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Short communication

A replication defect of pseudorabies virus induced by targeted α-helix distortion in the syntaxin-like bundle of glycoprotein H (V275P) is corrected by an adjacent compensatory mutation (V271A)

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Glycoprotein gH is essential for herpesvirus-induced membrane fusion during entry and cell-to-cell spread. Structural analyses of gH homologues revealed a conserved syntaxin-like bundle motif composed of three α-helices. Previous studies showed that targeted disruption of any of these helices strongly impaired maturation, cell surface expression and fusion activity of pseudorabies virus gH, as well as formation and spread of infectious virus. After passaging of one corresponding mutant (pPrV-gH-V275P) these replication defects were widely corrected by an adjacent spontaneous amino acid substitution (V271A). Although the doubly mutated gH was still non-functional in fusion assays, its targeted reinsertion into the cloned virus genome (pPrV-gH-V275P-V271A) led to a 200-fold increase in plaque sizes and 10 000-fold higher virus titres, compared with pPrV-gH-V275P. Thus, our results demonstrate that structural requirements for gH function in *in vitro* assays and virus replication are different, and that minor amounts of mature gH in virions are sufficient for productive replication.

Enveloped viruses utilize membrane fusion during entry into host cells, egress, cell-to-cell spread and induction of syncytia. The core fusion machinery required for entry and spread of viruses of the family *Herpesviridae* consists of three conserved viral envelope glycoproteins: gB and the heterodimeric gH/gL complex (Eisenberg et al., 2012).

Structural analyses of the gH/gL complexes from herpes simplex virus type 2 (HSV-2) and Epstein–Barr virus (EBV), and of a gH core fragment from the porcine alphaherpesvirus pseudorabies virus (PrV), revealed four structurally conserved domains (Fig. 1a) (Backovic *et al.*, 2010; Chowdary *et al.*, 2010; Matsuura *et al.*, 2010). Based on these results we performed targeted mutagenesis to analyse the function of domain IV (Fuchs *et al.*, 2012), as well as of domains II and III of PrV gH (Böhm *et al.*, 2015). Domain II contains a conserved bundle of three α-helices (Fig. 1a) which resembles functionally relevant elements in eukaryotic fusion proteins of the syntaxin family (Ungermann & Langosch, 2005), and has been designated syntaxin-like bundle (SLB) (Backovic *et al.*, 2010). In previous studies we interrupted any of the three helices in PrV gH by insertion of proline residues, which led to an almost complete loss of function in transient *in vitro* fusion assays, productive replication and cell-to-cell spread (Böhm *et al.*, 2015). Nevertheless, PrV recombinants obtained by mutagenesis of a bacterial artificial chromosome in *Escherichia coli* still exhibited residual replication competence which permitted limited propagation in non-complementing rabbit kidney (RK13) cells, and after several passages WT-like growth was restored due to spontaneous compensatory mutations leading to substitution of the introduced prolines by the original or similar amino acid residues (results not shown).

The hitherto only exception was observed after passage of pPrV-gH-V275P (Böhm *et al.*, 2015), which contains proline instead of valine at amino acid position 275 within the second α-helix of the SLB (α3) (Fig. 1). Whereas the original virus recombinant produced only foci of few infected RK13 cells, increasing numbers of larger plaques became visible from the third passage of pPrV-gH-V275P. The gH genes of two single plaque isolates were amplified by PCR and sequenced, revealing the continued presence of the original mutation, but a single additional mutation substituting valine to alanine at amino acid position 271 (Fig. 1). To verify whether this second site mutation was responsible for restoration of function, the two mutations were inserted into the eukaryotic gH expression plasmid pcDNA-PgHKDE (Böhm *et al.*, 2015).

