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Antibody-Induced and Cytoskeleton-Mediated Redistribution and Shedding of Viral Glycoproteins, Expressed on Pseudorabies Virus-Infected Cells

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Fluorescein isothiocyanate-labeled porcine pseudorabies virus (PrV) polyclonal antibodies were added to PrV-infected swine kidney cells in vitro at 37°C. In approximately 47% of the infected cells, the addition induced passive patching and subsequent energy- and microtubule-dependent capping of all viral envelope glycoproteins, expressed on the plasma membranes of the infected cells. Further contraction and extrusion of the capped viral glycoproteins occurred in approximately 30% of the capped cells 2 h after the addition of antibodies and was accompanied by a concentration of F-actin beneath the caps. At that time, about 18% of the extruded caps were shed spontaneously into the surrounding medium. Mechanical force released 85% of the extruded caps, leaving viable cells with no microscopically detectable levels of viral glycoproteins on their plasma membranes. Experiments with PrV deletion mutants showed that viral glycoproteins gE and gI are important in triggering viral glycoprotein redistribution. Since the PrV gE-gI complex exhibits Fc receptor activity which facilitates capping, the importance of gE and gI may be partially explained by antibody bipolar bridging.

Pseudorabies virus (PrV), a member of the *Alphaherpesvirinae*, also designated Aujeszky's disease virus or suid herpesvirus 1, is the causative agent of Aujeszky's disease. Pigs are the only natural reservoir for PrV. The disease is characterized by nervous signs, respiratory disorders, and reproductive failures, mainly abortions. Abortion is generally the result of transplacental infection and intrafetal replication of PrV. Infected mononuclear cells are detected in the blood of pigs after intranasal PrV inoculation and are thought to transport PrV from the upper respiratory tract to the pregnant uterus (29). Immunity raised by vaccination is not efficient enough for both complete inhibition of viral replication at the primary replication site and blocking of virus spread via the blood to prevent abortion (40). Nauwynck and Pensaert (28) were able to reproduce abortion in vaccinated sows by injecting in vitro-infected autologous blood mononuclear cells into the arteria uterina. Inoculation of cell-free PrV, on the other hand, did not result in abortion. From these observations, it was concluded that cell-associated virus plays a pivotal role in the immune evasion. Much research has already been done on the cell-associated spread of PrV in vitro (14, 33, 34, 36, 42). However, little is known about how infected blood mononuclear cells survive in the blood and how PrV crosses the maternal placenta in vaccinated sows without being eliminated by the immune system.

Viral envelope glycoproteins mediate virion attachment and penetration during infection. So far, eleven glycoproteins of PrV have been characterized: gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, and gN. All of these constitute homologs of proteins found in other herpesviruses (24). During infection by herpes simplex virus (HSV), which is the prototypic alphaherpesvirus, these proteins are glycosylated as they travel through the endoplasmic reticulum and Golgi apparatus (16). In PrV-infected cells, viral envelope glycoproteins are transported towards different cellular lipid bilayers and are abundantly present on the plasma membranes (27), where they are recognized by the immune system (4). Interactions between antibodies and membrane-anchored viral glycoproteins may induce cell- and complement-mediated lysis of the infected cells (4).

Given the importance of cell-associated spread in PrV pathogenesis, and the immune-evasive character of herpesviruses in general, we studied in detail the effect of PrV-specific antibodies on PrV-infected cells. We report on the antibody-induced, cytoskeleton-mediated redistribution of viral envelope glycoproteins on the plasma membranes of PrV-infected SK cells, which finally results in the shedding of these viral glycoproteins into the surrounding medium. Moreover, the data suggest an important role of the gE-gI complex in the observed processes, which may be explained partially by the Fc receptor activity of gE-gI.

MATERIALS AND METHODS

Viruses. PrV strains 89V87 and Kaplan [PrV (Ka)] and PrV (Ka) gB, gC, gD, gE, gI, gE-gI, gG, gH, and gM deletion mutants (PrV gB$^-$, PrV gC$^-$, PrV gD$^-$, PrV gE$^-$, PrV gI$^-$, PrV gE$^-$gI$^-$, PrV gG$^-$, PrV gH$^-$, and PrV gM$^-$) were used. All strains were described earlier (9, 18, 19, 24–26, 36, 37).

Antibodies. In most experiments, protein A-purified and fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) antibodies, derived from a pig inoculated with PrV (89V87) as described earlier (30), were used. Where indicated, protein G-purified IgG antibodies, derived from convalescent sera obtained from pigs surviving at 21 days after inoculation with virulent PrV (Kaplan and 75V19), were used. Dilutions of the antibodies were made in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.

Inoculation of cells. Monolayers of swine kidney (SK-6) cells were obtained by seeding 2×10^7 cells into a 150-cm^2 tissue flask containing Hanks minimal essential medium supplemented with 10% fetal calf serum (FCS), glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and kanamycin (0.1 mg/ml). After 2 and 4 days, fresh minimal essential medium, based on Earle's buffered salt solution and supplemented with 5% FCS, glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and kanamycin (0.1 mg/ml), was added. Seven-day-old confluent monolayers of SK-6 cells were trypsinized, in-

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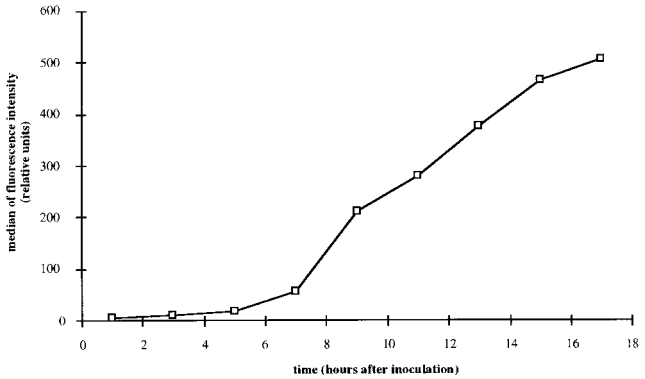

