Available online at www.sciencedirect.com

ScienceDirect

Virology 362 (2007) 60–66

VIROLOGY

www.elsevier.com/locate/yviro

Tyrosine phosphorylation and lipid raft association of pseudorabies virus glycoprotein E during antibody-mediated capping

Ann S. Desplanques [a], Hans J. Nauwynck [a], Kelly Tilleman [b], Dieter Deforce [b], Herman W. Favoreel [a,*]

[a] *Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium*
[b] *Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium*

Received 30 August 2006; returned to author for revision 4 October 2006; accepted 12 December 2006
Available online 22 January 2007

Abstract

In specific cell types infected with the alphaherpesviruses herpes simplex virus and pseudorabies virus (PRV), addition of virus-specific antibodies results in redistribution of cell-surface-anchored viral proteins. This redistribution is triggered by the viral protein gE and consists of the directional movement of the antibody–antigen complexes to one pole of the cell. This viral capping process has been associated with increased antibody-resistant virus spread and strongly resembles immunoreceptor capping, a process that is crucial in activation of different immune cells (e.g. capping of Fcγ-receptors, B and T cell receptors). Here, we report that the PRV gE-mediated viral capping process results in increased Src kinase-mediated tyrosine phosphorylation of the cytoplasmic domain of gE and that a fraction of gE associates with lipid rafts, all very reminiscent of immunoreceptor capping. These results provide evidence that gE-mediated capping is a viral mimicry of immunoreceptor capping.

Keywords: Alphaherpesviruses; Pseudorabies virus; Capping; Glycoprotein E; Tyrosine phosphorylation; Lipid rafts; Immunoreceptor

Introduction

The alphaherpesvirus subfamily of the herpesviruses consists of closely related members, including herpes simplex virus (HSV), the porcine pseudorabies virus (PRV), varicella-zoster virus (VZV), bovine herpesvirus type 1 (BoHV1) and the equine herpesvirus type 1 (EHV1). PRV is often used as a model for this subfamily (Pomeranz et al., 2005). Four morphologically different structural elements can be distinguished in the PRV virion: the core with the linear double stranded DNA genome, an icosahedral capsid, an amorphous protein mass called the tegument and the envelope with imbedded, often glycosylated envelope proteins. These viral envelope proteins are expressed in different membranes of the infected cell (including the plasma membrane). Viral envelope proteins on the plasma membrane can be recognized and bound by virus-specific antibodies, which may trigger antibody-dependent cell lysis.

In specific cell types (SK, HEL, HEp2) infected with PRV or HSV-1, binding of virus-specific antibodies to the viral cell surface proteins results in a particular redistribution of the antibody–antigen complexes. The antibody–antigen complexes aggregate quickly and undergo polarization to one pole of the cell, a process called capping (Favoreel et al., 1997; Rizvi and Raghavan, 2003). This capping process may impede efficient elimination of the infected cell by components of the immune system that can be activated by antibodies (complement, phagocytes, NK cells) (Favoreel et al., 1997). In addition, the capping process has been reported to be associated with enhanced cell-to-cell spread of HSV (Rizvi and Raghavan, 2003).

Capping of antibody–antigen complexes on the plasma membrane of PRV-infected cells strikingly resembles immunoreceptor capping, one of the initial steps in the activation of different leukocytes, e.g., Fcγ-receptor capping in monocytes, macrophages and neutrophils; B-cell receptor capping in B lymphocytes, and T-cell receptor capping in T-lymphocytes

* Corresponding author. Fax: +32 9 264 74 95.
E-mail addresses: Ann.Desplanques@UGent.be (A.S. Desplanques), Hans.Nauwynck@UGent.be (H.J. Nauwynck), Kelly.Tilleman@UGent.be (K. Tilleman), Dieter.Deforce@UGent.be (D. Deforce), Herman.Favoreel@UGent.be (H.W. Favoreel).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.12.018

(Pizzo and Viola, 2004). Tyrosine phosphorylations by Src family tyrosine kinases and small, dynamic, sterol- and sphingolipid-enriched domains on the plasma membrane, which are called lipid rafts, are crucial signaling factors involved in capping of these different immunoreceptors. In general, upon triggering of the immunoreceptor (e.g., by IgG binding, antigen binding, MHC interaction), the immunoreceptor oligomerizes and its affinity for lipid rafts increases. The immunoreceptor is subsequently tyrosine phosphorylated by Src kinases, generally in its immunoreceptor tyrosine-based activation motif (ITAM). An ITAM motif consists of two YXXΦ sequences, where Y is a tyrosine, X can be every amino acid and Φ is a large hydrophobic residue (often leucine). Tyrosine phosphorylation of the immunoreceptor initiates a signaling cascade, eventually leading to capping of the immunoreceptor-containing lipid rafts and activation of the leukocyte (Kwiatkowska et al., 2003; Pierce, 2002).