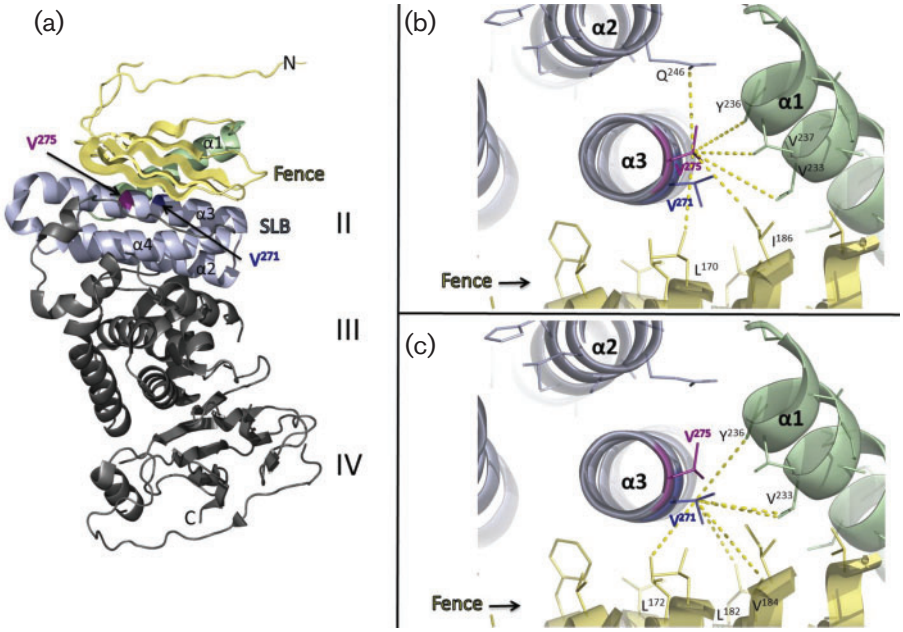

Fig. 1. Location of the analysed mutations in PrV gH. (a) The crystal structure of the gH core fragment (residues 107–639, PDB code 2XQY) (Backovic *et al.*, 2010) with the locations of N and C termini. Domains II to IV, 'fence' (yellow), SLB (pale blue), helix α1 (green), and residues V275 (magenta) and V271 (dark blue) are indicated. In detailed views, the Cα atoms of V275 (b) and V271 (c) in helix α3 are connected by dotted yellow lines with proximate (distance <7 Å) hydrophobic side chains of residues within the fence and helices α1 or α2, to illustrate the network of van der Waals interactions established to allow hydrophobic packing.

Our previous studies revealed that the mutation V275P, like proline insertions in the other SLB-helices, abrogates maturation of PrV gH in plasmid-transfected transiently gH-expressing RK13 cells, and that its transport to the cell surface is strongly inhibited (Böhm *et al.*, 2015). These defects of gH-V275P were not detectably corrected by the additional mutation V271A, as shown by Western blot analyses of plasmid-transfected cell lysates (Fig. 2a) and comparative indirect immunofluorescence (IIF) tests of permeabilized and non-permeabilized cells (Fig. 2b). Although in PrV gL is not required for transport of gH (Klupp *et al.*, 1997), cotransfection of cells with gH and gL expression plasmids enhanced the proportion of the mature, fully glycosylated, approximately 95 kDa form of WT gH (Klupp *et al.*, 1992) compared to cells transfected with the gH plasmid alone (Fig. 2a). In contrast, only smaller immature gH was detected in cells transfected with plasmids encoding gH-V275P or gH-V275P-V271A irrespective of the presence or absence of gL (Fig. 2a). This might indicate that the mutations in gH domain II inhibit gL binding to domain I, or that misfolding prevents gH maturation. Processing of integral membrane proteins usually parallels transport from the endoplasmic reticulum to the Golgi apparatus and eventually to the cell surface. Consistently, only WT gH was abundantly detected at the plasma membrane by IIF analyses of non-permeabilized plasmid-transfected RK13 cells, whereas gH-V275P and gH-V275P-V271A were barely visible (Fig. 2b, upper panel). However, total amounts of WT and mutant gH were similar as shown by analysis of permeabilized cells (Fig. 2b, lower panel). Co-expression of gL did not significantly improve surface localization of mutated or WT gH (results not shown).

