Involvement of Cellular Cytoskeleton Components in Antibody-Induced Internalization of Viral Glycoproteins in Pseudorabies Virus-Infected Monocytes

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Addition of pseudorabies virus (PrV)-specific polyclonal immunoglobulins to PrV-infected monocytes induces internalization of plasma membrane-anchored viral glycoproteins and this may interfere with antibody-dependent cell lysis. We investigated the role of actin, microtubules, clathrin, and dynein, the major cellular components involved in physiological endocytosis during this virological internalization. Porcine monocytes were infected in vitro for 13 h and afterward treated with different concentrations of colchicine, cytochalasin D, latrunculin B, and amantadine-HCL, which inhibit polymerization of microtubules, actin/clathrin, actin, and clathrin, respectively. This resulted in a significant reduction of internalization compared to the nontreated control, indicating that these components are involved in the process. A double labeling was performed during the internalization process and a clear colocalization of actin, microtubules, clathrin, and dynein with the viral glycoproteins was observed at different stages during the internalization process. We conclude that these cellular components are used by PrV to generate the antibody-induced internalization of viral glycoproteins.

Key Words: antibodies; pseudorabies virus; endocytosis; cytoskeleton.

INTRODUCTION

Pseudorabies virus (PrV) is a member of the Alphaherpesvirinae, which causes Aujeszky’s disease in its natural host, the pig. The clinical signs of the disease depend on the age of the pig and are characterized by nervous signs in newborn pigs, respiratory disorders in fattening pigs, and reproductive failure in sows. Abortion may be an important consequence of PrV infection in susceptible pregnant sows (Pensaert and Kluge, 1989). In the presence of vaccination immunity, PrV inoculation may result in infection of the respiratory tract, involving mononuclear cells in draining lymph nodes. These cells may enter the bloodstream, resulting in a restricted viremia (Wittman et al., 1980). The restricted replication in immune animals generally does not cause problems. However, abortion may occur as a result of cell-mediated transplacental spread and intrafetal replication. It has been shown that blood monocytes are essential to transport the virus in vaccination-immune pigs (Nauwynck and Pensaert, 1992, 1995a). Little is known on the exact mechanism how these PrV-infected monocytes survive in the blood without being eliminated by antibody-dependent cell lysis or cytotoxic T-lymphocytes.

Favoreel et al. (1999) investigated the effect of PrV-specific antibody addition to PrV-infected porcine blood monocytes in vitro. This study showed that addition of antibodies resulted in an aggregation of the membrane-bound viral glycoproteins, followed by the internalization of these glycoprotein-antibody complexes. This antibody-induced internalization is a fast and efficient process and may interfere with the antibody-dependent cell lysis.

The antibody-induced internalization of viral glycoproteins resembles the well-studied bivalent ligand-induced endocytosis of cellular receptors (Favoreel et al., 2000). It is known that binding of such a ligand to its receptor induces aggregation of the receptors. Because of the resulting change in conformation, adaptor proteins (AP) can bind to certain highly conserved amino acid sequences in the cytoplasmic tail of the receptor. These AP will then bind to clathrin molecules, the driving force behind membrane invagination, with formation of clathrin-coated pits (Goldstein et al., 1985; Hirst and Robinson, 1998). The clathrin-coated pits are then pinched off into the cytoplasm as clathrin-coated vesicles. It is assumed that actin plays an important role during this stage of endocytosis (as reviewed by Qualmann et al., 2000). Clathrin-coated vesicles will then deliver the receptor-ligand complexes to early endosomes. The receptors are directed back to the plasma membrane via a recycling pathway, while the ligand proteins are sorted into a lysosomal pathway (Ghosh et al., 1994).
port of the lysosomes to the center of the cell is performed by the motor protein dynein, which mediates vesicle or organelle transport and moves along microtubules (as reviewed by Goodson et al., 1997; Hamm-Alvarez, 1998).

The main purpose of this study was to investigate whether several components of the cellular cytoskeleton (clathrin, actin, dynein, and microtubules), known to play a role in the process of bivalent ligand-induced endocytosis of cellular receptors, are being used in PrV-infected monocytes to generate the antibody-induced internalization of viral glycoproteins.

