Brief Report

Characterization of equine arteritis virus particles and demonstration of their hemolytic activity

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Summary

Equine arteritis virus (EAV), a member of the newly established family Arteriviridae, is a small, positive-stranded RNA virus. It carries two protein complexes in its envelope, gp5/M and the recently described gp2b/gp3/gp4 complex. We report here on several basic features of EAV replication in cell culture and on the protein composition of virus particles. We have also characterized gp2b, gp3, and gp4 expressed using a baculovirus system in insect cells. Finally, we provide evidence that EAV possess hemagglutinating and hemolytic activity. The hemolysis assay might be useful for determining which of the surface proteins carries the receptor-binding and membrane fusion activity of EAV.

EAV encodes seven structural proteins: the nucleocapsid protein N and six membrane proteins, the glycoproteins gp2b, gp3, gp4, gp5 and the unglycosylated proteins E and M. The membrane proteins are present as monomers (E) or form disulfide-linked heterodimeric (gp5/M) or heterotrimeric complexes (gp2b/gp3/gp4) [3, 4, 6, 15, 18–20]. All of the structural proteins are essential for virus infectivity, but their function during the replicative cycle of EAV is not known. Gp5 and M, but not E, gp2b, gp3, or gp4, are required for budding of virus particles at internal membranes, implicating the latter in virus entry [21]. Likewise, exchanging the ectodomains of gp5 and M of EAV with those of other arteriviruses does not change the cell tropism of the recombinant virus [5, 17]. Thus, the recently described gp2b/gp3/gp4 complex is currently a prime candidate for the receptor-binding and probably also the membrane-fusion activity of EAV.

Here, we have investigated several basic features of EAV, such as virus growth in cell culture and preparation of virus particles, and have analysed their structural proteins. Furthermore, we report for the first time that EAV particles exhibit hemolytic activity. This classical feature of many enveloped viruses [1] has been very useful in the
past for identifying proteins involved in cell entry of viruses.

We first compared several procedures for growth of EA V in BHK cells. Confluent monolayers were infected at a multiplicity of infection of 10, and cells were incubated at 37°C or 39.5°C for 14 or 22 h. Aliquots of the supernatant were then removed and the virus titer was determined using a plaque assay (Fig. 1A). Virus release was more efficient at 37°C compared to 39.5°C and more infectious virus was present when cell culture supernatants were harvested at 22 h postinfection. DEAE-dextran, which is thought to enhance virus binding to cells in the initial adsorption phase, slightly decreased vi-

**Fig. 1.** Protein composition of virus particles and expression of gp2b, gp3, and gp4 using baculovirus. (A) Confluent monolayers of BHK cells were infected with EA V (Bucyrus) at a multiplicity of infection of 10 and were incubated for 14 or 22 h, either at 37°C or 39.5°C. The cell culture supernatants were removed and centrifuged (3000 RPM, 15 min), and the virus titer was determined using a plaque assay according to reference 8 except that plates were stained with neutral red 48 h after infection. Samples labeled with “Dex” contained DEAE-dextran (50 µg/ml) during the two-hour adsorption period. (B) BHK cells were infected with EA V and incubated for 22 h at 37°C. Cell debris was removed from cell culture supernatants by low-speed centrifugation (3000 × g, 15 min), and virus particles were pelleted (28,000 RPM, 2 h). The virus pellets were resuspended in phosphate-buffered saline, and aliquots were subjected to SDS-PAGE (12% gel) using reducing or non-reducing conditions and staining with Coomassie blue. MW Molecular weight markers. (C) EA V-infected cells were labeled with 10 µCi/ml [35S]-methionine/cysteine starting at 5 h postinfection. Virus particles prepared as described above were subjected to SDS-PAGE and fluorography. (D) [35S]-labeled EA V particles were lysed in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 20 mM Tris, 10 mM EDTA, and 10 mM iodoacetamide), and were subjected to immuno-precipitation with 5 µl of peptide antiserum α-gp2b [2], followed by SDS-PAGE under non-reducing conditions and fluorography. (E) Gp2b equipped with a His- and a FLAG-tag at the C-terminus and authentic gp3 and gp4 were expressed in Sf9-cells by recombinant baculovirus, which was produced as described by the manufacturer (“BaculoDirect”, Invitrogen). Cells labeled with [35S]-methionine/cysteine (10 µCi/ml) for 4 h were lysed with RIPA buffer, and cellular extracts were subjected to immunoprecipitation with 5 µl of peptide antiserum α-gp2b or peptide antiserum α-gp3 or antiserum α-gp4 [18]. Samples were then split into two aliquots, which were digested with 1 µl Endo-H (+, New England Biolabs) or mock-digested (−) for 1 h prior to SDS-PAGE and fluorography. Control: cells infected with non-recombinant baculovirus.
rus production. The highest virus titer, i.e. $1 \times 10^8$ plaque-forming units/ml, was achieved with virus growth for 22 h at 37°C.