Earlier studies have shown that cotransfection of RK13 cells with expression plasmids for PrV glycoproteins gB, gD, gH and gL induces cell fusion leading to formation of syncytia, and that this process was enhanced by substitution of WT gB by a C-terminally truncated variant (gB-008), which accumulates at the plasma membrane (Klupp *et al.*, 2000; Nixdorf *et al.*, 2000). In agreement with our previous results (Böhm *et al.*, 2015), evaluation of *in vitro* fusion assays including gH-V275P revealed no detectable fusion activity of the mutated protein, i.e. 36 h after cotransfection, only very few and small spontaneously formed polykaryocytes were detectable, like in the absence of any gH (Fig. 2c). In the presence of gH-V275P-V271A, the areas of the syncytia were only slightly increased, and remained far below the sizes obtained with WT gH (Fig. 2c). This finding correlated with the inhibited maturation and transport of both mutated proteins, and did not indicate a compensatory effect of the second site mutation V271A.

To exclude the possibility that the improved replication of passaged pPrV-gH-V275P had been caused by compensatory

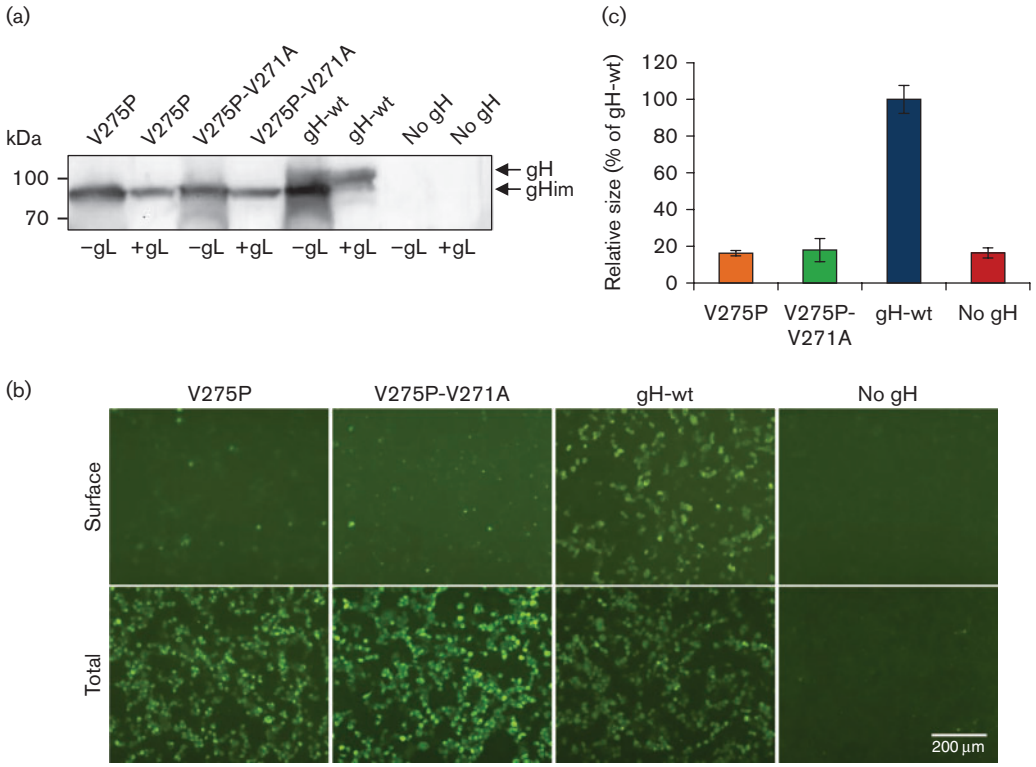

Fig. 2. *In vitro* characterization of mutated gH. (a, b) For Western blotting (a) and IIF tests (b), RK13 cells were (co)transfected with expression plasmids for WT gH, gH-V275P or gH-V275P-V271A with or without a gL expression plasmid, and analysed after 48 h using a gH-specific antiserum. (a) After separation of proteins, blots were probed for presence of mature and immature gH (gHim). (b) IIF tests were performed to compare the amounts of surface-expressed gH on cells fixed with 3 % paraformaldehyde (upper panel) to total amounts of gH in cells which were additionally permeabilized with 0.5 % Triton X-100 (lower panel). Bar, 200 µm. (c) Cell fusion was analysed 36 h after cotransfection of RK13 cells with expression plasmids for PrV glycoproteins gB-008, gD, gL, WT or mutated gH (V275P, V275P-V271A), and GFP for visualization. The areas of 50 fluorescing syncytia per virus were measured in three experiments. The mean relative (% of WT gH) sizes and SD are shown. For experimental protocols, see Böhm *et al.* (2015).