**RESULTS**

Kinetics of antibody-induced internalization of PrV-infected monocytes in adhesion vs suspension and the importance of viral glycoproteins gB and gD

In the study of Favoreel et al. (1999), monocytes in suspension were used to investigate the kinetics of the antibody-induced redistribution and internalization of viral glycoproteins expressed on PrV-infected monocytes. To obtain better confocal images, monocytes in adhesion were used in this study. Identical experiments as described by Favoreel et al. (1999) were performed first to evaluate whether monocytes in adhesion behave similar to monocytes in suspension. The evolution of redistribution of the viral glycoproteins after addition of PrV-specific antibodies did not differ between PrV-infected monocytes in adhesion and monocytes in suspension (Fig. 1A). Shortly after antibody addition, viral glycoproteins became aggregated in patches and then rapidly internalized. The only visible difference between monocytes in adhesion and monocytes in suspension was that the internalization process was more efficient when monocytes were in adhesion. After 1 h of incubation with PrV-specific IgG, the percentage of cells that showed internalized viral glycoproteins was 85% in adhesion, which is higher than the value previously obtained by Favoreel et al. (1999) with monocytes in suspension (65%).

In the study of Favoreel et al. (1999), it has been shown, by the use of PrV (Ka) and isogenic deletion mutants, that the viral glycoproteins gB and gD are very important for the viral glycoprotein internalization process in suspension. Similar experiments with PrV (Ka) and the gB and gD deletion mutant strains were performed with monocytes in adhesion. From Fig. 1B it can be concluded that viral glycoproteins gB and gD are also of significant importance in the internalization process for monocytes in adhesion ($P \leq 0.001$, one-way ANOVA). As an extra control, experiments using the PrV (Ka) deletion mutants gc, ge, gh, and gm were performed and none of these mutants had an impaired ability to induce internalization, which is also consistent with previously described results with monocytes in suspension (Favoreel et al., 1999) (data not shown). Therefore, in all further experiments adherent monocytes were used.

Influence of chemical cellular inhibitors on the antibody-induced internalization

During different steps of the well-studied process of ligand-induced endocytosis of cellular receptors, microtubules, actin, clathrin, and dynein are of critical importance (Goldstein et al., 1985; Hirst and Robinson, 1998; Merrifield et al., 1999). Experiments with different chemical inhibitors were performed to investigate whether these components of the cellular cytoskeleton are of importance in the process of internalization of viral plasma membrane glycoproteins. The concentrations of colchicine, cytochalasin D, and amantadine-HCl are similar as those used in several other studies (Bourguignon and Bourguignon, 1984; Cooper, 1987; Everitt and Rodriguez, 1999; Favoreel et al., 1997). For latrun-
lin B, an actin-disrupting agent, a concentration of 50 μM was used based on the effect of different concentrations of the agent on the monocyte actin filaments (determined via phalloidin-Texas Red staining) and on cell viability (determined via propidium iodide staining).

None of the reagents caused significant decreases in the expression of viral glycoproteins on the plasma membrane, nor did they affect patching of plasma membrane glycoproteins after the addition of antibodies, as analyzed by fluorescence microscopy (data not shown).

All the different concentrations of the chemical inhibitors, which were used in the experiments, caused no significant decrease in cell viability, as analyzed by flow cytometry after incubation with propidium iodide. As shown in Fig. 2, all the inhibitors reduced the antibody-induced internalization of viral glycoproteins in PrV-infected monocytes in a concentration-dependent manner.

Addition of 500 μM colchicine resulted in a significant reduction of internalization of 60.0 ± 4.5% (P < 0.001, one-way ANOVA) when compared to the nontreated monocytes (relative internalization level of 100%), indicating the importance of microtubules during the antibody-induced internalization of viral glycoproteins. Addition of 30 μM cytochalasin D, 50 μM latrunculin B, and 500 μM of amantadine–HCl resulted in a significant reduction of internalization of 79.0 ± 3.5, 56.0 ± 1.0, and 59.0 ± 4.4%, respectively (P < 0.001, one-way ANOVA) compared to nontreated cells. These results suggest that actin polymerization, clathrin-coated pit formation, and vesicle budding and tubulin polymerization are all involved in the process of antibody-induced internalization of viral glycoproteins.

**Visualization of the cellular component microtubules, actin, clathrin, and dynein during the different stages of antibody-induced internalization of viral glycoproteins**

To further elucidate the role of the different cellular components in the observed process, microtubules, actin, clathrin, and dynein were visualized by fluorescence labeling in PrV-infected monocytes at different time points after addition of porcine PrV-specific antibodies.

**Microtubules**

Monocytes were infected with 89V87 for 13 h and incubated with FITC-labeled PrV polyclonal antibodies. After fixation at different time points postantibody addition (p.Ab.a), mouse anti-α-tubulin was added, followed by goat anti-mouse IgG-Texas Red.

