Localization of pestiviral envelope proteins Erns and E2 at the cell surface and on isolated particles

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The glycoproteins Erms of classical swine fever virus (CSFV) and Erns and E2 of bovine viral diarrhoea virus (BVDV) are shown to be located at the surface of infected cells by the use of indirect immunofluorescence and by cytofluorometric analysis. The positive immunostaining of the cell surface was further analysed by immunogold electron microscopy and it could be shown that only extracellular virions were labelled. Gold granules were not seen at the cellular plasma membrane. In contrast to BVDV E2, the CSFV E2 of virions sticking to the plasma membrane was not accessible to the respective monoclonal antibodies. However, CSFV particles isolated from culture supernatant were able to bind both monoclonal anti-Erns and anti-E2 antibodies. For CSFV and BVDV, binding of anti-Erns antibodies to the virions was more pronounced than that of anti-E2. This finding was unexpected since E2 is considered to be the immunodominant glycoprotein.

Introduction

Pestiviruses are classified as members of the Flaviviridae (Wengler, 1995). They occur worldwide and are the causative agents of economically important animal diseases such as classical swine fever, bovine viral diarrhoea and border disease of sheep. Like other members of the family, pestiviruses are plus-stranded RNA viruses whose genome comprises one long open reading frame (Collett et al., 1988; Meyers et al., 1989; Moormann et al., 1990; Deng et al., 1992). Translation into one hypothetical polyprotein is accompanied by proteolytic processing through host cell and virus-encoded proteases. The structural proteins of pestiviruses include a putative nucleocapsid protein (C) and three envelope glycoproteins Erns, E1 and E2 (Thiel et al., 1991). The boundaries of the structural proteins have been directly determined by N-terminal sequencing (Rümneapf et al., 1993; Stark et al., 1993; Elbers et al., 1996). Two of the envelope proteins, namely Erns and E2, are capable of inducing neutralizing antibodies (Bolin et al., 1988; Donis et al., 1988; Wensvoort et al., 1989; Corapi et al., 1990; Greiser-Wilke et al., 1990; Weiland et al., 1990, 1992).

Investigations on structure and morphogenesis of pestiviruses have progressed more slowly than the analysis of the genome and its organization (Meyers & Thiel, 1996). Until now, little information has been available on the assembly and release of pestiviruses from infected cells. A prerequisite for virus release by budding from the extracellular membrane is expression of viral glycoproteins. Convincing evidence for a budding process of pestiviruses has so far not been reported (Gray & Nettleton, 1987), and indeed Greiser-Wilke et al. (1991) actually reported that viral glycoproteins are not present at the surface of cells infected with pestiviruses.

The present study shows that monoclonal antibodies (MAbs) directed against Erns of CSFV and BVDV mediated immunofluorescence at the surface of pestivirus-infected cells detectable by conventional microscopy and flow cytometric (FCM) analysis. This observation was unexpected and required further investigation by a separate approach. Accordingly, immunoelectron microscopic studies were performed. With this method it could be shown that the MAbs against CSFV and BVDV envelope glycoproteins bound exclusively to the surface of virions accumulated outside the cell.

Methods

- **Viruses and cells.** The origin of the CSFV strain (Alfort Tübingen) and the BVDV strain (NADL Tübingen) as well as their propagation in STE, 38A1D and MDBK cells, respectively, is described in previous reports (Weiland et al., 1989, 1992). The adherently growing STE cells
were used for all immunobinding studies; the 38A1D cells, growing in suspension, were utilized for production of virus.

### Preparation of MABs

In previous papers the preparation and characterization of MABs against BVDV E2, formerly designated BVDV gp53 (Weiland et al., 1989), and CSFV E\textsuperscript{en} and E2, formerly designated HCV gp44/48 and HCV gp55, respectively (Weiland et al., 1992), have been described. Whereas anti-CSFV fusions led to the detection of both anti-E\textsuperscript{en} as well as anti-E2 antibodies, screening of the anti-BVDV fusion led only to anti-E\textsuperscript{en} MABs. Since parts of the fusion products between SP2/0 myeloma cells and spleen cells of the BVDV-immunized mouse were cryoconserved immediately after fusion in the presence of polyethylene glycol, it was possible to go back to this material to search again for anti-BVDV E\textsuperscript{en} hybridomas. From studies with MABs against CSFV E\textsuperscript{en} we had learned that their immunostaining of fixed infected cells was always weaker and more diffuse than that observed with anti-CSFV E2 MABs (Weiland et al., 1992). Therefore, the studies were focused especially on those hybridomas which showed a weak and diffuse reactivity pattern in the indirect immunoperoxidase assay of fixed BVDV-infected cells as already observed with anti-CSFV E\textsuperscript{en} antibodies.