FIG. 1. Kinetics of viral glycoprotein expression on the plasma membranes of PrV-infected cells. SK-6 cells were inoculated with PrV (89V87) for different time periods. Cells were fixed, stained with FITC-labeled porcine PrV antibodies, and analyzed by flow cytometry.

oculated with PrV at a multiplicity of infection of 20 50% tissue culture infective doses per cell, and further incubated in a siliconized bottle on a tilting platform at 37°C in minimal essential medium based on Earle's buffered salt solution and supplemented with 2% FCS, glutamine (0.9 mg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and kanamycin (0.1 mg/ml).

Incubation of PrV-infected cells with porcine anti-PrV polyclonal antibodies. In general, cells inoculated for 13 h were centrifuged at 400 × *g* for 10 min, washed, and resuspended in PBS. The cells were incubated at 0.6×10^7 cells/ml with FITC-conjugated PrV polyclonal antibodies (0.5 mg of IgG/ml, except where indicated otherwise) for 2 h at 37°C. Every 10 min, the cells were shaken gently to avoid sedimentation. At different times (0, 5, 10, 20, 30, 60, 90, and 120 min) during antibody incubation, the cells were fixed with 0.4% formaldehyde. Finally, the cells were washed thoroughly, mounted in a glycerin-PBS solution (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo[2.2.2]octane (Janssen Chimica, Beerse, Belgium), excited with an Osram HBO 50-W bulb and an I3 filter, and observed with a Leitz DM RBE microscope (Wild Leitz GmbH, Heidelberg, Germany).

To determine the energy dependence of the observed processes, 40 mM NaN_3 was added 0.5 h before and during antibody incubation.

To determine the involvement of F-actin and microtubules, 25 μM cytochalasin B (inhibitor of G-actin polymerization to F-actin) or 0.1 mM colchicine (inhibitor of tubulin polymerization) was added immediately after inoculation of the cells and during antibody incubation.

Definition of different viral glycoprotein distributions. The viral glycoprotein distribution was scored as a "rim" when the fluorescence label exhibited a homogeneous cell surface cover. The cells were scored as "patched" when the labeled viral glycoproteins formed randomly distributed aggregates on the cellular surface. The cells were scored as "capped" when the viral glycoproteins formed one large aggregate at one pole of the cell, occupying less than 50% of the cell surface. Caps were defined as "extruded" when the fluorescence-labeled viral glycoproteins formed an extracellular vesicle, becoming detached from the cellular surface.

Quantitative results were obtained by examining the fluorescence distribution on at least 200 cells. The number of cells displaying a cap or an extruded cap was expressed as a percentage of the viral antigen-positive cells. All assays were run independently at least three times.

Fluorescence labeling of different cytoskeletal components. FITC-labeled cells were washed in a cytoskeleton stabilizing buffer (CSB) containing 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM glucose, and 20% FCS and fixed in a 3% solution of paraformaldehyde in CSB for 10 min at room temperature. After being washed in CSB, the cells were permeabilized for 1 min in a 0.1% solution of Triton X-100 in CSB and washed in CSB.

Microtubules were stained by incubation of cells with mouse anti-α-tubulin (Amersham International PLC, Buckinghamshire, United Kingdom) and by a 1:20 dilution in CSB at 37°C for 2 h. Afterwards, the cells were washed in washing buffer (WB) containing 200 mM Tris, 1.54 M NaCl, 20 mM EGTA, 20 mM $MgCl_2$, and 10% FCS, incubated with goat anti-mouse IgG-Texas red (Amersham International PLC) diluted 1:10 in CSB at 37°C for 1 h, and washed twice in WB.

F-actin was stained by incubating the cells with 200 nM phalloidin-Texas red (Molecular Probes, Eugene, Oreg.) in CSB for 1 h at 37°C. Afterwards, the cells were washed twice in WB.

Confocal laser scanning microscopy. Fluorescent samples were examined on a Bio-Rad MRC 600 confocal laser scanning system (Bio-Rad House, Hertfordshire, United Kingdom) linked to a Nikon Diaphot 300 microscope (Nikon Corporation, Tokyo, Japan) and interfaced to an AST Premmia SE 4/66d computer (AST Computer, Irvine, Calif.). Krypton-argon laser light was used to excite FITC (488-nm line) and Texas red (568-nm line) fluorochromes with a K1 and a K2 emission filter, respectively. Extended-focus images were obtained with Bio-Rad COMOS Software. Images were printed on a Kodak XLS 8600 PS printer (Eastman Kodak Company, Rochester, N.Y.).

Flow cytometric analysis. Flow cytometric analysis was conducted with a Becton-Dickinson (San Jose, Calif.) FACScalibur equipped with a 15-mW air-cooled argon ion laser and interfaced to a Macintosh Quadra 650 computer (Apple Computer, Inc., Cupertino, Calif.) using BD Cellquest software. Acquisition rates were maintained at 300 to 500 cells/s. At least 10,000 cells were analyzed for each sample and forward-scattered light versus side-scattered light dot plots were used to identify SK-6 cell populations.

Flow cytometric determination of cell viability was performed by adding 100 μl of a 0.1% solution of ethidium bromide to 500 μl of a suspension of approximately 10^6 cells/ml and analyzing fluorescence intensity after 3 min.