Capping of antibody–antigen complexes in PRV and HSV-infected cells shows some striking similarities to this process. Interestingly, capping in both PRV and HSV-infected cells is mediated by the viral glycoprotein gE (Favoreel et al., 1997; Rizvi and Raghavan, 2003), a protein that shows IgG binding Fc-receptor activity in different alphaherpesviruses (Favoreel et al., 1997; Johnson et al., 1988; Litwin et al., 1992). The cytoplasmic domain of gE contains ITAM-like YXXΦ sequences, and mutation of these sequences has been shown to strongly reduce capping (Favoreel et al., 1999). In addition, inhibition of tyrosine phosphorylation and disruption of lipid rafts impairs the viral capping process (Favoreel et al., 1999, 2004). This led us to hypothesize that gE-mediated capping of antibody–antigen complexes may constitute a viral mimicry of immunoreceptor capping. To further dissect this hypothesis, in the current study, we investigated whether gE becomes tyrosine phosphorylated in its cytoplasmic domain/YXXΦ motifs during capping, whether Src kinases are involved, and whether gE associates with lipid rafts during capping.

Results

Tyrosine phosphorylation of PRV gE

As a first step in characterizing the parallel between viral capping and immunoreceptor capping, we analyzed whether gE is tyrosine phosphorylated in PRV infected cells. Western blot analysis of SK cells infected with wild type PRV (Be wt) revealed a tyrosine phosphorylation band that corresponds with the size of gE (Fig. 1). Intensity of this band increased significantly (2.74±0.54 fold), based on three independent experiments, upon induction of antibody-mediated capping and the band was not present in cells infected with isogenic gE null PRV (Fig. 1), indicating that it represents gE. Immunoprecipitation of gE from cells infected with Be wt confirmed that this tyrosine phosphorylation band represents gE (Fig. 2) and the strong tyrosine phosphorylation band below the gE band in Fig. 1 was identified by electrospray tandem mass spectrometry as the UL47 tegument protein (Desplanques et al., unpublished data). Thus, gE is tyrosine phosphorylated in PRV infected cells and tyrosine phosphorylation increases upon induction of capping.

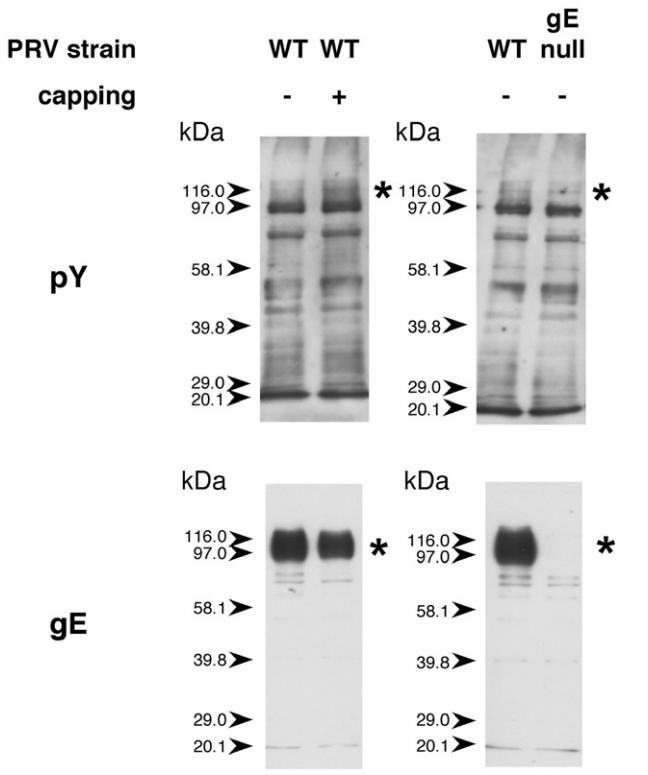

Fig. 1. Tyrosine phosphorylation of PRV gE. Tyrosine phosphorylation status (pY) of PRV-infected SK cell lysate (WT, no capping induction; WT, capping induction; gE null virus, no capping induction) was assessed on Western blot. gE expression level was analyzed as a control. *Size of gE.

Tyrosine phosphorylation of the cytoplasmic domain of PRV gE

To determine whether tyrosine phosphorylation of gE occurs in the cytoplasmic domain/YXXΦ motifs, gE was immunoprecipitated from cells inoculated with either Be wt, an isogenic virus strain lacking the cytoplasmic domain of gE (Be 107) or a virus strain with mutations in the two YXXΦ motifs in the cytoplasmic domain of gE (Be 105: Y478S-Y517S). The gE tyrosine phosphorylation band was completely absent in cells infected with the virus lacking the cytoplasmic domain of gE, while it was strongly reduced in intensity in cells infected with the virus containing point mutations in the YXXΦ motifs in gE (Fig. 2). To exclude the possibility that the slight remaining gE phosphorylation signal in cells infected with Be 105 could be due to recognition of threonine or serine phosphorylation by the phosphotyrosine-specific antibody, specificity of the phosphotyrosine specific monoclonal antibody was tested. The antibody did not show any reactivity with phosphothreonine-BSA or phosphoserine-BSA, while it reacted strongly with an identical amount of phosphotyrosine-BSA (data not shown). This indicates that the gE phosphorylation signal in cells inoculated with Be 105 is not due to an aspecific binding of the antibody, but probably to phosphorylation of additional tyrosine residues in the cytoplasmic domain of gE.