### Infection of cells

MDBK as well as STE and 38A1D cells were infected in suspension with BVDV and CSFV, respectively. 10\textsuperscript{5} cells were suspended in 1 ml of the virus solution at an m.o.i. of 0.1. After incubation at room temperature with gentle shaking for 20 min appropriate cell dilutions were performed and the cells were plated either on Cooke multitest slides or in culture bottles.

### Immunofluorescence on unfixed pestivirus-infected adherent cells

Forty-six hours after plating BVDV-infected cells, noninfected MDBK cells, CSFV-infected and non-infected STE cells were incubated with the MAbs against E\textsuperscript{en} and E2, respectively, for 20 min at 37 °C. Binding of antibody to the cells was visualized by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibody (Dianova) after a further incubation at 37 °C for 20 min. To stabilize the incubated vital cells for microscopic evaluation they were fixed with freshly prepared paraformaldehyde (3.5%) for 5 min.

### Flow cytometric analyses

Forty-six hours after plating pestivirus-infected cells were removed from the plate by incubation for 10 min with 0.02% EDTA in PBS without divalent cations. Surface expression of glycoproteins CSFV E\textsuperscript{en}, CSFV E2, BVDV E\textsuperscript{en} and BVDV E2 was analysed in a fluorescence-activated cell sorter (FACStar Plus; Becton Dickinson) using MAbs a18 (anti-CSFV E2), 24/16 (anti-CSFV E\textsuperscript{en}) (Weiland et al., 1992), D5 (anti-BVDV E2) (Weiland et al., 1989) and the newly established VI-12-6 (anti-BVDV E\textsuperscript{en}), respectively. As a control antibody, MAb 1A12, directed against a surface molecule of various mammalian cells including cells of porcine and bovine origin, was used. The binding of all MAbs was detected by using FITC-labelled goat anti-mouse antisera (Jackson Laboratories). Dead cells were excluded from the analysis by electronic gating using scatter parameters.

### Preparation of virus

Supernatant from CSFV-infected 38A1D cells was first centrifuged at 5400 g for 20 min to remove cell debris. After the virions had been pelleted according to a sedimentation coefficient described for pestiviruses through a sucrose cushion (20% sucrose, 16 h, 52,200 g, 4 °C, rotor SW 27) they were resuspended in TEN buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl) and further separated by the use of a sodium potassium tartrate step gradient (11 h, 274,000 g, 4 °C) (Unger, 1993).

### Immunogold electron microscopy

STE cells and MDBK cells were infected in suspension with CSFV and BVDV, respectively, and grown as monolayers on Cooke multitest slides. At 48 h after infection they were incubated without previous fixation with MABs 24/16 (anti-CSFV E\textsuperscript{en}), MAB a18 (anti-CSFV E2), MAB VI-12-6 (anti-BVDV E\textsuperscript{en}) or MAB D5 (anti-BVDV E2), respectively. Incubation with rabbit anti-mouse IgG (Dianova) was followed by incubation with protein A–colloidal gold (8 nm in diameter) (Weiland et al., 1992). Cell monolayers were then fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated and in situ embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

CSFV particles isolated by ultracentrifugation from culture supernatant were incubated with the respective antibodies and protein A–colloidal gold (Weiland, 1981). They were then negatively stained with unbuffered uranyl acetate.

### Results

#### Characterization of MABs against BVDV

The first MAbs against BVDV established in our lab reacted exclusively with E2. A modified screening focused on hybridoma supernatants exhibiting weak and diffuse staining in an immunoperoxidase assay (Fig. 1A). Identification of its target antigen was performed as described before (Weiland et al., 1989) by radioimmunoprecipitation experiments with extracts of metabolically labelled BVDV-infected cells (unpublished results). Using this approach, only one anti-BVDV E\textsuperscript{en} MAB could be established (VI-12-6). An additional fusion led exclusively to the isolation of hybridomas secreting anti-BVDV E2 antibodies, thereby confirming the statement of Corapi et al. (1990) that establishment of anti-BVDV E\textsuperscript{en} hybridomas is a rare event.