The organization of the microtubules in the cell at the different stages during antibody incubation was analyzed by confocal microscopy and scored as an “intact network” if a clear network with a clear microtubule organizing center (MTOC) was visible and as a “disorganized network” if only short microtubules and only a vague MTOC were visible. Figure 3 shows six different configurations of viral glycoproteins and microtubules, observed by confocal microscopy at different time points p.Ab.a.

At 0 min p.Ab.a (13 h postinfection), 96 ± 2.6% of the
cells had an intact network with microtubules organized as perinuclear fibers originating from the MTOC. Of the cells in rim and patch, 92 ± 2.6 and 94 ± 2.1% had a disorganized network at 2 and 5 min p.Ab.a, respectively. The disorganization of the microtubules was accompanied by a rounding of the cells. Over 99% of the cells with internalized viral glycoproteins showed an intact microtubule network and an intact cell morphology again from 10 up to 60 min p.Ab.a. At these time points (10 up to 60 min p.Ab.a), 94 ± 2.1 and 92 ± 2.4%, respectively, of the cells that remained in rim and patch still had a disorganized network. It can be concluded from these results that a depolymerization of the microtubules occurs shortly after antibody binding to the infected cells and that a reorganization of the microtubule network seems to be important for the subsequent internalization of the viral glycoproteins to take place.

Actin

No visible change in the organization of the actin filaments was observed on the different redistribution

FIG. 3. Double immunofluorescence labeling of viral glycoproteins (FITC) with microtubules (Texas Red) at different time points during antibody-induced internalization, observed by confocal microscopy. The images are middle sections of each cell. Arrow indicates the position of the nucleus. Bar, 5 μm.
stages of the viral glycoproteins during PrV-specific antibody incubation. A cortex of actin fibers, lying just beneath the plasma membrane, was observed in over 95% of the cells at all time points.

Clathrin and dynein

With a double immunofluorescence labeling, viral glycoproteins (FITC-labeled) and clathrin or dynein (Texas Red-labeled) were stained to see if and when a colocalization could be observed.

First, a positive control with 10 μg of BODIPY FL-LDL (Molecular Probes, Eugene, OR) was enclosed, because endocytosis of low-density lipoprotein (LDL) is known to be clathrin- and dynein-dependent (Kibbey et al., 1998; Ichikawa et al., 2000). A colocalization between LDL and clathrin dynein could be observed, indicating that the
Figure 4A shows the viral glycoproteins (FITC) with clathrin (Texas Red) at different time points p.Ab.a. An accumulation of clathrin was seen in the regions where the viral glycoproteins were located at 2 and 5 min p.Ab.a. A clear colocalization of vesicles which contain the viral glycoprotein-antibody complexes with clathrin was seen from 10 until 30 min p.Ab.a. From 30 min p.Ab.a, clathrin started to recycle back to the plasma membrane and the vesicles were further transported toward the nucleus. At 60 min p.Ab.a, a colocalization of the viral glycoproteins with clathrin was no longer observed.

Figure 4B shows the viral glycoproteins (FITC) with dynein (Texas Red) at different time points p.Ab.a. At 5 min p.Ab.a, dynein started to colocalize with certain internalized viral glycoproteins and during the later stages of internalization (from 10 until 60 min p.Ab.a), a clear colocalization of the cellular protein dynein with the internalized viral glycoproteins was observed.

**DISCUSSION**

PrV-infected porcine blood monocytes have been shown to be capable of transporting the virus to different internal organs in pigs with specific antibodies in the blood circulation (Wittman et al., 1980; Nauwynck and Pensaert, 1992, 1995a). Since viral glycoproteins become expressed on the plasma membrane of PrV-infected monocytes, one would expect that antibody-dependent cell lysis will eliminate these cells. However, some cells avoid such destruction. One possible explanation for the inefficient antibody-dependent lysis of PrV-infected monocytes has been described recently: the fast and efficient antibody-induced internalization of viral plasma membrane glycoproteins (Favoreel et al., 1999).

The data presented here show that this antibody-induced internalization process shows strong similarities to the bivalent ligand-induced endocytosis of cellular receptors (Goldstein et al., 1985). To generate the antibody-viral antigen internalization process, the PrV-infected monocyte mobilizes different cellular components such as clathrin, actin, dynein, and microtubules as shown by the use of different chemical inhibitors and by colocalization studies between the different cellular components and the viral glycoproteins.