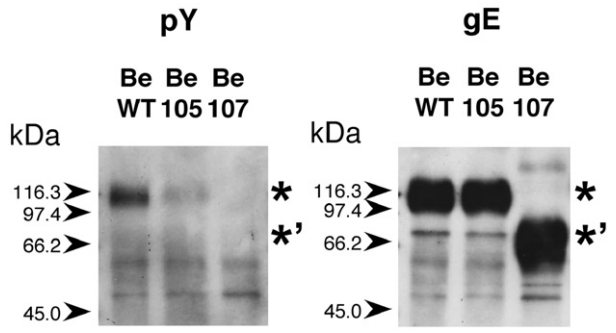

Fig. 2. Tyrosine phosphorylation of PRV gE occurs in the cytoplasmic domain, at least partly in the YXXΦ motifs. gE tyrosine phosphorylation (pY) was assessed on Western blot after gE immunoprecipitation out of cell lysate of SK cells infected with PRV Be WT, PRV Be 105 (Y478S-Y517S) or PRV Be 107 (gE cytoplasmic domain minus). gE expression level was analyzed as a control. *Size of gE, *′Reduced size of truncated gE (without cytoplasmic domain).

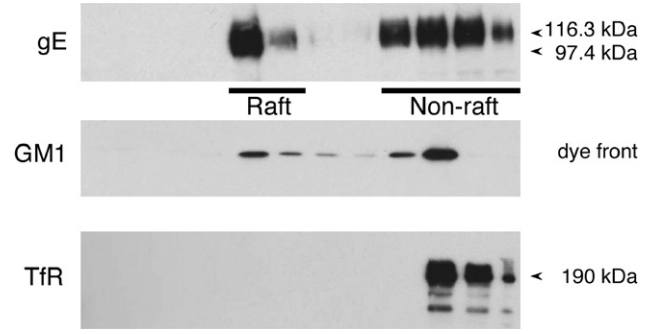

Fig. 4. A fraction of PRV gE associates with lipid rafts. Lipid rafts were isolated from PRV infected SK cells by density ultracentrifugation and fractions were analyzed on Western blot with an anti-gE antibody, with cholera toxin subunit B hrp conjugate (detection of the raft marker GM1) or an anti-TfR (detection of the non-raft marker TfR) antibody.

Together, these data indicate that tyrosine phosphorylation of gE occurs in the cytoplasmic domain, at least partly on the YXXΦ motifs.

Src kinases play a role in tyrosine phosphorylation of PRV gE

Since Src kinases are pivotal kinases in many signaling cascades, and since they are responsible for immunoreceptor tyrosine phosphorylation during immunoreceptor capping and activation, we assessed whether they are involved in gE tyrosine phosphorylation.

Capping was induced in the presence of increasing concentrations of the Src kinase inhibitor PP2 or its negative control PP3. Treatment of the infected cells with increasing concentrations of PP2 decreased gE tyrosine phosphorylation in a dose dependent manner, whereas PP3 treatment did not reduce tyrosine phosphorylation of gE (Fig. 3). This shows that Src kinases are critically involved in tyrosine phosphorylation of gE.

Lipid raft association of PRV gE

Lipid rafts play a very important role in immunoreceptor capping: they form zones of confinement where immunoreceptors become tyrosine phosphorylated and further signaling is induced. Moreover, capping of immunoreceptors and signaling molecules towards one pole of the cell occurs by the coalescence of the lipid rafts containing these molecules (Pierce, 2002). We therefore assessed whether gE associates with lipid rafts and if so, how this lipid raft association evolves during the capping process.

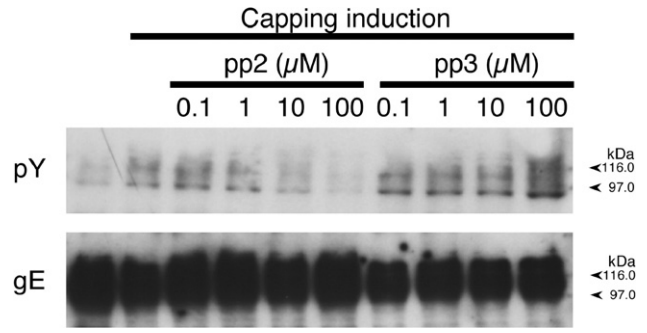

Fig. 3. Src kinases play a role in tyrosine phosphorylation of gE. gE tyrosine phosphorylation status (pY) was analyzed after treatment with the Src kinase inhibitor PP2 or its negative control PP3. gE expression level was analyzed as a control.