We analyzed the virus particles pelleted from supernatants of infected BHK cells by SDS-PAGE, both by Coomassie staining (Fig. 1B) and by metabolic labeling of their proteins (Fig. 1C). Especially the Coomassie staining of the virus preparations showed that they were contaminated by cellular proteins. Attempts to purify the virus preparation further by precipitation of cell culture supernatants with polyethylenglycol (10% PEG 6000) or by centrifugation through a 20% sucrose cushion yielded identical results. Furthermore, loading the virus preparation onto a continuous sucrose gradient did not generate a detectable virus band (data not shown).

Nevertheless, the viral gene products are the major proteins in our preparation, which resemble those published previously [9]. Two major protein bands were present at the bottom of the gel, with molecular weights of 15 and 19 kDa, corresponding to the nucleocapsid protein N (predicted MW of 12,300) and the matrix protein M (predicted MW 17,700). Another major band was visible at 42 kDa, which likely represents the fully glycosylated form of gp5 [2]. M and gp5 are known to form disulfide-linked complexes in virus particles, whereas N is monomeric. Indeed, separation of the virus preparation under non-reducing conditions shifted the M and gp5 bands (but not N) to positions corresponding to higher molecular weights not resolved in the gel. Between gp5 and M, a variety of bands are apparent, representing gp2b, gp3, and gp4. These bands also shifted to higher-molecular-weight positions when SDS-PAGE was employed under non-reducing conditions. To unequivocally demonstrate the presence of disulfide-linked complexes, we subjected aliquots of $[^{35}S]$-methionine-labeled EAV particles to immunoprecipitation with antibodies against gp2b. SDS-PAGE under non-reducing conditions revealed bands with molecular weights of 45 and 66 kDa (Fig. 1D), which represent gp2b/gp4 dimers and gp2b/gp3/gp4 trimers, as described previously [20].

In order to set up a system allowing high-yield production of viral proteins, we characterized the gp2b, gp3, and gp4 proteins further by expressing their genes using a baculovirus system in insect cells. Gp3 and gp4 were expressed as native, full-length proteins, whereas gp2b was equipped with a FLAG-His-tag at its cytoplasmic tail to possibly allow its purification. SF9 cells infected with recombinant baculovirus were labeled with $[^{35}S]$-methionine, and cellular extracts were subjected to precipitation with antibodies against the viral glycoproteins. An aliquot of the immunoprecipitate was digested with endoglycosidase H (+) to analyze proteins for glycosylation (Fig. 1E). SDS-PAGE and fluorography showed that gp3 was visible as multiple bands with molecular weights between 28 and 38 kDa. They shifted after Endo-H digestion to a single band with a molecular weight of 18 kDa, indicating that the protein with its five putative glycosylation sites is heavily and heterogeneously glycosylated, as has been observed before after expression of the gp3 gene in BHK-cells [18]. Gp4 was expressed as a single band with a molecular weight of 30 kDa, which is present as an 18-kDa band after Endo-H cleavage, indicating that all of its four potential glycosylation sites are filled. Due to the attached His-FLAG tag, gp2b is expressed with a molecular weight of 34 and 6 kDa heavier than the native protein. Upon Endo-H digestion its molecular weight was slightly reduced, consistent with the presence of only one glycosylation site. These results are in agreement with the sizes of gp2b, gp3 and gp4 in virus particles (compare with Fig. 1C) and with published studies on the expression of gp2b, gp3, and gp4 in mammalian cells [4, 18], indicating that the baculovirus system is able to generate authentic EAV glycoproteins. However, their expressions levels were disappointingly low, and neither gp2b alone nor – upon triple infection of SF9 cells – a gp2b/gp3/gp4 complex could be purified by nickel-affinity chromatography (data not shown).

