of insect cells with VHSV gave rise to syncytia when the pH of the cell culture medium was 6.2 (Lorenzen & Olesen 1995), and insect cells transfected with plasmids expressing the G protein also gave rise to a pH 6 dependent fusion (Lecocq-Xhonneux et al. 1994). Most probably, a pH of ~6 is needed to induce conformational changes in the G protein of VHSV, thus exposing some regions for fusion, as suggested by the results obtained from binding experiments of anti-p2 to G (Estepa & Coll 1996). It is also known that exposure of isolated rabies G trimers (Gaudin et al. 1993) to low pH induces conformational changes that expose hydrophobic inner regions (Bourhy et al. 1993). However, for fusion to occur, several stretch(es) of G, an intact G trimer structure, collaboration of other viral proteins, and/or the presence of the viral membrane anchorage of the G are also most probably needed.

The temperature-dependent step for fusion has been described both for VSV and for rabies (Gaudin et al. 1995). However, whereas 37°C is required for mammalian rhabdoviral fusion, only 14°C was required for VHSV, a finding in agreement with the respective viral optimal growth temperatures.

The inhibition of cell fusion by anti-G PAbs and MAbs suggested that fusion was dependent on the surface expression of the G-protein (Figs. 3 & 4). Surface expression of the VHSV-G antigen had already been demonstrated by flow cytometry (Estepa et al. 1992) and by immunofluorescence (Lorenzen et al. 1990, Lorenzen & Olesen 1995). Because anti-p2 antibodies inhibited the fusion reaction, a correlation between the p2 region and fusion was indicated. Similarly the putative fusion peptide of VSV, aa 123-137 (Zhang & Ghosh 1994, Gaudin et al. 1995), lies adjacent to the p2 analogous regions (Coll 1995a, b, c). A similar case was found in rabies viruses (Gaudin et al. 1995). Thus, all these results suggest that both p2-like regions and fusion must be functionally related in rhabdoviruses. According to the model of rhabdoviral fusion described by Puri et al. (1988), the glycoprotein G is involved in at least 3 major steps in the process: binding to receptors in the host membrane at 4°C, adhesion strengthening by conformational changes after lowering the pH at 4°C, and membrane fusion by additional conformational changes after increasing the temperature to physiological values (Haywood 1994). All the predictions of this model seem to be met by the fish rhabdovirus VHSV

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