To determine cytoskeletal association of viral glycoproteins, the plasma membranes of the cells were disrupted before flow cytometric analysis by a modified version of the method described by Martin and Sugden (22). Briefly, approximately 10^6 cells were suspended in 100 μl of a PBS solution containing 79% PBS, 20% FCS, and 1% Triton X-100 and incubated for 5 min with rocking every 30 s. Afterwards, the cells were diluted by addition of 400 μl of PBS and analyzed by flow cytometry. All assays were run independently at least three times.

RESULTS

Antibody-induced redistribution and release of plasma membrane-bound viral glycoproteins in PrV-infected cells. To determine the kinetics of viral glycoprotein expression on the plasma membranes of PrV-infected cells, SK cells were inoculated with PrV (89V87) for different time periods and fixed with formaldehyde and viral glycoproteins were labeled as described in Materials and Methods. Expression of viral glycoproteins on the plasma membrane started 5 h after inoculation and increased gradually (Fig. 1).

Preliminary results in our laboratory suggested a complicated cascade of events occurring at the level of plasma membrane-bound viral glycoproteins in PrV-infected cells after the addition of porcine PrV polyclonal antibodies at 37°C. The chronology of these events was studied by incubating cells which had been infected with PrV for 13 h with FITC-labeled porcine anti-PrV polyclonal antibodies for different time periods and monitoring the cells by fluorescence microscopy. Starting 5 min post-antibody addition (pAA), viral glycoproteins redistributed from a rather homogeneous cell surface cover (rim) into large aggregates (patches) which were randomly distributed over the cellular surface (Fig. 2, 3a, and 3b). The aggregates started to translocate to one side of the cell at 10 min pAA (cap). Caps became detached in 17% of the cells

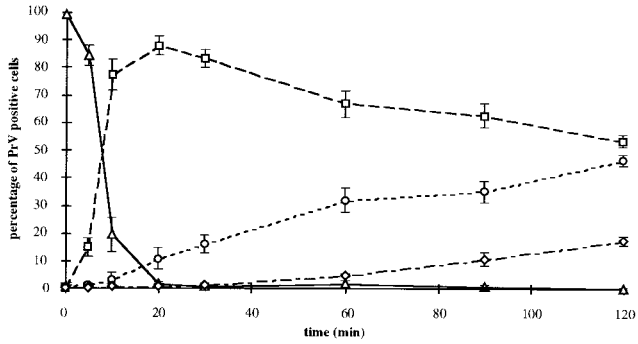

FIG. 2. Antibody-induced redistribution of viral glycoproteins expressed on the plasma membranes of PrV-infected cells. PrV-infected SK-6 cells were incubated with FITC-labeled porcine PrV polyclonal antibodies for 2 h at 37°C and fixed at different time points during this incubation. Distribution of viral glycoproteins was observed by fluorescence microscopy and scored as described in Materials and Methods. Curves indicate percentages of PrV-positive cells with rims (△), patches (□), caps (○), and extruded caps (◇) of viral glycoproteins. The data are means ± standard deviations of triplicate assays.

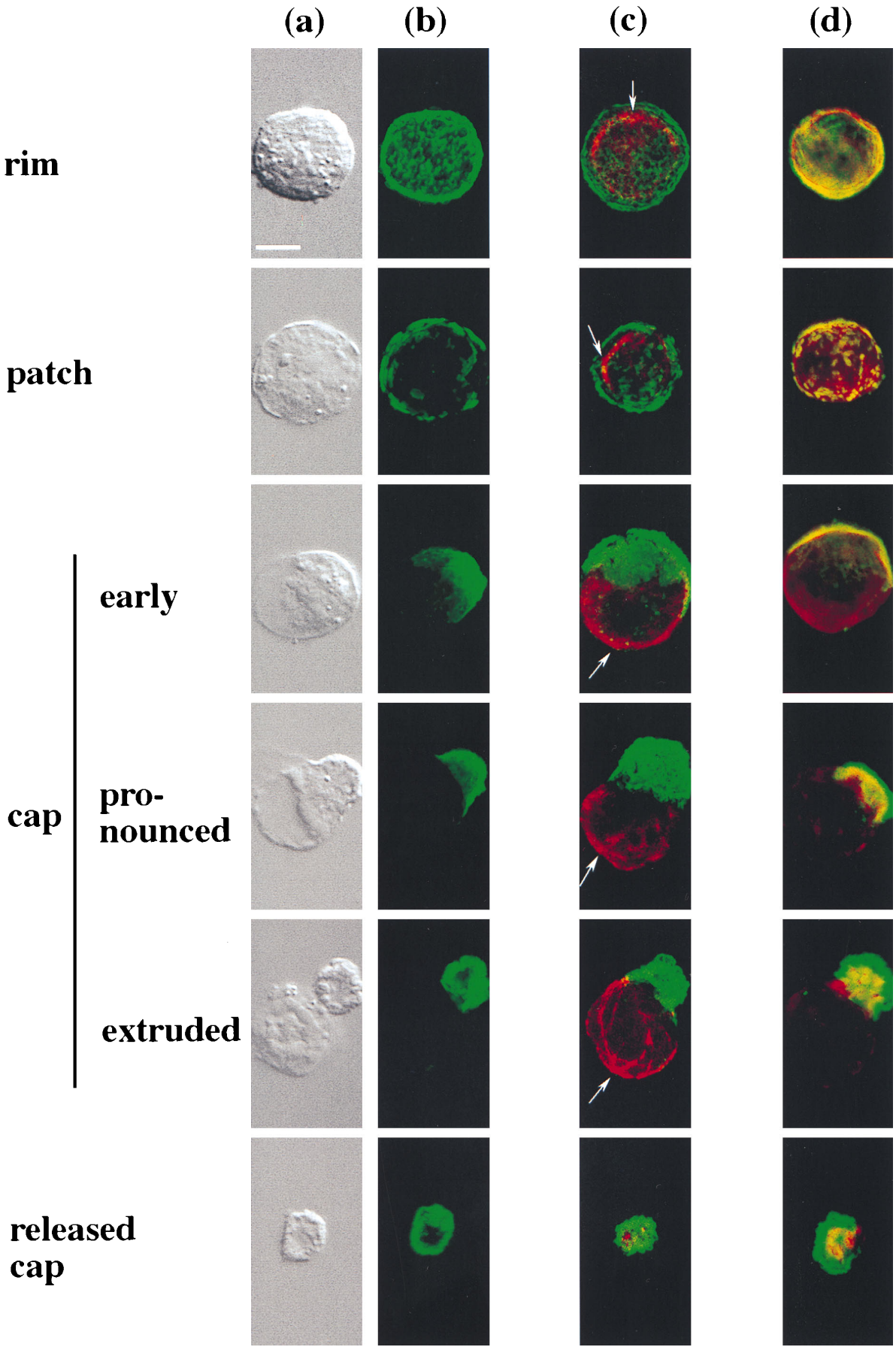
(a)
(b)
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FIG. 3. Antibody-induced redistribution of viral glycoproteins and accompanying changes in F-actin and microtubules. PrV-infected SK cells were incubated with FITC-labeled porcine PrV polyclonal antibodies for 2 h at 37°C and fixed at different time points during this incubation. (a) Differential interference-contrast image of cells at different times after antibody incubation. (b) Confocal laser scanning image of viral glycoprotein distribution of cells shown in panel a. (c) Double labeling of viral glycoproteins (green) and microtubules (red) of cells with different viral glycoprotein distributions. Arrows indicate localization of MTOCs. (d) Double labeling of viral glycoproteins (green) and F-actin (red) of cells with different viral glycoprotein distributions. Bar, 5 μm.