It has been described that extracts prepared from EAV-infected cells, especially when they were treated with Tween/ether, can hemagglutinate chicken erythrocytes with titers up to 64 [10, 13]. Our virus preparations (50µl [1–10µg/µl protein] incubated with 50µl of 1% erythrocytes at room temperature) usually exhibited HA titers between 8 and 16. Does EAV also possess hemolytic activity, which
would indicate that it carries surface proteins with membrane fusion activity? Incubation of erythrocytes with EAV indeed caused release of hemoglobin, and we observed a linear dependency between the virus concentration and hemoglobin release (Fig. 2A). It was not possible to increase the virus concentration further to analyze whether hemoglobin release reaches saturation. The hemolytic activity of EAV is rather low compared to that of other viruses. Electron microscopy of virus preparations showed many virus particles with disrupted membranes, as described before [9]. Likewise, the virus preparations exhibited lower titers (1 x 10^9 pfu/ml) than one would expect for a more than 100-fold concentrated virus pellet (compared to the cell culture supernatant). Thus, EAV particles are fragile, and their infectivity and membrane fusion activity is partly destroyed during centrifugation.

We asked whether conditions known to denature proteins might inhibit the hemolytic activity of EAV. Shifting the pH to acidic (1.5) or to basic values (12.5) prior to performing the assay eliminated or clearly reduced hemolysis (Fig. 2B). Likewise, pre-treatment of virus particles at 90°C abolished their hemolytic activity, although it is remarkably stable at 70°C (Fig. 2C). Furthermore, trypsin treatment (1 µg/µl, 10 min) of the virus preparation prior to incubation with erythrocytes reduced release of hemoglobin to 50% (not shown). Thus, hemolytic activity of the virus preparations requires proteins. Incubation of EAV with increasing erythrocyte concentration did not increase the amount of released hemoglobin (Fig. 3A), indicating that the erythrocyte concentration is not limiting. Because the absolute values for OD 450 are rather low, we calculated the percentage of erythrocytes lysed by EAV relative to complete hemolysis accomplished with a detergent. At a 1% erythrocyte concentration this percentage is low (10%), but it increases up to 70% at an erythrocyte concentration of 0.1% (Fig. 3B). Finally, we asked whether performing the assay at mildly acidic pH might increase the hemolytic activity of EAV. This has been described for many viruses, such as influenza virus and togaviruses, which enter cells via the endocytic pathway, and this phenomenon reflects activation of the viral fusion protein at the acidic pH of the endosome [7, 12, 16]. However, we did not observe an increase in the hemolytic activity of EAV at acidic pH (Fig. 3C). Thus, in this respect, EAV resembles paramyxoviruses, which fuse with the plasma membrane [11]. Nevertheless, we do not want to exclude that EAV enters cells via the endo-

![Fig. 2. EAV exhibits hemolytic activity. (A) Increasing amounts of EAV prepared as described in Fig. 1b were incubated with 0.5% chicken erythrocytes in a final volume of 50 µl citrate-saline buffer (20 mM citrate (pH 7), 120 mM NaCl) at 37°C for 60 min. Erythrocytes were then pelleted at 600 g for 10 min, and the optical density (OD) of the supernatant was measured at 450 nm. The OD 450 of samples incubated without virus was subtracted. The mean of triplicate samples analyzed in parallel is shown. Twenty microgram of EAV corresponds to a protein concentration of 400 µg/ml in the assay. (B) Virus preparations (20 µg) were incubated with 1 µl 1 M NaOH (pH 12.5) or 1 µl 1 M HCl (pH 1.5) for 15 min at room temperature. The pH was then adjusted to neutral values by adding 1 µl HCl and 1 µl 1 M NaOH, respectively, prior to performing the assay. The mean of three independent experiments (duplicates) including the standard deviation is shown. The highest OD 450 was set to 1 for each experiment. (C) Virus preparations (20 µg) were incubated for 10 min at 24°C, 70°C, 80°C or 90°C, briefly centrifuged (1000 x g, 1 min), and cooled on ice prior to performing the assay. The highest OD 450 was set to 1]
cystic pathway. We have observed that pre-treatment of cells with lysosomotropic agents inhibits virus replication (Nitzschke et al., in preparation). The membrane proteins of EA V involved in virus entry as well as their receptor on the target cell have not been identified. It might be that EA V, in principle, can fuse at neutral pH, but its cellular receptor is rapidly endocytosed, and subsequent acidification in the endosome is required to release virus particles from the receptor. Furthermore, we have to note that hemolytic activity may not correlate with membrane fusion activity. Once surface proteins of EA V have been purified, the hemagglutination and hemolysis assay described here might be useful for determining which complex (e.g. gp5/M or gp2b/gp3/gp4) contains the receptor-binding and membrane fusion activity of EA V.

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