The first step after the addition of PrV-specific antibodies to PrV-infected monocytes is the aggregation of the viral plasma membrane glycoproteins. This aggregation (patching) is followed by an invagination of the plasma membrane and the formation of vesicles containing the viral glycoprotein-antibody complexes. The importance of clathrin during these early steps was shown by inhibition of clathrin-coated pit invagination at the plasma membrane using amantadine-Cl and inhibition of clathrin-coated vesicle budding using cytochalasin D, which resulted in a strong decrease in internalization, as well as by immunofluorescence double staining. A clear colocalization of vesicles which contain the viral glycoprotein-antibody complexes and clathrin was seen from 5 until 30 min p.Ab.a. From that time point on, clathrin recycled back to the plasma membrane and the vesicles were further transported toward the nucleus. A very similar role for clathrin has been shown during the spontaneous endocytosis of certain herpes viral glycoproteins, such as varicella-zoster virus gE (Olson and Grose, 1997) and human cytomegalovirus virus gb (Tugizov et al., 1999). Spontaneous endocytosis of PrV gb and gE has also been described and is likely to be clathrin-dependent as well (Tirabassi and Enquist, 1998). Despite these similarities, it is, in the current context, important to clearly discriminate spontaneous gb and gE endocytosis and the antibody-induced internalization of the major viral glycoproteins. PrV gb and gE endocytosis are spontaneous processes (antibody-independent) that affect gb and gE alone and that occur during early stages of infection (<6 h postinfection) (Tirabassi and Enquist, 1998). Antibody-induced internalization of PrV glycoproteins, on the other hand, is antibody-dependent, results in internalization of all major viral glycoproteins, and can be induced from 7 up to 17 h postinfection (Favoreel et al., 1999).

Actin filaments have also been shown to be of major importance during the initial stages of the receptor-mediated endocytosis process, but a clear role for actin in endocytic processes has not been established (as reviewed by Qualmann et al., 2000). Two major hypotheses have been described. The first hypothesis proposes an active role of actin during the initial stages of the endocytosis process, by which actin modulates vesicle budding and mediates vesicle transport or fusion (Lamaze et al., 1997; Merrifield et al., 1999). The second hypothesis suggests a more passive role for actin filaments, by which actin provides the integrity of the cell, necessary for the endocytosis process to take place (Gaidarov et al., 1999). Since a significant reduction in endocytosis was observed with both the inhibitors cytochalasin D and latrunculin B, but no change in distribution of actin filaments was observed during the different stages of the internalization process by fluorescence labeling, our results tend to favor the second hypothesis.

During bivalent ligand-induced endocytosis of cellular receptors, the clathrin-coated pits are pinched off into the cell as clathrin-coated vesicles, followed by shedding of the clathrin coat and fusion of the endocytic vesicles with early endosomes. Further transport of these vesicles toward the nucleus is mediated by dynein, a microtubule motor protein that moves along microtubules (Goodson et al., 1997; Hamm-Alvarez, 1998). In the present study, involvement of dynein in the transport of
the vesicles containing the viral glycoprotein–antibody complexes was demonstrated by a clear colocalization of the internalized viral glycoproteins with dynein from 5 until 60 min after antibody treatment. To have an indication whether the microtubules function as “highways” during this transport of the vesicles toward the cell nucleus, experiments with a chemical inhibitor of microtubule polymerization (colchicine) and double immunofluorescence labeling of microtubules with the viral glycoproteins were performed. Addition of colchicine resulted in a significant decrease in internalization, which suggests the involvement of microtubules during the process of antibody-induced internalization of viral glycoproteins. A remarkable observation was made when microtubules and viral glycoproteins were analyzed by double immunofluorescence staining. At 0 min after antibody treatment, the cells had an intact network and normal cell morphology, indicating that an infection with PrV had no influence on the organization of the microtubule network. This observation is consistent with earlier experiments performed at our laboratory, which revealed that the microtubule network remains intact from 0 until 16 h after infection with 89V87 (data not shown). During the stages of rim and patch (2 and 5 min after antibody treatment, respectively), the microtubule network became disorganized, which was accompanied by a loss of cell shape. It has already been described that a disorganization of the microtubule network affects the cell shape of certain cell types, including macrophages (Rodinov et al., 1993; Rosania and Swan- son, 1996). During the subsequent stages of internalization (10, 30, and 60 min after antibody treatment), the microtubule network appeared to be intact again, accompanied by a convalescent normal cell shape and transport of the vesicles toward the nucleus. It has been shown that certain membrane proteins are prevented from patching and subsequent endocytosis because they are linked to microtubules. These integral membrane proteins are immobilized at specialized microdomains on the cell surface, which are linked to the microtubules via the spectrin–ankyrin network, a member of the membrane-associated cytoskeleton. The model can also be used to study viral infection of rim and patch that remained in these configurations during these later stages after antibody treatment. It is generally accepted that microtubules are being used by cells for efficient transport of proteins and lipids to various sites within the cell (Cole and Lippincott-Schwartz, 1995). Therefore it is reasonable to suggest that the microtubule network needs to be intact again before transport of the internalized viral glycoproteins toward the center of the cell can take place. This is consistent with the results of the present study where it was shown that the microtubules regained their structure at approximately 10 min after antibody treatment, during the later stages of the internalization process. The low percentage of cells in rim and patch that remained in these configurations during these later stages after antibody treatment still had a disorganized microtubule network, supporting the idea that an intact network is necessary for later stages in the internalization process to occur.