starting at 30 to 60 min pAA (extruded cap). Approximately 10 to 15% of the extruded caps were shed spontaneously into the surrounding medium at 2 h pAA. Shaking of the cells for 1 min removed 68% of the extruded caps without noticeable effects on cell viability, as assessed by ethidium bromide staining and flow cytometry. Cells which shed viral glycoproteins did not retain visually detectable levels of viral glycoproteins on their plasma membranes.

The use of antibodies derived from hyperimmune sera from pigs inoculated with different PrV parental strains (75V19 and Kaplan) did not alter the percentage of cells with caps (40.6% ± 2.3% [mean ± standard deviation] and 41.7% ± 0.4%, respectively, at 2 h pAA) or extruded caps (12.7% ± 2.2% and 11.1% ± 3.2%, respectively, at 2 h pAA). In all further experiments, antibodies derived from a PrV (89V87)-inoculated pig were used.

Influence of infection kinetics and antibody concentration on the antibody-induced viral glycoprotein redistribution process. The amount of viral glycoprotein expressed on the plasma membrane of the infected cell gradually increases during PrV infection (Fig. 1). Thus, infection status might be an important parameter in the antibody-induced processes. SK cells were incubated with porcine PrV polyclonal antibodies for 2 h at several time points after PrV inoculation. Figure 4 shows that percentages of cells with both capped viral glycoproteins and an extruded cap increased with cells inoculated for 5 to 9 h. This increase was accompanied by a gradual enlargement of the formed patches. Inoculation periods longer than 9 h did not affect either capping of viral glycoproteins or subsequent extrusion. To summarize the results shown in Fig. 1 and 4, once a minimum amount of viral glycoprotein is present on the plasma membrane, antibody addition may trigger capping and subsequent extrusion. All subsequent experiments were performed with cells 13 h after inoculation.

Another parameter with a potential effect on antibody-induced processes may be the concentration of antibodies added. Therefore, percentages of capping and extrusion of the caps were examined in PrV-infected cells incubated with different concentrations of antibodies for 2 h (Fig. 5). Addition of 0.01 to 0.2 mg of IgG/ml resulted in an increase in percentages of both capped viral glycoproteins and extruded caps (Fig. 5), again accompanied by gradual enlargement of the formed patches. Higher antibody concentrations did not affect either capping or subsequent extrusion.

We hypothesize that the patch size, which is influenced by both the infection status of the cell and the concentration of antibodies added, has to exceed a minimal threshold size before viral glycoprotein capping and subsequent extrusion can occur.

Energy, microtubule, and actin dependence of the antibody-induced viral glycoprotein redistribution process. Capping of viral glycoproteins resembles a morphologically different phenomenon: the antibody-induced capping and internalization of (Fc) receptors in lymphocytes and mast cells during activation, which is an active process mediated by the cytoskeleton (35, 39, 41). Experiments in the presence of the ATPase inhibitor NaN_3, the tubulin polymerization inhibitor colchicine, and cytochalasin B, which abolishes the polymerization of G-actin to F-actin, were performed to investigate the energy, microtubule, and F-actin dependence of both capping of viral glycoproteins and extrusion of the caps.

None of the reagents caused significant decreases in the expression of viral glycoproteins on the plasma membranes of the infected cells, nor did they affect patching of plasma membrane glycoproteins after the addition of antibodies. Table 1 shows the effect on capping of viral glycoproteins and extrusion of the caps at 2 h pAA. Addition of NaN_3 caused a significant decrease in the capping ($P < 0.01$) of viral glycoproteins and in the ratio of extruded caps to total caps ($P < 0.05$), indicating an energy requirement for both capping of viral glycoproteins and subsequent extrusion of the caps. Addition of colchicine caused a significant decrease in capping ($P < 0.01$), highlighting the importance of microtubules in the initial polarization of patches toward a cap but also suggesting a microtubule-independent contraction and extrusion of the cap. Addition of cytochalasin B caused a significant reduction of the ratio of extruded caps/total caps ($P < 0.01$), indicating an F-actin-independent polarization of patches toward a cap but an F-actin-dependent contraction and extrusion of the cap.