#### Demonstration of pestiviral glycoproteins at the surface of infected cells

Immunity to virus infections largely depends on the development of an immune response to antigens present on the surface of either virions or virus-infected cells. In immunofluorescence (IF) studies adherent unfixed CSFV-infected STE cells showed faint distinct fluorescent granula after incubation with anti-CSFV E\textsuperscript{en} MAbs (Fig. 2A). This was observed with all 15 hybridoma supernatants containing anti-CSFV E\textsuperscript{en} antibodies directed against at least 11 different E\textsuperscript{en} epitopes of CSFV (Kosmidou et al., 1995). Using the same assay, none of 41 anti-CSFV E2 MAbs (directed against at least 12 different E2 epitopes of CSFV) mediated binding of FITC-labelled secondary antibodies (Fig. 2B). However, when BVDV-infected cells were used, immunofluorescence was detected after incubation with MAbs against BVDV E\textsuperscript{en} as well as with those against E2 (Fig. 2C, D). Interestingly, the immune reaction was more intense with the anti-BVDV E\textsuperscript{en} MAbs.

As the fluorescent cell sorting allows exact quantification of antibody binding to cell surfaces this method was used in addition to the standard fluorescence microscopy. In the case of CSFV-infected STE cells incubation with anti-E\textsuperscript{en} MAB 24/16 clearly induced an increase in fluorescence intensity, whereas after incubation with anti-E2 MAB a18 fluorescence
was not above background levels (Fig. 3, upper row). BVDV-infected MDBK cells also showed E<sub>rns</sub> expression at their surface. However, as already observed in the standard IF studies, these cells also exhibited clear surface expression of the E2 protein (Fig. 3, lower row). Thus the results obtained by standard fluorescence microscopy were confirmed by FCM analysis.

These studies showed in addition that the target antigens of the anti-glycoprotein MAb were so firmly connected to the cell surface that they resist the EDTA treatment used for detachment of the cells.

**Immuno-electron microscopy of pestivirus-infected cells**

All enveloped RNA viruses which acquire an outer lipid envelope by budding from the host plasma membrane induce expression of viral glycoproteins at the cell surface. For pestiviruses, budding from the cell surface has never been reported. Thus, binding of anti-glycoprotein MAb to the surface of CSFV- as well as of BVDV-infected cells represented an unexpected finding. We sought to confirm the antibody reaction with the surface of pestivirus-infected cells as shown by immunofluorescence by an analysis at the electron microscopic level.

Using CSFV- as well as BVDV-infected cells, labelling by immunogold was investigated. A strong binding of anti-E<sub>rns</sub> MAb exclusively to virions at the cell surface could be demonstrated by an intense accumulation of the electron dense marker colloidal gold (Figs 4A, C and 5A). In contrast, all 12 anti-CSFV E2 MAb tested up to now did not show any reaction with CSFV particles in ultrathin sections (Fig. 4B, D). However, using anti-BVDV E2 MAb, a distinct, but faint reaction could be seen with BVDV particles (Fig. 5B). Thus, the differences in the reactivity of anti-CSFV E2 MAb and anti-BVDV E2 MAb with infected cells as shown in standard IF and FCM analyses were confirmed by immune electron microscopic studies. Interestingly, binding of the MAb directed against the structural glycoproteins of the pestiviruses was exclusively restricted to extracellular virions. Binding to the plasma membrane of infected cells was never observed. An antibody (MAb 1A12) directed against a so far undefined surface molecule of various mammalian cells mediated binding of immunogold to the whole cell surface (not shown).

For CSFV- as well as BVDV-infected cells detection of virus particles was a rare event. Obviously, virions were released from the cell only in small numbers. Sometimes, however, virions were found lined up, building a chain of virus particles (Fig. 4A). This phenomenon was only seen with CSFV-infected cells.