In conclusion, the current study provides strong indications that actin, microtubules, clathrin, and dynein, important components during physiological endocytosis, are being mobilized by PrV-infected monocytes to generate the antibody-induced internalization of viral glycoproteins, a potential immune evasion mechanism of PrV. Furthermore, the present findings establish an interesting in vitro model for (biochemical) investigations on the possible interactions between certain viral glycoproteins, expressed on the plasma membrane of a PrV-infected cell, and different components of the cellular cytoskeleton. The model can also be used to study viral and cellular endocytosis processes in general, since the antibody-induced internalization of viral glycoproteins in PrV-infected monocytes is easily reproducible and is a fast, very efficient and coordinated process.

MATERIALS AND METHODS

Isolation of blood monocytes

Pigs from a PrV-negative farm were used as blood donors. Blood was collected from the vena jugularis on heparin (15 U/ml) (Leo, Zaventem, Belgium). Blood mononuclear cells were separated on Ficoll–Paque (Pharma-
cicia Biotech AB, Uppsala, Sweden) following the manufacturer’s instructions. Mononuclear cells were then resuspended in medium A, based on RPMI 1640 (Gibco-BRL, Life Technologies Inc., Gaithersburg, MD), supplemented with 10% fetal calf serum (FCS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate, 1% nonessential amino acids 100X (Gibco-BRL), and 10 U/ml heparin. Afterward, cells were seeded on a four-well chambered coverglass (Nunc A/S, Roskilde, Denmark) at a concentration of 2.5 × 10⁶ cells/ml and cultivated at 37°C with 5% CO₂. After 48 h, nonadherent cells, mainly consisting of lymphocytes, were removed by washing the chambered coverglass three times with RPMI 1640.

Infection of the monocytes

The adherent cells, consisting of ≥70% of monocytes (as assessed by flow cytometric analysis after incubation with 74.22.15 (Pescovitz et al., 1984)), were infected with the PrV strain 89V87 (Nauwynck and Pensaert, 1992) at a m.o.i. of 20 in 0.5 ml medium A without heparin. Monocytes were incubated at 37°C with 5% CO₂ for 13 h. In some experiments, PrV strain Kaplan (Ka) and PrV (Ka) deletion mutants gB⁻, gC⁻, gD⁻, gE⁻, gH⁻, and gM⁻ were used. All strains have been described earlier (Dijkstra et al., 1996, Kaplan and Vater, 1959, Klupp et al., 1992, Mettenleiter et al., 1987, 1988; Rauh and Mettenleiter, 1991). Strains carrying deletions in the genes encoding essential glycoproteins were grown on complementing cell lines.

Incubation of PrV-infected monocytes with porcine anti-PrV polyclonal antibodies

Monocytes, 13 h after incubation, were washed three times with RPMI 1640 and incubated with FITC-conjugated PrV polyclonal antibodies (0.33 mg/ml) (as described by Favoreel et al., 1999) for 1 h at 37°C. These FITC-labeled, protein A-purified IgG antibodies were derived from a PrV (89V87)-inoculated pig, originating from a PrV-negative farm as described earlier (Nauwynck and Pensaert, 1995b). At different time points (0, 2, 5, 10, 30, and 60 min) pAb.a, cells were fixed with 0.4% formaldehyde (except where indicated otherwise). Finally, the cells were washed thoroughly, mounted in a glycerin-phosphate buffered saline solution (PBS) (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo(2.2.2)octane (DABCO; Janssen Chimica, Beerse, Belgium), excited with an Osram HBO 50-W bulb using a 13 filter, and observed with a Leica DM IL inverted fluorescence microscope (Leica, Germany).