Purification of lipid rafts by density ultracentrifugation revealed that PRV gE shows affinity for lipid rafts: a significant fraction of the gE protein floats to the lighter fractions of the gradient (Fig. 4). Western blot analysis of the light and heavy fractions of the gradient confirmed efficient separation of raft fractions from non-raft fractions, as a significant portion of the lipid raft marker GM1 floated to the lighter fractions and nearly all of the non-lipid raft marker TfR remained at the bottom of the gradient (Fig. 4).

Although giving a good indication whether a protein has affinity for lipid rafts or not, purification of lipid rafts by density ultracentrifugation cannot be used quantitatively to determine the fraction of individual proteins that is present in rafts (Shogomori and Brown, 2003). Therefore, to determine whether or not lipid raft association of gE increases during the capping process, an alternative fluorescence-based assay was used (Stuermer et al., 2001), based on colocalization between gE and the raft marker GM1 during capping. Confocal analysis of gE and GM1 localization throughout the capping process revealed that gE colocalizes with the raft marker GM1, especially during capping (Fig. 5). A quantitative colocalization analysis using the CoLocalizer Pro software confirmed this: the colocalization coefficient m1 was found to increase substantially during the capping process. This indicates that a fraction of gE associates with lipid rafts and that lipid raft association of gE increases during antibody-mediated oligomerization and capping.

Discussion

Our results show that tyrosine phosphorylation of gE increases during the viral capping process, that tyrosine phosphorylation occurs in the cytoplasmic domain, at least partly on the YXXΦ motifs, that Src kinases play a crucial role

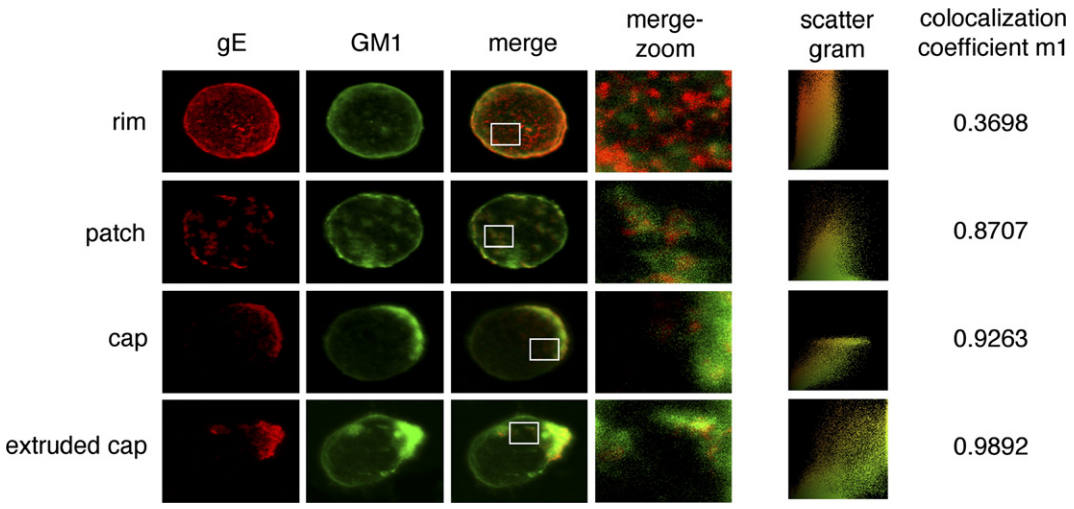

Fig. 5. gE (Texas red staining) and GM1 (FITC staining) localization were assessed throughout the capping process (rim, patch, cap, extruded cap) by confocal analysis and colocalization analysis. Colocalized pixels are located along the diagonal of the scatter gram and the colocalization coefficient m1 (value between 0 and 1) indicates the fraction of red pixels that colocalize with green pixels, in other words, the fraction of gE that colocalizes with GM1 and thus with rafts.

in this tyrosine phosphorylation, that a fraction of gE associates with lipid rafts and that lipid raft association of gE increases during capping. These findings strongly suggest that alphaherpesvirus gE-mediated capping constitutes a viral mimicry of immunoreceptor capping.