The diameter of the pestivirus particles as measured in ultrathin sections after embedding in epoxy resin was about 40–50 nm. A characteristic feature of CSFV particles was their
Fig. 2. Indirect IF tests. Unfixed CSFV-infected STE cells show faint, distinct fluorescent granula after incubation with anti-CSFV E\(^\text{mAb}\) 24/16 (A), no reaction could be detected with anti-CSFV E2 MAb a18 (B). After incubation with anti-BVDV E\(^\text{mAb}\) VI-12-6 (C) and anti-BVDV E2 MAb D5 (D), respectively, fluorescent granula were seen on the cell surface of unfixed BVDV-infected MDBK cells; the immune reaction was more distinct when anti-E\(^\text{mAb}\) MAb were used (C).

rather large virus core closely surrounded by the envelope (Fig. 4 A–D). With BVDV particles the envelope appeared to be more loosely arranged (Fig. 5). Only in a few cases could the icosahedral symmetry of the particles, the structure of most spherical viruses (Caspar & Klug, 1962), as recognizable by their hexagonal outlines, be demonstrated (Fig. 4 D).
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Investigation of isolated virions by immunoelectron microscopy

E2 is generally considered to be the immunodominant glycoprotein which represents the primary target of neutralizing antibodies. Surprisingly, CSFV particles located close to the cell surface reacted only with anti-E\textsuperscript{rns} antibodies but not with anti-E2 antibodies. For BVDV a stronger reaction with E\textsuperscript{rns} than with E2 was observed. It was speculated that better accessibility of E2 to the respective antibodies may develop after release of virions from the cell surface. To test this hypothesis virions were isolated from the supernatant of CSFV-infected cells by combined ultracentrifugation steps. Incubation with anti-E\textsuperscript{rns} as well as with anti-E2 MAbs caused decoration of the virus particles with gold granules as can be seen in Fig. 4(E, F). It was striking that – counting 80 virus particles of each preparation – anti-E\textsuperscript{rns} MAbs mediated the binding of three times more gold granules than anti-E2 antibodies. On average, 4–4 gold granules per virus particle were seen with anti-E\textsuperscript{rns} MAbs; however, only 1–5 were seen with anti-E2 antibodies. After negative staining CSFV particles had a diameter of 55–70 nm. Due to flattening on the film of the grid during preparation the isolated virions appear to be larger than in ultrathin sections.

Discussion

IF microscopy on live adherent cells as well as FCM analysis of viable cells suspended after detachment by EDTA treatment led to the detection of two pestiviral glycoproteins at the cell surface. This finding was surprising since budding at the cell surface has not been reported for pestiviruses (Gray & Nettleton, 1987; Laude, 1987) and in previous studies viral glycoproteins were not detected at the cell surface (Greiser-Wilke et al., 1991). Further analysis by immune electron microscopy showed that immunogold granules exclusively decorated virions which had accumulated at the cell surface after their release in aggregates. Interestingly, the plasma membrane remained completely unlabelled. Thus, the positive immunofluorescence was mediated by binding of the MAbs to virions and not to proteins integrated in the plasma membrane. A similar observation was described by Gould et al. (1985) for yellow fever virus (YFV), a member of another genus of the Flaviviridae. A MAb directed against the envelope protein of this virus allowed the detection of surface fluorescence on infected unfixed cells. Interestingly, aggregates of virus particles coated with antibody molecules were found by electron microscopy in the extracellular space. The authors concluded that fluorescent granula seen on the surface of infected cells were actually due to virions. Schlesinger et al. (1990) detected on the surface of cells infected with YFV both the virion envelope protein (E) and the non-structural glycoprotein NS1. However, evidence for integration into the plasma membrane was only obtained for the NS1 protein, not for the E protein.

For CSFV, expression of glycoproteins at the surface of cells has been shown by immunofluorescence after infection with vaccinia virus recombinants expressing either E\textsuperscript{rns} or E2 (Konig et al., 1995). It has not been determined whether integration into the plasma membrane occurred. In any case, it is difficult to directly compare cell surface expression of single
Fig. 4. Immunogold electron microscopy of CSFV-infected STE cells incubated with anti-CSFV E\textsuperscript{\textit{rns}} MAb 24/16 (A, C) and anti-CSFV E2 MAb a18 (B, D) before embedding in Araldite. In (A) and (C) decoration of virus particles with colloidal gold can be seen. Binding of MAbs to the cellular plasma membrane was not observed. No immunostaining was detected with the anti-E2 MAb (B, D). In (D) the hexagonal structure of the virion is demonstrated (arrows). When CSFV particles isolated from culture supernatant were immunostained with anti-CSFV E\textsuperscript{\textit{rns}} MAb 24/16 (E) and anti-CSFV E2 MAb a18 (F), both MAbs decorated isolated virus particles; there was a stronger reaction with anti-E\textsuperscript{\textit{rns}} MAb (E). Bar, 100 nm (A, B, E, F); 50 nm (C, D).
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Detection of extracellular virions at the cell surface as observed after treatment with MAb s indicated firm binding of the released virus particles to the cell surface as the particles remained attached to the cells in spite of several steps of treatment used during the performance of our immunological assays. In particular, during preparation of samples for FCM analysis the binding withstood EDTA-mediated detachment of the cells and also mechanical stress during the incubation with primary/secondary antibody and washing procedures with centrifugation of the suspended cells. It was observed almost 30 years ago that cultured pestiviruses remain cell-associated to a large extent (Danner & Bachmann, 1970) and this has been confirmed many times. Our studies suggest that this phenomenon is based in part on the firm adhesion of virions to the cell surface.