Definition of viral glycoprotein distribution

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 viral glycoproteins were considered “internalized” when all visible viral glycoproteins were located in vesicles inside the cell, without any remaining on the plasma membrane.

Quantitative results were obtained by examining the fluorescence distribution on at least 200 cells. All assays were run independently at least three times.

Chemical inhibitors of the different cellular components

To determine the involvement of the cellular components during the observed process of antibody-induced internalization, different concentrations of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; inhibitor of tubulin polymerization; 0 μM–500 μM), cytochalasin D (Sigma-Aldrich Chemie GmbH; inhibitor of G-actin polymerization to F-actin and inhibitor of budding of clathrin-coated vesicles; 0 μM–100 μM), latrunculin B (ICN Biochemicals Inc., OH; actin-disrupting agent; 0 μM–50 μM), and amantadine–HCl (Sigma-Aldrich Chemie GmbH; inhibitor of clathrin-coated-pit invagination at the plasma membrane; 0 μM–500 μM) were added 0.5 h before and during antibody incubation. For each concentration of the chemical inhibitors, the viability of the monocytes was analyzed by flow cytometry after the addition of 10 μg propidium iodide (Molecular Probes). Only concentrations with no significant effect on cell viability were used.

Double immunofluorescence labeling of viral glycoproteins with microtubules or actin

After incubation with FITC-conjugated PrV antibodies, as described above, the cells were washed in a cytoskeleton-stabilizing buffer (CSB), containing 10 mM Pipes buffer, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, and 5 mM glucose, and afterward fixed with a 3% solution of paraformaldehyde in CSB for 10 min at 37°C. After washing in CSB, cells were permeabilized for 2 min in a 0.1% solution of Triton X-100 in CSB and then washed again in CSB.

Microtubules were stained by incubation of cells with mouse anti-α-tubulin IgG1 antibodies 1:20 in CSB (Sigma-Aldrich Chemie GmbH), for 1 h at 37°C. Afterward, cells were washed in washing buffer (WB) containing 200 mM Tris, 1.45 M NaCl, 20 mM EGTA, and 20 mM MgCl₂ and subsequently incubated with goat anti-mouse IgG-Texas Red 1:40 in CSB (Molecular Probes) at 37°C for 1 h and washed twice in WB.

To stain actin, cells were incubated with 200 nM phalloidin-Texas Red (Molecular Probes) in CSB at 37°C for 1 h and afterward washed twice in WB.
Double immunofluorescence labeling of viral glycoproteins with clathrin or dynein

After incubation with FITC-conjugated PrV antibodies, as described above, the cells were washed with Tris-buffered saline (containing 20 mM Tris–HCl and 150 mM NaCl) + 4.5% sucrose (w/v) + 2% heat-inactivated goat serum (TBS-SG), fixed in a 3% solution of paraformaldehyde and permeabilized with methanol 100% as described by Raccoosin and Swanson (1994).

Clathrin was stained by incubation of cells with mouse anti-clathrin IgM antibodies (ICN Biomedicals Inc.) 1:100 in PBS with 0.3% gelatin and dynein by incubation with mouse anti-dynein IgM antibodies (Sigma-Aldrich Chemie GmbH) 1:100 in PBS with 0.3% gelatin for 1 h at 37°C. Afterward, cells were washed in TBS-SG and incubated with rat anti-mouse IgM-biotin (Serotec Ltd., Oxford, U.K.) 1:250 in PBS with 0.3% gelatin for 1 h at 37°C. After washing, cells were incubated with streptavidin-Texas Red 1:100 in PBS with 0.3% gelatin (Molecular Probes) at 37°C for 1 h and washed twice in TBS-SG.

Confocal laser scanning microscopy

Fluorescent double stained samples were examined on a Bio-Rad Radiance 2000 confocal laser scanning system (Bio-Rad House, Hertfordshire, U.K., www.microscopy.bio-rad.com), linked to a Nikon Diaphot 300 microscope (Nikon Corporation, Tokyo, Japan) and interfaced to an AST Premmia SE 4/66d computer (AST Computer, Irvine, CA). Krypton/Argon laser light was used to excite FITC (488 nm line) and Texas Red (568 nm line) fluorochromes using 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 Co., Rochester, NY).

REFERENCES