In addition, our findings shed a new light on a well-known property of gE of different alphaherpesviruses (HSV, PRV and VZV): its Fc receptor activity (Favoreel et al., 1997; Johnson et al., 1988; Litwin et al., 1992). Fc receptor activity of alphaherpesvirus gE has been shown to result in binding of the Fc domain of IgG antibodies and has been shown to protect virus and virus-infected cells from antibody-mediated elimination (Lubinski et al., 2002; Van de Walle et al., 2003). For different Fc receptors of host organisms, such as Fcγ receptor II (FcγRII) and the Fcα receptor, both primarily present on monocytes, macrophages and neutrophils, IgG and IgA binding, respectively, have been shown to lead to capping of the Fc receptor and activation of the immune cell (Kwiatkowska et al., 2003; Lang et al., 1999). During this capping process, the Fc receptor is tyrosine phosphorylated on ITAM motifs in its cytoplasmic domain, a process that relies on lipid rafts, all very reminiscent to our current observations for gE-mediated capping. In addition, the capping of FcγRII relies on a rearrangement of the actin cytoskeleton, similar to what has been described before for gE-mediated capping (Favoreel et al., 1997).

Crosslinking and capping of FcγRII lead to activation of the leukocytes, and culminate in initiation of effector mechanisms, including generation of superoxide and oxidative burst, chemotaxis, phagocytosis, degranulation of monocytes and neutrophils (Katsumata et al., 2001; Lang et al., 1999; Scott-Zaki et al., 2000). Seen the striking mechanistic similarity between natural Fc receptor-mediated capping and capping initiated by the alphaherpesvirus Fc receptor gE that we describe here, it will be interesting to determine whether the gE-mediated viral capping process may initiate similar effector mechanisms as natural Fc receptors, which perhaps may allow the virus to influence, interfere with or maybe even destabilize the immune response.

We found that PRV gE is tyrosine phosphorylated in infected SK cells. Tyrosine phosphorylation of BoHV1 and VZV gE has been reported before (Olson et al., 1997; Shaw et al., 2000). Interestingly, tyrosine phosphorylation of VZV gE was only found in gE dimers (Olson et al., 1997). This is in line with our current findings, which indicate that 'artificial' oligomerization of PRV gE (by crosslinking via the addition of antibodies) leads to increased tyrosine phosphorylation of gE. It will be interesting to determine whether, similar to our current observations, Src kinases may be involved in tyrosine phosphorylation of BoHV1 and VZV gE. Not much is known on the role of Src kinases in the herpesvirus life cycle. Recently, the general state and role of Src family kinases in replication of HSV1 was investigated (Liang and Roizman, 2006). It was concluded that Src kinases play significant, both positive and negative, roles in HSV1 replication and that HSV1 proteins (e.g., ICP0, US3 and UL13) regulate the activities of these kinases to achieve optimal viral yields in the course of viral replication (Liang and Roizman, 2006). Our finding that Src kinases play a role in tyrosine phosphorylation of PRV gE during the capping process reveals an additional role of these kinases during alphaherpesvirus infection.

Our finding that a fraction of PRV gE fractionates in lipid rafts is also in agreement with recent reports that mention lipid raft association of a fraction of gE of PRV (Favoreel et al., 2004; Riteau et al., 2006).

We found that both gE association with lipid rafts and gE tyrosine phosphorylation increased during capping. In addition, we found that Src tyrosine kinases are critically involved in gE tyrosine phosphorylation, and it has been reported earlier that Src kinases Lck and Fyn exhibit optimal activity in raft microdomains but encounter inhibitory conditions in non-raft surrounding membrane areas (Ilangumaran et al., 1999). Combining these data makes it tempting to speculate that gE tyrosine phosphorylation predominantly occurs in lipid rafts. Further experiments are planned to investigate this possibility.

The fraction of gE that we found, after density ultracentrifugation, to localize to lipid rafts may perhaps be an underestimation of the actual fraction of gE that localizes to rafts since a significant fraction of the lipid raft marker GM1 localized to the non-raft region (Fig. 4). It is possible that, due to the rather stringent detergent lysis conditions, some raft components may be removed from the rafts during the purification protocol. Alternatively, a fraction of GM1 and possibly PRV gE may be associated with the so-called high-density detergent-resistant membranes (DRM-H), which have also recently been described during raft-purification in Newcastle disease virus-infected cells (Laliberte et al., 2006). The DRM-H fraction is a class of detergent-resistant membranes with higher buoyant densities than those of classical DRM, such as classical lipid rafts. The higher density is due to their association with the underlying membrane cytoskeleton.

Fractions of different other viral structural components (envelope and tegument proteins) have also been reported to associate with lipid rafts in alphaherpesvirus-infected cells, such as PRV gB, HSV UL56, HSV vhs and HSV gH (Favoreel et al., 2004; Koshizuka et al., 2002; Lee et al., 2003). Based on the three-dimensional structure of isolated virions determined by cryo-electron tomography, Grunewald and colleagues (2003) proposed that lipid rafts in trans-Golgi network vesicles may function as concentrating platforms for tegument proteins and envelope proteins, thereby promoting successful assembly of alphaherpesvirus particles.