One crucial step in the development of virus vaccines is the identification of the antigen(s) which induce(s) protective immunity. For pestiviruses, E2 was defined as the immunodominant envelope protein. It induces effective neutralizing antibodies and is able to mediate protection after inoculation of pigs with either recombinant live virus expressing E2 or purified E2 (Rumenapf et al., 1991; van Zijl et al., 1991; Hulst et al., 1993; König et al., 1995; van Rijn et al., 1996). It was assumed that this protein is located at the surface of the virions. It was therefore surprising that CSFV particles that accumulated on the surface of the infected cell were not labelled with anti-E2 MAbs, but with anti-E^{rns} MAbs. In the case of BVDV both corresponding MAbs mediated binding of gold granules to virions adhering to the cellular surface. Interestingly, the binding was less intense with anti-E2 MAbs(162,865),(550,891). One may assume that the lack of binding as observed for CSFV is due to the antibodies used. However, 12 different anti-CSFV E2 MAbs failed to bind to the surface of CSFV-infected cells. Since it could also be argued that the E2-specific MAbs have a low affinity to the respective epitopes, the anti-E2 MAbs were directly compared with anti-E^{rns} MAbs by means of virus neutralization, immunostaining, immunoprecipitation and immunoblotting; there was no indication of a lower affinity of anti-E2 MAbs (Weiland et al., 1992).

Interestingly, CSFV particles isolated from culture supernatant exhibited surface labelling with both MAbs, anti-E^{rns} and anti-E2, as shown in Fig. 4(E, F). It is suggested that the CSFV E2 protein becomes accessible to anti-E2 MAbs only after the final release of the virus particle from the cell surface. This may be explained by a further maturing process of virus particles when they are released from the cellular plasma membrane. During this process the E2 reactive epitopes may become accessible on the surface of the virions. Another possibility is that the E2 epitopes are hidden by cellular material as long as the virions are stuck to the cell surface. A further possible explanation is that E2 epitopes, normally masked, may become un-masked during virion preparation, i.e. treatment with high salt by purification through sucrose and potassium tartrate. This, however, is unlikely as native virus prior to ultracentrifugation is already reactive to anti-E2 antibodies, resulting in neutralization of the virus.
According to our studies, although the E2 glycoprotein is less exposed at the surface of virions, it still represents the crucial antigen for virus neutralization and induction of protective immunity. Studies on the mechanism of neutralization by MAbs against E\textsubscript{NS} and E2 proteins may help to elucidate this finding.

Essential questions with regard to the assembly and release of pestiviruses remain unanswered. The present study shows that pestiviral glycoproteins can be demonstrated at the cell surface. Interestingly, the respective antigens belong to complete virions associated with the plasma membrane. Accordingly, the glycoproteins are not integrated into the plasma membrane. In BVDV-infected cells we found virus particles in distended portions of the rough endoplasmic reticulum as already described by Gray & Nettleton (1987). In accordance with these authors, however, we neither detected budding nor any of the stages of virus release.

As described for members of the genus Flavivirus (Ishak et al., 1988; Rice, 1996), release of pestiviruses probably occurs by budding of nascent virions through intracellular membranes into cytoplasmic vesicles and by exocytic fusion of virus-containing vesicles with the plasma membrane. However, in spite of intense studies we could not demonstrate any defined stages of pestivirus morphogenesis like intracellular budding or virus release. It is therefore assumed that the intracellular processes of virus replication take place over a very short time period.

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References


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