mutations in other genes, the open reading frame of gH-V275P-V271A was inserted into gH-deleted pPrVΔgH-ABF as previously done with gH-V275P and WT gH genes (Böhm *et al.*, 2015). The resulting isogenic recombinants pPrV-gH-V275P-V271A, pPrV-gH-V275P and pPrV-gHK were characterized by protein analyses and *in vitro* replication studies.

Western blotting was performed with infected cell lysates and purified virus particles (Fig. 3a). Whereas 18 h after infection with pPrV-gHK, a major portion of intracellular WT gH exhibited a molecular mass of 95 kDa, only an immature approximately 80 kDa form of gH-V275P was detectable, and only a minor portion of gH-V275P-V271A was fully processed (Fig. 3a, upper panel). Sucrose-gradient purified virus particles of pPrV-gH-V275P-V271A contained traces of mature gH, whereas gH was undetectable in virions of pPrV-gH-V275P, like in pPrV-ΔgHABF particles (Fig. 3a, upper panel). Remarkably, the amount of gH-V275P-V271A in virions was considerably lower than that of WT gH, whereas the amounts of gB were similar (Fig. 3a, middle panel). Absence of the non-structural PrV protein pUL31 (Fuchs *et al.*, 2002) proved purity of the virion preparations (Fig. 3a, lower panel). This demonstrates that the doubly mutated gH, despite inefficient processing, is transported to the probable site of final herpesvirus envelopment in the *trans*-Golgi network (TGN) (Mettenleiter, 2002), although still quite inefficiently.

To investigate to what extent the altered structure, the impaired maturation and the inefficient virion incorporation of gH affected virus entry, penetration kinetics of pPrV-V275P-V271A and pPrV-gHK were compared in RK13 cells as described (Böhm *et al.*, 2015). After synchronized virus attachment, the majority of infectious WT particles were internalized within 10 min at 37 °C, and after 40 min more than 90 % had entered the cells (Fig. 3b). In contrast, only roughly 20 and 50 % of pPrV-gH-V275P-V271A had penetrated after 10 and 40 min, respectively (Fig. 3b). Although this delay was significant, it was much less pronounced than what was observed after introduction of artificial disulfide bonds within the SLB, or