In conclusion, we found that gE-mediated capping of antibody–antigen complexes shows remarkable mechanistic similarity to immunoreceptor capping, consisting of Src-mediated tyrosine phosphorylation of gE and lipid raft association of gE.

Materials and methods

Antibodies and reagents

Mouse monoclonal antibody against gE (18E8) was described earlier (Nauwynck and Pensaert, 1995) and was used at a dilution 1:100. Polyclonal rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark) were used at 1:50. Mouse anti-transferrin receptor (TfR) was purchased from Zymed Laboratories, Inc. (San Francisco, CA) and diluted 1:300. Texas red-labeled goat anti-mouse antibodies, fluorescein isothiocyanate (FITC)-labeled streptavidin (both used at a dilution 1:50) and cholera toxin subunit B (CT-B) hrp conjugate (dilution 1:200) were purchased from Molecular Probes (Eugene, OR). Complete protease inhibitor cocktail was purchased from Roche Diagnostics Gmbh, Mannheim, Germany. Biotinylated cholera toxin B subunit (diluted 1:100), Optiprep density gradient medium, Triton X-100, sodium orthovanadate, sodium fluoride, phosphothreonine-BSA, phosphotyrosine-BSA and phosphoserine-BSA were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). PP2 was purchased from Biomol (Plymouth meeting, Pa), PP3 from Calbiochem (San Diego, CA). The phospho-tyrosine mouse monoclonal antibody (P-Tyr-100, Cell Signaling Technology, Danvers, MA) was used at 1:200. Biotinylated anti-mouse Ig (dilution 1:300), streptavidin–biotin hrp complex (dilution 1:300), ECL Western blotting detection kit and rProtein A Sepharose Fast Flow beads were all purchased from Amersham Biosciences (Uppsala, Sweden).

Virus mutants

PRV strains Becker WT (Be WT) (Becker, 1967), a gE null mutant (Whealy et al., 1993), a gE cytoplasmic domain minus mutant (Be 107) and a strain with mutations in the YXXΦ motifs of the cytoplasmic domain (Be 105: Y478S-Y517S) (Tirabassi and Enquist, 1999) were used. Src kinase inhibitor experiments were performed with the PRV-UL47 minus mutant (Kopp et al., 2002).

Induction of capping

Capping was induced as described previously (Favoreel et al., 1997), with some modifications in the antibodies that were used. Briefly, SK cells were inoculated with PRV at a multiplicity of infection (m.o.i.) 10 and 13 h post-inoculation (p.i.), gE-specific mouse monoclonal antibodies (18E8) were added to induce capping. Cells were incubated at 37 °C for 45 min, followed by a second antibody incubation with polyclonal rabbit anti-mouse.

Tyrosine phosphorylation analysis

At 13 h p.i., capping was either or not induced in PRV infected SK cells. Cells were lysed for 1 h at 37 °C in TNE buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 6.8), containing 1% Triton X-100, complete protease inhibitor cocktail, 10 mM sodium fluoride and 1 mM sodium orthovanadate. Nuclei were removed by centrifugation and samples were dissolved 1:2 in a 2× concentrated reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. 15 µl of each fraction was subjected to SDS–PAGE (BioRad Mini Protean 3) and Western blot (BioRad Mini Trans Blot). Blots were incubated with the P-Tyr-100 antibody in Tris-buffered solution with 0.1% Tween-20 (TBS/Tween) and 5% BSA overnight at 4°, followed by biotinylated anti-mouse Ig and streptavidin–biotin hrp complex. gE expression was analyzed by incubation of the blot with the mouse monoclonal 18E8 antibody in PBS, followed by the same secondary and tertiary conjugate as for the phosphorylation analysis. Blots were developed with the ECL Western blotting detection kit. Quantity One Analysis Software (Bio-Rad) was used to determine the average increase in gE tyrosine phosphorylation after capping induction on blots from 3 independent experiments.

Immunoprecipitation of gE

After induction of capping with the 18E8 gE antibody and polyclonal rabbit anti-mouse, gE was immunoprecipitated out

of the lysate by incubating the lysate overnight at 4 °C with prewashed rProtein A Sepharose Fast Flow beads. After three PBS washings, immunoprecipitated gE was released from the beads by boiling in 2× concentrated reducing SDS–PAGE loading buffer and gE tyrosine phosphorylation was analyzed on Western blot as described above.

Specificity testing of P-Tyr-100 antibody

0.1 μg of phosphotyrosine-BSA, phosphothreonine-BSA or phosphoserine-BSA were loaded on an SDS–PAGE gel and blotted. The blot was incubated with the P-Tyr-100 antibody, biotinylated anti-mouse Ig and streptavidin–biotin hrp, followed by ECL development.