Redistribution of microtubules and F-actin during the antibody-induced viral glycoprotein redistribution process. To elucidate the role of different cytoskeletal components in the observed processes, microtubules and F-actin were visualized by fluorescence labeling in PrV-infected cells at different time points after addition of porcine PrV polyclonal antibodies.

Microtubules were organized as perinuclear fibers originating from the microtubule organizing center (MTOC) in cells with the rim and patch configurations of viral glycoproteins. In capped cells and cells with extruded caps, the MTOC was located at the opposite side of the cap and microtubules extended toward the cap (Fig. 3c).

F-actin fibers were distributed homogeneously beneath the plasma membrane in cells with the rim, patch and "early" cap configurations of viral glycoproteins. In cells with more pronounced caps, F-actin fibers colocalized with the viral glycoproteins. In cells with extruded caps, F-actin was concentrated within the extrusion, with very low levels of F-actin remaining in the attached cell (Fig. 3d). These data are consistent with the results obtained by the addition of cytochalasin B before and during antibody incubation and stress the importance of

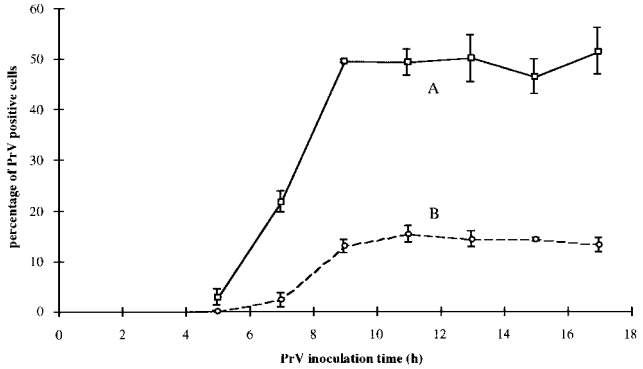


FIG. 4. Effect of infection status on the redistribution process. At different time points after PrV inoculation, SK cells were incubated for 2 h with FITC-labeled porcine PrV polyclonal antibodies. Line A shows the percentage of capped cells, and line B shows the percentage of cells with extruded caps. The data are means ± standard deviations of triplicate assays.

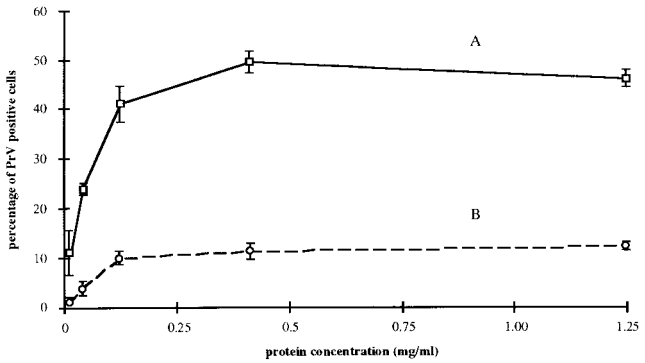


FIG. 5. Effect of antibody concentration on the redistribution process. PrV-infected SK cells were incubated with different concentrations of FITC-labeled porcine PrV polyclonal antibodies for 2 h. Line A shows the percentage of capped cells, and line B shows the percentage of cells with extruded caps. The data are means ± standard deviations of triplicate assays.

F-actin in contraction and extrusion of the capped viral glycoproteins. Moreover, they suggest that the cytoskeletal connection between an extruded cap and its attached cell is weak.

Role of different viral envelope glycoproteins in the redistribution process. Experiments with PrV (Ka) and isogenic gB, gC, gD, gE, gI, gE-gI, gG, gH, and gM deletion mutants were performed to investigate which viral envelope glycoprotein(s) plays a role in antibody-induced redistribution of viral glycoproteins. None of the mutants used altered the expression of viral glycoproteins on the plasma membranes significantly (data not shown).

Addition of antibodies to PrV (Ka)-infected SK cells showed redistribution kinetics of viral glycoproteins similar to those observed with strain PrV (89V87) (data not shown). Table 2 shows the percentage of cells with caps and the percentage of cells with extruded caps at 2 h pAA, after infection with different PrV strains. Infection by the PrV (Ka) gE and gE-gI deletion mutant resulted in a significant decrease in the percentages of cells with capped viral glycoproteins ($P < 0.01$) and with extruded caps ($P < 0.01$), suggesting an involvement of viral glycoprotein gE in triggering viral glycoprotein capping. The PrV (Ka) gI deletion mutant did not alter the total percentage of capped cells, but it significantly reduced the percentage of extruded caps ($P < 0.01$). These data, therefore, indicate an important role for the gE-gI complex during antibody-induced redistribution of the viral glycoproteins, with distinct roles for gE and gI.

Fc receptor activity of the gE-gI complex. A possible role for gE-gI during antibody-induced redistribution might be the formation of large patches by antibody bipolar bridging. Antibody bipolar bridging has been described for HSV, in which HSV-specific antibodies may bind viral glycoproteins by their Fab portions and the Fc receptor complex gE-gI with their Fc portions.

To examine if the PrV gE-gI complex also possesses Fc receptor activity, uninfected cells and cells infected with PrV (Ka), gE^-, gI^-, or gE^-gI^- for 13 h were incubated for 1 h at 37°C with 0.4 mg of swine IgG per ml which had been purified from PrV-negative swine serum. Afterwards, the cells were washed, fixed, washed again, and incubated for 45 min with 1/30 diluted rabbit α-swine-FITC at room temperature. Finally, cells were washed and the fluorescence intensity was analyzed by flow cytometry (Fig. 6). None of the mutants used caused a reduction in expression of the viral glycoproteins gB, gC, or gD on the plasma membrane, as assessed by indirect immunofluorescence with monoclonal and monospecific rabbit polyclonal antibodies directed against gB, gC, and gD (data not shown).