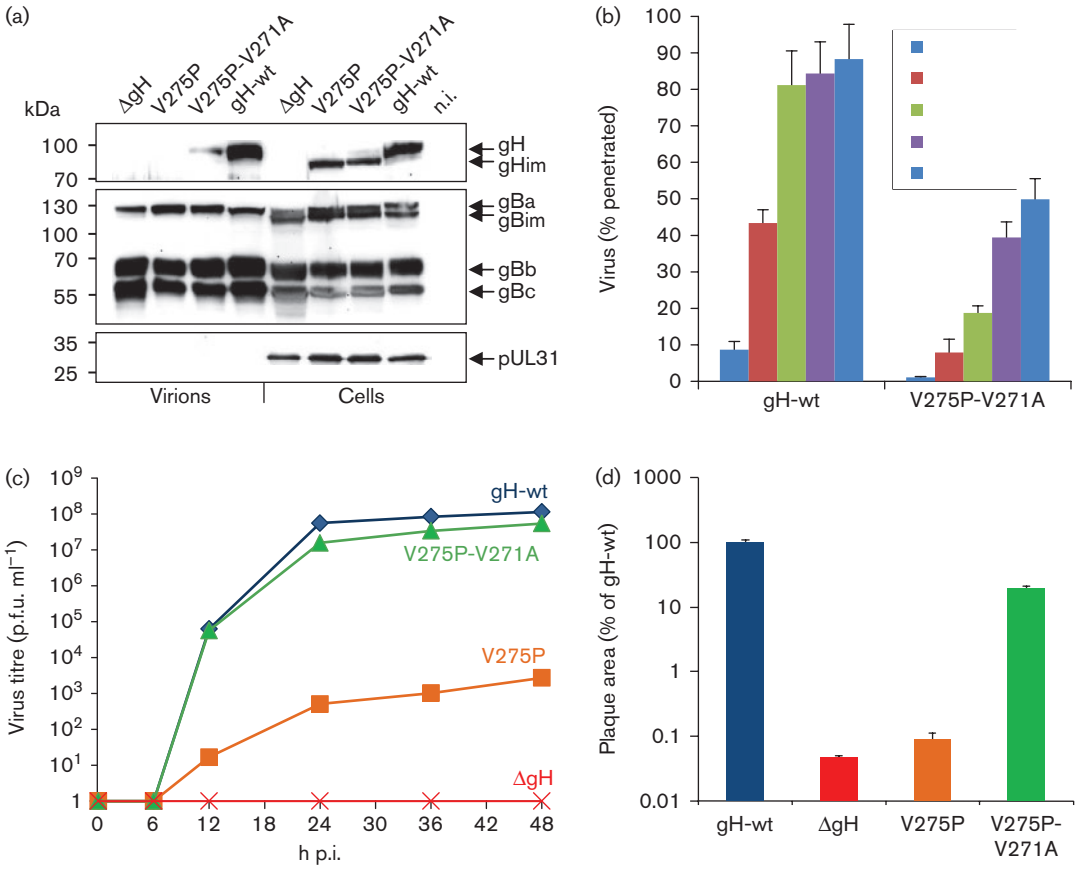

Fig. 3. Replication of PrV recombinants in RK13 cells. (a) Western blot analyses of infected (m.o.i. 2, 18 h p.i.) and uninfected cells, and of sucrose gradient-purified virions of pPrV-ΔgHABF, -gH-V275P, -gH-V275P-V271A and -gHK (gH-wt). Blots were probed with monospecific sera against PrV proteins. Mature and immature gH (gHim), mature and immature full-length gB (gBim, gBa), furin-cleaved gB (gBb, gBc) and pUL31 are indicated. (b) For determination of penetration kinetics, cell monolayers were infected on ice with approximately 500 p.f.u. per well of the indicated virus. After 0, 5, 10, 20 and 40 min at 37 °C, non-penetrated virus was inactivated by acid treatment, and 2 days later plaque numbers were compared with those in untreated wells. The mean results of three experiments and SD are shown. (c) Growth kinetics were analysed after PrV infection at an m.o.i. of 0.1. Mean progeny virus titres after the indicated times (h post-infection, h p.i.) were determined by plaque assays in three independent experiments. (d) The average areas of 50 plaques per virus were determined 48 h after infection. The percentages of WT (pPrV-gHK) sizes and SD derived from three experiments are shown. (e) Representative fluorescence images were recorded 24 h after infection of RK13 cells with the GFP expressing PrV recombinants. Bar, 200 μm. For experimental protocols, see Böhm *et al.* (2015).

between SLB and gH domain III (Böhm *et al.*, 2015). Due to the severe replication defects and the genetic instability of pPrV-gH-V275P in non-complementing cells, investigation of its penetration kinetics did not provide coherent results (not shown).

To avoid these problems, phenotypically complemented PrV-gH-V275P and pPrV-ΔgHABF isolated from WT gH-expressing RK13-gH/gL cells (Klupp *et al.*, 2008), but non-complemented pPrV-gH-V275P-V271A and pPrV-gHK, were used to compare *in vitro* replication and spread on non-complementing RK13 cells (Fig. 3c–e). Growth kinetic studies confirmed that gH-deleted PrV was unable to produce infectious progeny, and that maximum titres of pPrV-gH-V275P were less than 10^4 p.f.u. ml^{-1} (Böhm *et al.*, 2015). In contrast, the double mutant pPrV-gH-V275P-V271A exhibited similar replication kinetics and maximum infectious titres (nearly 10^8 p.f.u. ml^{-1}) as WT pPrV-gHK (Fig. 3c). Thus, as observed with other gH mutants (Böhm *et al.*, 2015), the delay in penetration of pPrV-gH-V275P-V271A did not significantly prolong the replication cycle.