Src kinase inhibitor treatment

At 13 h p.i., PRV-inoculated cells were incubated 30 min at 37 °C with 0, 0.1, 1, 10 or 100 μM of the Src kinase inhibitor PP2 or its negative control PP3. Afterwards, capping was induced as described above in the presence of the same concentration of the inhibitors and tyrosine phosphorylation analysis was performed as described above.

Purification of lipid rafts by density ultracentrifugation

Lipid rafts were purified as described previously (Favoreel et al., 2004), with some small modifications. Briefly, cells were lysed at 4 °C in TNE buffer (25 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 6.7) containing 1% Triton X-100 and complete protease inhibitor cocktail for 30 min. After homogenization with a 25-G syringe, the lysate was mixed with ice-cold iodixanol (Optiprep) up to 40% iodixanol and overlayed with 5 mL 30% iodixanol (ice-cold) and 3 mL 5% iodixanol (ice-cold). Gradients were subjected to ultracentrifugation at 200000 g, 4 °C for 20 h in the SW41Ti rotor of a Beckman ultracentrifuge (Beckman, Munich, Germany). Ten to twelve fractions were collected from top to bottom, diluted 1:2 in 2× concentrated non-reducing SDS–PAGE loading buffer and subjected to SDS–PAGE and Western blot. Blots were incubated with the appropriate antibodies or conjugates for detection of gE (18E8), TfR and GM1 (CT-B hrp) and developed with ECL.

Fluorescence staining for assessing lipid raft association

For fluorescence experiments, capping was induced only with the 18E8 anti-gE monoclonal antibody. Thereafter, cells were fixed with 3% paraformaldehyde at room temperature and washed with PBS. After a 1-h incubation with biotinylated cholera toxin B subunit, cells were washed with PBS again and stained subsequently with streptavidin–FITC and goat anti-mouse Texas red. After washing in PBS, cells were 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) and analyzed by confocal microscopy.

Confocal microscopy

Cells were analyzed by using a TCS SP2 laser scanning spectrum confocal system (Leica Microsystems, GmbH, Heidelberg, Germany), using an Argon 488 nm laserline and a Gre/Ne 543 nm laser line to excite FITC and Texas red, respectively. To avoid signal overlap, FITC and Texas red images were taken separately. Images were merged using Leica confocal and Confocal Assistant software (Todd Clark Brelje). Colocalization was quantified by the CoLocalizer Pro software (CoLocalization Research Software).

Acknowledgments

Ann Desplanques is supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). We thank Lynn Enquist, Rebecca Tirabassi and Thomas Mettenleiter for the kind gift of virus mutants and Chantal Vanmaercke for excellent technical assistance.

References

Becker, C.H., 1967. On primary damage of autonomic ganglia following infection with Herpes suis virus in different animal species. Experientia 23, 209–210.

Favoreel, H.W., Nauwynck, H.J., Van Oostveldt, P., Mettenleiter, T.C., Pensaert, M.B., 1997. Antibody-induced and cytoskeleton-mediated redistribution and shedding of viral glycoproteins, expressed on pseudorabies virus-infected cells. J. Virol. 71, 8254–8261.

Favoreel, H.W., Nauwynck, H.J., Pensaert, M.B., 1999. Role of the cytoplasmic tail of gE in antibody-induced redistribution of viral glycoproteins expressed on pseudorabies-virus-infected cells. Virology 259, 141–147.

Favoreel, H.W., Mettenleiter, T.C., Nauwynck, H.J., 2004. Copatching and lipid raft association of different viral glycoproteins expressed on the surfaces of pseudorabies virus-infected cells. J. Virol. 78, 5279–5287.

Grunewald, K., Desai, P., Winkler, D.C., Heymann, J.B., Belnap, D.M., Baumeister, W., Steven, A.C., 2003. Three-dimensional structure of herpes simplex virus from cryo-electron tomography. Science 302, 1396–1398.

Ilangumaran, S., Arni, S., van Echten-Deckert, G., Borisch, B., Hoessli, D.C., 1999. Microdomain-dependent regulation of Lck and Fyn protein-tyrosine kinases in T lymphocyte plasma membranes. Mol. Biol. Cell 10, 891–905.

Johnson, D.C., Frame, M.C., Ligas, M.W., Cross, A.M., Stow, N.D., 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. 62, 1347–1354.

Katsumata, O., Hara-Yokoyama, M., Sautes-Fridman, C., Nagatsuka, Y., Katada, T., Hirabayashi, Y., Shimizu, K., Fujita-Yoshigaki, J., Sugiya, H., Furuyama, S., 2001. Association of FcgammaRII with low-density detergent-resistant membranes is important for cross-linking-dependent initiation of the tyrosine phosphorylation pathway and superoxide generation. J. Immunol. 167, 5814–5823.

Kopp, M., Klupp, B.G., Granzow, H., Fuchs, W., Mettenleiter, T.C., 2002. Identification and characterization of the pseudorabies virus tegument proteins UL46 and UL47: role for UL47 in virion morphogenesis in the cytoplasm. J. Virol. 76, 8820–8833.