TABLE 1. Effects of different additives on the antibody-induced viral glycoprotein redistribution process[a]

Treatment	Cells with capped viral glycoproteins (%)	Ratio of extruded caps/total caps
Control	35.8 ± 2.0	0.295 ± 0.046
40 mM NaN_3	8.1 ± 0.6**	0.071 ± 0.024*
0.1 mM colchicine	11.7 ± 3.2**	0.263 ± 0.046
25 μM cytochalasin B	28.1 ± 3.0	0.004 ± 0.008**

[a] PrV-infected cells were incubated with FITC-labeled porcine PrV polyclonal antibodies for 2 h at 37°C in the presence or absence of different reagents. Viral glycoprotein distribution was scored by fluorescence microscopy. The values represent means ± standard deviations of triplicate assays. Significant differences from the control within a column are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

From the data shown in Fig. 6a, it can be concluded that the PrV gE-gI complex does exhibit Fc receptor activity towards swine IgG.

To test whether the Fc receptor activity is important for the antibody-induced redistribution of viral glycoproteins, it was blocked before the addition of PrV-specific antibodies to PrV-infected cells. PrV (Ka)-infected cells were preincubated with 0.4 mg of PrV-negative IgG per ml for 1 h at 37°C followed by incubation of the cells with PrV-specific antibodies. Figure 6b shows that blocking of the Fc receptor activity significantly reduces capping and extrusion of the caps ($P < 0.05$).

Flow cytometric analysis of cells with extruded caps and released caps. Lymphocytes with capped receptors can be distinguished from other lymphocytes by flow cytometric analysis, as they appear as a subpopulation of cells with low fluorescence intensity (8). Using similar data acquisition settings, we were not able to distinguish cells with capped viral glycoproteins from cells with patched viral glycoproteins. However, a correlation ($r^2 = 0.997$) between the percentage of a subpopulation of cells with low fluorescence intensity and the visually determined percentage of cells with an extruded cap of viral glycoproteins was found (Fig. 7a, section β).

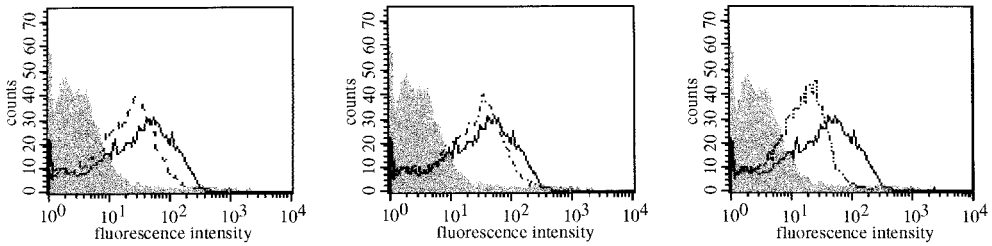
After shaking of capped cells at 2 h pAA for 1 min, cells were pelleted and removed by sequential centrifugation for 3 min at 350 × *g*, 5 min at 350 × *g*, and 5 min at 550 × *g*. This resulted in an accumulation of released caps in the supernatant. In comparison to the subpopulation of cells with extruded caps, flow cytometric analysis showed released caps as a subpopulation of smaller particles with lower granularity and

TABLE 2. Roles of different PrV envelope glycoproteins in the antibody-induced redistribution process[a]

Strain	Capped viral glycoproteins (%)	Extruded caps (%)
PrV (Ka)	45.5 ± 3.7	14.4 ± 0.7
PrV gB^-	38.8 ± 1.1	14.8 ± 1.3
PrV gC^-	44.2 ± 4.0	13.8 ± 1.1
PrV gD^-	43.3 ± 1.2	13.9 ± 1.2
PrV gE^-	18.3 ± 2.3*	3.7 ± 0.9*
PrV gI^-	41.1 ± 2.0	5.1 ± 0.6*
PrV gE^-gI^-	20.0 ± 1.1*	2.8 ± 0.5*
PrV gG^-	39.1 ± 5.3	12.0 ± 1.8
PrV gH^-	40.3 ± 3.2	12.1 ± 0.7
PrV gM^-	39.4 ± 6.9	11.7 ± 3.0

[a] PrV-infected SK cells were incubated with FITC-labeled porcine PrV polyclonal antibodies for 2 h at 37°C after inoculation for 13 h with different PrV strains. Viral glycoprotein distribution was scored by fluorescence microscopy. The values represent means ± standard deviations of triplicate assays. Significant differences from the Kaplan parental strain within a column are indicated by asterisks (*, $P < 0.01$).