Cell-to-cell spread was analysed by plaque assays, and cells infected with the GFP-expressing PrV mutants were identified by fluorescence microscopy (Fig. 3e). After 2 days only single pPrV-ΔgHABF-infected RK13 cells were found, and small groups of positive cells were detected with pPrV-gH-V275P, corresponding to mean 'plaque' areas of less than 0.1 % of the WT size (Fig. 3d). However, the compensatory mutation of pPrV-gH-V275P-V271A led to an increase in plaque areas to 20 % of the WT size (Fig. 3d). Remarkably, unlike in WT plaques, all cells infected with pPrV-gH-V275P-V271A remained separated and did not form syncytia (Fig. 3e). This finding was in line with the absence of detectable cell fusion activity of gH-V275P-V271A in transient assays (Fig. 2c).

Our findings demonstrate that the requirements for gH in transient *in vitro* fusion assays and during productive virus replication and spread are different. Obviously, sufficient amounts of gB, gD and gH/gL at the plasma membrane are required to induce cell fusion. This is emphasized by the observation that C-terminal truncation of gB enhances surface localization and fusion activity (Baghian *et al.*, 1993; Klupp *et al.*, 2000; Nixdorf *et al.*, 2000), as well as by the frequent correlation between surface expression and fusion activity of previously described gH mutants (Böhm *et al.*, 2015; Galdiero *et al.*, 1997). However, recent *in vitro* studies with HSV-1 glycoproteins indicated that only the core fusion protein gB is required in higher quantities, whereas catalytic amounts of surface gD and gH/gL are sufficient for fusion (Atanasiu *et al.*, 2013).

For formation of infectious herpesvirus particles, the envelope glycoproteins do not have to be translocated to the plasma membrane, but need to be present in the TGN. Together with a catalytic function of gH in membrane fusion, this could explain why several mutations which severely impair gH maturation and transport have only minor effects on virus infectivity (Böhm *et al.*, 2015; Fuchs *et al.*, 2012; Schröter *et al.*, 2014). Although gH is essential for entry and cell-to-cell spread of PrV (Peeters *et al.*, 1992), few functional gH molecules per particle might be sufficient to activate gB for virus entry, and direct spread at cell junctions. Remarkably, the latter process does not require complete fusion between infected and uninfected cells, occurs in the absence of gD and, to some extent, gL in PrV (Klupp & Mettenleiter, 1999; Rauh & Mettenleiter, 1991), but is supported by the non-essential gE/gI complex (Johnson & Huber, 2002; Mettenleiter, 2003).

It remains to be elucidated whether the compensatory mutation V271A only enhances maturation and virion incorporation of gH-V275P, or whether it also directly improves gH function during fusion. In either case partial reconstitution of the original structure of gH, in particular of the highly conserved SLB, would be a plausible explanation. Certain unconfirmed protein secondary structure predictions (Garnier *et al.*, 1996) indicate that gH mutation V275P causes an extensive interruption of the α-helix, which is partly repaired by the additional mutation V271A. The crystal structure of WT PrV gH (Backovic *et al.*, 2010) revealed that amino acids V275 and V271 are located on the same side of helix α3, packing closely against the opposing β-sheet termed the fence (Fig. 1a). The hydrophobic side chains of V275 and V271 form a network of van der Waals interactions with other hydrophobic

side chains coming from the fence and helices α1 and α2 (Fig. 1b, c). The interruption or distortion of helix α3 by proline 275 presumably affects these interactions, causing steric clashes and less favourable packing of the SLB against the fence. This might also impair *N*-glycosylation of the fence at asparagine 162, and/or binding of gL which, at least in HSV-2 and EBV (Chowdary *et al.*, 2010; Matsuura *et al.*, 2010), is associated with gH domain I on the opposite side of the fence. It is conceivable that substitution of valine 271 by alanine, which has a smaller side-chain, alleviates the impact of the V275P mutation on packing of SLB and fence, thus improving maturation, transport and function of PrV gH.

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