Koshizuka, T., Goshima, F., Takakuwa, H., Nozawa, N., Daikoku, T., Koiwai, O., Nishiyama, Y., 2002. Identification and characterization of the UL56 gene product of herpes simplex virus type 2. J. Virol. 76, 6718–6728.

Kwiatkowska, K., Frey, J., Sobota, A., 2003. Phosphorylation of FcgammaRIIA is required for the receptor-induced actin rearrangement and capping: the role of membrane rafts. J. Cell Sci. 116, 537–550.

Laliberte, J.P., McGinnes, L.W., Peeples, M.E., Morrison, T.G., 2006. Integrity of membrane lipid rafts is necessary for the ordered assembly and release of infectious newcastle disease virus particles. J. Virol. 80, 10652–10662.

Lang, M.L., Shen, L., Wade, W.F., 1999. Gamma-chain dependent recruitment of tyrosine kinases to membrane rafts by the human IgA receptor Fc alpha R. J. Immunol. 163, 5391–5398.

Lee, G.E., Church, G.A., Wilson, D.W., 2003. A subpopulation of tegument protein vhs localizes to detergent-insoluble lipid rafts in herpes simplex virus-infected cells. J. Virol. 77, 2038–2045.

Liang, Y., Roizman, B., 2006. State and role of SRC family kinases in replication of herpes simplex virus 1. J. Virol. 80, 3349–3359.

Litwin, V., Jackson, W., Grose, C., 1992. Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. J. Virol. 66, 3643–3651.

Lubinski, J.M., Jiang, M., Hook, L., Chang, Y., Sarver, C., Mastellos, D., Lambris, J.D., Cohen, G.H., Eisenberg, R.J., Friedman, H.M., 2002. Herpes simplex virus type 1 evades the effects of antibody and complement in vivo. J. Virol. 76, 9232–9241.

Nauwynck, H.J., Pensaert, M.B., 1995. Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. Arch. Virol. 140, 1137–1146.

Olson, J.K., Bishop, G.A., Grose, C., 1997. Varicella-zoster virus Fc receptor gE glycoprotein: serine/threonine and tyrosine phosphorylation of monomeric and dimeric forms. J. Virol. 71, 110–119.

Pierce, S.K., 2002. Lipid rafts and B-cell activation. Nat. Rev., Immunol. 2, 96–105.

Pizzo, P., Viola, A., 2004. Lipid rafts in lymphocyte activation. Microbes Infect. 6, 686–692.

Pomeranz, L.E., Reynolds, A.E., Hengartner, C.J., 2005. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiol. Mol. Biol. Rev. 69, 462–500.

Riteau, B., de Vaureix, C., Lefevre, F., 2006. Trypsin increases pseudorabies virus production through activation of the ERK signalling pathway. J. Gen. Virol. 87, 1109–1112.

Rizvi, S.M., Raghavan, M., 2003. Responses of herpes simplex virus type 1-infected cells to the presence of extracellular antibodies: gE-dependent glycoprotein capping and enhancement in cell-to-cell spread. J. Virol. 77, 701–708.

Scott-Zaki, P., Purkall, D., Ruddy, S., 2000. Neutrophil chemotaxis and superoxide production are induced by cross-linking FcgammaRII receptors. Cell. Immunol. 201, 89–93.

Shaw, A.M., Braun, L., Frew, T., Hurley, D.J., Rowland, R.R., Chase, C.C., 2000. A role for bovine herpesvirus 1 (BHV-1) glycoprotein E (gE) tyrosine phosphorylation in replication of BHV-1 wild-type virus but not BHV-1 gE deletion mutant virus. Virology 268, 159–166.

Shogomori, H., Brown, D.A., 2003. Use of detergents to study membrane rafts: the good, the bad, and the ugly. Biol. Chem. 384, 1259–1263.

Stuermer, C.A., Lang, D.M., Kirsch, F., Wiechers, M., Deininger, S.O., Plattner, H., 2001. Glycosylphosphatidyl inositol-anchored proteins and Fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. Mol. Biol. Cell 12, 3031–3045.

Tirabassi, R.S., Enquist, L.W., 1999. Mutation of the YXXL endocytosis motif in the cytoplasmic tail of pseudorabies virus gE. J. Virol. 73, 2717–2728.

Van de Walle, G.R., Favoreel, H.W., Nauwynck, H.J., Pensaert, M.B., 2003. Antibody-induced internalization of viral glycoproteins and gE–gI Fc receptor activity protect pseudorabies virus-infected monocytes from efficient complement-mediated lysis. J. Gen. Virol. 84, 939–948.

Whealy, M.E., Card, J.P., Robbins, A.K., Dubin, J.R., Rziha, H.J., Enquist, L.W., 1993. Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J. Virol. 67, 3786–3797.