(a)



strain	mean fluorescence intensity	% reduction of fluorescence intensity compared to Kaplan (mean ± SD of triplicate assay)
Mock	13.0	
Kaplan	51.3	
Kaplan gE$^-$	29.4	56.3 ± 8.2
Kaplan gI$^-$	38.2	48.2 ± 13.1
Kaplan gE$^-$gI$^-$	20.7	82.4 ± 4.5

(b)

treatment	% cells with a cap	% cells with an extruded cap
2 h incubation with PrV specific antibodies (1)	43.2 ± 2.6	11.7 ± 2.2
1 h incubation with PrV negative antibodies, followed by (1)	22.4 ± 5.1*	4.6 ± 0.7*

FIG. 6. PrV gE-gI complex exhibits Fc receptor activity, which facilitates capping. (a) Flow cytometric histogram plots showing fluorescence intensity of cells mock infected (shaded plot) and infected with PrV (Ka) (solid line), PrV gE$^-$ (broken line, left-hand diagram), PrV gI$^-$ (broken line, middle diagram), and PrV gE$^-$gI$^-$ (broken line, right-hand diagram) for 13 h, washed, and incubated with 0.4 mg of PrV-negative IgG per ml for 1 h at 37°C, followed by fixation and incubation with rabbit α-swine-FITC. The accompanying table shows the reduction in fluorescence intensity of different mutant virus-infected cells toward cells inoculated with PrV (Ka). SD, standard deviation. (b) Percentage of PrV (Ka)-infected cells displaying a cap or an extruded cap at 2 h pAA, with and without preincubation of the cells with 0.4 mg of PrV-negative IgG per ml for 1 h. Significant differences from the control within a column are indicated by asterisks ($P < 0.05$).

higher fluorescence intensity (Fig. 7b and c, sections γ). The percentages of spontaneously (13%) (Fig. 7a, section γ) and inducibly (85%) (Fig. 7b, section γ) released caps were somewhat different from the visually determined percentages. Since flow cytometric quantitation was performed directly after antibody incubation and shaking, whereas cells were washed prior to visual observation, the differences might be explained by a preferential loss of released caps during washing steps.

In a number of studies, cytoskeletal association of plasma membrane proteins has been operationally defined as the inability of nonionic solvents to solubilize these proteins (25). Membrane solubilization of PrV-infected cells, incubated with FITC-labeled porcine PrV polyclonal antibodies for 2 h in the presence or absence of 40 mM NaN_3, caused a release of the extruded caps from the attached cells (Fig. 7d, section γ), indicating that the extruded caps do not possess cytoskeletal associations with the attached cell. Furthermore, membrane solubilization resulted in the disappearance of the original low-fluorescence-intensity subpopulation containing PrV-positive cells with extruded caps (Fig. 7d, section β). A possible explanation for this observation may be that cells with extruded caps contain very low levels of filamentous actin. This difference might render the cells more sensitive to disruption after membrane removal.

DISCUSSION

The data presented here show that the addition of virus-specific antibodies to PrV-infected cells in vitro results in the shedding of viral envelope glycoproteins from the plasma membranes into the surrounding medium.

The envelope of a PrV virion contains at least 10 different glycoproteins. During infection, newly synthesized viral envelope glycoproteins are transported to and incorporated into different cellular lipid bilayers, including the plasma membrane (27). These plasma membrane-bound viral glycoproteins render the infected cell recognizable to the immune system (4). Specific antibodies may bind to these glycoproteins, inducing cell- and complement-mediated lysis of the infected cell (4).

Addition of polyclonal antibodies from hyperimmune sera of pigs to PrV-infected porcine SK-6 cells in vitro condenses the plasma membrane-bound viral glycoproteins into clusters (patches). Patches then move toward one side of the cell, forming a cap. A correlation between the size of the patches and both the time after virus inoculation and the amount of antibodies added was found. Addition of antibodies at a minimum concentration of 0.2 mg of IgG/ml to cells infected for at least 9 h resulted in a maximal conversion of patched to capped cells. We assume that a minimum size of the formed patches has to be exceeded before capping may occur. This hypothesis

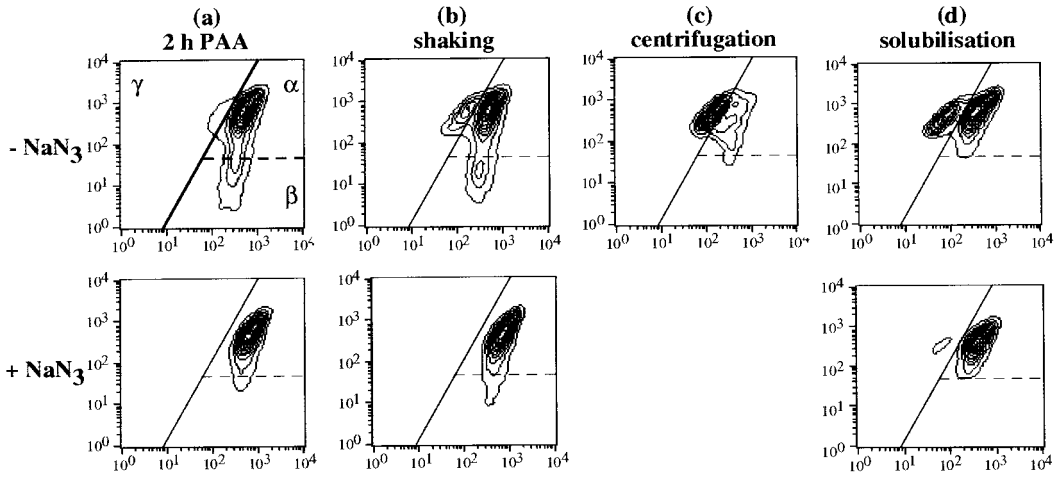


FIG. 7. Flow cytometric contour plots showing fluorescence intensity (*y* axis) versus side-scattered light intensity (*x* axis). (a) PrV-infected SK cells were incubated with FITC-labeled porcine PrV polyclonal antibodies for 2 h at 37°C in the absence (−) or presence (+) of 40 mM NaN_3 and analyzed by flow cytometry. Cells with rim, patch, and cap configurations appear in section α; cells with extruded caps appear in section β. (b) Cells represented in panel a were shaken for 1 min and analyzed. Released caps appear as a subpopulation of particles with high fluorescence and lower granularity (section γ). (c) Released caps (section γ) were concentrated as described in the text. (d) Cells represented in panel a were solubilized with Triton X-100, as described in the text, resulting in release of the extruded caps (section γ).

is comparable to a morphologically different phenomenon: the capping and internalization of antigen receptors in lymphocytes after the addition of bivalent antigens, leading to lymphocyte activation (5, 10). Since animal sera may contain up to 10 mg of IgG/ml (which contains at best 10% specific IgG) (15), the observed processes may also happen in vivo.

Polarization of the patches is energy dependent, as shown by inhibition with sodium azide, and seems to be mediated by a complicated cascade of alterations in the cellular cytoskeleton. Fluorescence labeling of α-tubulin and inhibition of tubulin polymerization by colchicine showed that microtubule fibers are of critical importance during the initial polarization of the patches and the formation of an early cap. Patches move in the opposite direction of the MTOC, with microtubule strands directed from this center toward the formed cap. The development of the early cap into a more pronounced cap is accompanied by a colocalization of F-actin filaments and is inhibited by the addition of cytochalasin B, an inhibitor of G-actin polymerization to F-actin. Although localization of myosin in cells with different viral glycoprotein distribution has not yet been performed, we hypothesize that the development of a more pronounced cap is mediated by an actin-myosin sliding mechanism, basically similar to the mechanism responsible for, e.g., the constriction of a cell during cell division, resulting in the formation of two daughter cells (1). Ongoing actin-mediated contraction of the capped viral glycoproteins then leads to the detachment of the cap from the cell, resulting in the formation of a vesicle-like extruded cap. Plasma membrane disruption experiments revealed that these extruded caps do not possess remaining cytoskeletal associations with the attached cell and can easily be removed without affecting cell viability. The remaining cells do not retain visually detectable levels of viral glycoproteins on their plasma membranes. Since the experiments were performed in the continuous presence of fluorescence-labeled antibodies, reexpression of viral glycoproteins during antibody incubation may have been blocked or specifically directed towards the cap.

In order to determine if specific viral glycoproteins might be important in triggering viral glycoprotein capping, experiments with PrV strains containing deletions in the genes coding for different envelope glycoproteins were performed. Infection with a gE- and a gE–gI-deleted mutant resulted in a significant decrease in caps and extruded caps, whereas the use of a gI-deleted mutant did not alter the percentage of capped cells but significantly reduced contraction of the caps. These data indicate an important role for the gE-gI complex during the observed processes. An attractive hypothesis could be that, in analogy with the HSV (17) and varicella-zoster virus (20) gE-gI complex, the PrV gE-gI complex exhibits Fc receptor activity. This Fc receptor activity might cause antibody bipolar bridging, as described for HSV (13). This mechanism consists of the binding of HSV-specific antibodies to viral glycoproteins with their Fab portions and to the gE-gI complex via their Fc portions. If a similar mechanism exists for PrV, it might facilitate the formation of large patches, thereby initiating capping. Zuckermann et al. (43) showed that PrV gE-gI does not exhibit Fc receptor activity for human IgG. The present study demonstrates Fc receptor activity of PrV gE-gI for swine IgG. Blocking of the Fc receptor activity by preincubation of PrV-infected cells with normal swine IgG, followed by incubation of the cells with swine anti-PrV antibodies, resulted in a significant decrease in capping. These data suggest that PrV antibodies bound to different viral glycoproteins on the plasma membrane may undergo antibody bipolar bridging with the PrV gE-gI complex, thereby improving patch formation and facilitating capping. However, given the different effect on antibody-induced capping obtained by deleting either gE or gI, and since deletion of gE causes a more severe decrease in capping than does blocking of the Fc receptor activity, it seems unlikely that antibody bipolar bridging is the only function of gE and/or gI during the antibody-induced redistribution process.

Capping and subsequent extrusion of the caps may be the result of a signal transduction response induced by the cross-linking of certain viral glycoproteins, including gE and gI and presumably facilitated by the gE-gI Fc receptor activity. Of possible importance to fully elucidate the function of gE in the observed processes may be the presence of two YXXL sequences in the amino acid sequence of the cytoplasmic tail of PrV gE (with Y standing for tyrosine, X for any amino acid, and L for leucine). Capping and internalization of receptors in mast, B, and T cells is initiated by the activation of a tyrosine-

signaling pathway by antibody-induced tyrosine phosphorylation of two highly conserved YXXL regions in the cytoplasmic tails of the receptors (21, 32, 38). Olson et al. (31) showed that rare dimeric forms of gE in varicella-zoster virus-infected cells are tyrosine phosphorylated, presumably on the single YXXL sequence in the cytoplasmic tail, while monomeric forms are not. Similarly, artificial "oligomerization" of gE by PrV-specific antibodies and antibody bipolar bridging might cause tyrosine phosphorylation of the cytoplasmic tail of gE and subsequent signal transduction, leading to capping of the viral glycoproteins.

The present findings establish an interesting model for investigating virus-cell interactions, since they suggest a possible interaction between viral glycoproteins and the cellular cytoskeleton, as well as the capability of viral glycoproteins to function in signal transduction to the infected cell.

Moreover, the findings may have significance for the pathogenicity of the virus. Although the lack of visually detectable levels of viral glycoproteins does not exclude the possible presence of enough molecules for complement activation, it may be important to note in this context that the observed processes strongly resemble the antibody-induced shedding of antigens from the surface of the parasitic protozoan *Entamoeba histolytica* (2, 7, 11). In *Entamoeba*, this mechanism is thought to play a major role in the immune evasion of the protozoan (6, 7, 12). This may be of interest, given the highly immune-evasive character of herpesviruses in general. It is known that herpesviruses have developed multiple ways to evade the immune response (for a review, see reference 3). Future research will show whether the current model reflects a new immune-evasive strategy by PrV.

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