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**Herpes simplex virus type 1 glycoprotein B sorting  
in hippocampal neurons**

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**Herpes simplex virus type 1 (HSV-1) is a neuroinvasive human pathogen that spreads in the nervous system in functionally connected neurons. Determining how HSV-1 components are sorted in neurons is critical to elucidate the mechanisms of virus neuroinvasion. By using recombinant viruses expressing glycoprotein B (gB) tagged with green fluorescent protein (GFP), the subcellular localization of this envelope protein was visualized in infected hippocampal neurons in culture. Results obtained using a fully infectious recombinant virus containing GFP inserted into the ectodomain of gB support the view that capsids and gB are transported separately in neuron processes. Moreover, they show that during infection gB is sorted to the dendritic tree and the axons of polarized hippocampal neurons. However, GFP insertion into the cytoplasmic tail of gB impaired the maturation of the resulting fusion protein and caused its retention in the endoplasmic reticulum. The defective protein did not gain access to axons of infected neurons. These results suggest that the cytoplasmic tail of gB plays a role in maturation and transport and subsequently in axonal sorting in differentiated hippocampal neurons.**

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## INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a neurotropic and neuroinvasive human alphaherpesvirus. After replication in epithelial cells of the skin or mucosa, infection is mostly restricted to peripheral sensory and autonomous neurons, where latency is established. However, HSV-1 occasionally reaches the brain by transneuronal transport from an as yet undetermined portal of entry, resulting in acute necrotizing

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encephalitis (Whitley & Gnann, 2002). The virion particle consists of a capsid which contains the viral genome, surrounded by the tegument, which is made up of more than 15 proteins. In addition, surrounding the tegument is a cell-derived membrane envelope containing at least 11 viral glycoproteins. The viral factors which determine neuroinvasion remain unknown. Moreover, the process of transport and assembly of HSV-1 in neurons is not completely understood, although it is known to differ from that observed in other cell types. In infected cells, the present view is that capsids newly synthesized in the nucleus undergo a process of envelopment and de-envelopment through the nuclear membrane, before final envelopment occurs by budding into the trans-Golgi network (Mettenleiter, 2002). However, recent studies in neurons suggest that unenveloped nucleocapsids move long distances in axons separately from envelope proteins, which suggests that assembly and egress occurs at the axon terminus (Holland *et al.*, 1999; Miranda-Saksena *et al.*, 2000; Penfold *et al.*, 1994). Therefore, a preliminary step in determining the molecular basis of virus migration along complex neuro-anatomic pathways is the characterization of viral factors which determine the directional transport of structural components of the virus in individual neurons (Tomishima *et al.*, 2001). The development of HSV-1 recombinant viruses containing various fluorescence-tagged structural proteins has paved the way for the investigation of the sites of synthesis and transport of viral proteins in various cell cultures (Desai & Person, 1998; Donnelly & Elliott, 2001; Elliott & O'Hare, 1999). Using this approach, we generated HSV-1 recombinants carrying green fluorescent protein (GFP) in-frame with glycoprotein B (gB). HSV-1 gB is a major envelope protein highly conserved among herpesviruses, with multiple biological functions during the virus life-cycle, such as entry, cell fusion, cell-to-cell spread (Pereira, 1994; Spear, 1993), maturation and egress (Peeters *et al.*, 1992). In addition, HSV-1 gB has been involved in neuroinvasion (Mitchell & Stevens, 1996; Sivadon *et al.*, 1998; Yuhasz & Stevens, 1993). It is a type I membrane glycoprotein composed of 904 amino acids (aa), including a 30 aa signal sequence, a 696 aa ectodomain, a 69 aa transmembrane domain and a 109 aa cytosolic domain (Bzik *et al.*, 1984; Cai *et al.*, 1988b; Pellett *et al.*, 1985). We have previously reported that insertion of GFP within the amino-terminus of gB leads to a fully infectious HSV-1 (Potel *et al.*, 2002). In this study, we designed a recombinant HSV-1 containing GFP fused in-frame within the cytoplasmic tail of gB. We then analysed and compared the biochemical properties, subcellular localization and processing of each fusion protein in the context of transfection and during infection in Vero cell lines and in primary neuron cultures.

## METHODS

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**Cells and viruses.** African green monkey kidney Vero cells were grown in minimum essential medium supplemented with 5 % foetal calf serum (FCS) at 37 °C in a 5 % CO<sub>2</sub> incubator. Vero-derived D6 cells which are stably transfected with the HSV-1 UL27-gB gene (Cai *et al.*, 1987) were grown in Dulbecco's modified essential medium plus 10 % FCS and 0.5 mg ml<sup>-1</sup> of G418 (geneticin; Gibco-BRL).

HSV-1 KOS strain was used as the reference virus. The gB-null K082 virus that carries a stop codon at position 43 of gB was propagated on D6 cells (Cai *et al.*, 1987). Both the D6 cells and the K082 virus were kindly provided by P. Desai and S. Person (Johns Hopkins University, Baltimore). Recombinant HSV-1 KGFP-gB (Potel *et al.*, 2002) was propagated in Vero cells. Extra-cellular and intra-cellular virions were purified after infection of Vero or D6 cells. Approximately  $3 \times 10^8$  cells were infected at an m.o.i. of 0.005 p.f.u. per cell. Extra-cellular virions were concentrated 3–4 days later by pelleting the cleared infected cell culture supernatant through a cushion of 25 % glycerol in 10 mM Tris/HCl pH 7.5, 50 mM NaCl, 1 mM EDTA for 1 h at 27 000 r.p.m. in a Beckman SW28 rotor. Intra-cellular virions were liberated by three cycles of freezing and thawing and by sonication of the infected cell pellet. Virions were concentrated as above after a low-speed centrifugation to clear the cell debris.

**Hippocampal cell cultures.** Hippocampal neuron cultures were obtained as previously described (Goslin & Banker, 1989). Briefly, hippocampi were dissected from embryonic D18 rats, in HBSS-HEPES (1× HBSS, 20 mM HEPES). Cell suspensions were treated with trypsin 0.25 % for 15 min at 37 °C, followed by 0.9 g DNase I  $\Gamma^{-1}$  (Roche) for 5 min at 37 °C. Cell dissociation was obtained by trituration using a fire-polished Pasteur pipette. Cells were then plated onto polyornithine-treated glass coverslips in culture medium (Eagle's modified essential medium: 1 % L-glutamine, 0.6 % glucose, 0.2 %  $\text{NaHCO}_3$ ,  $10^{-2}$  M HEPES, 5 UI penicillin and streptomycin  $\text{ml}^{-1}$ ) with 10 % horse serum. Approximately 20 000 cells were plated on a coverslip. After attachment of cells, coverslips were inverted and transferred onto a confluent monolayer of astroglial cells, and maintained in B27 Neurobasal medium supplemented by 1 % L-glutamine, 5 UI penicillin and streptomycin  $\text{ml}^{-1}$ . Coverslips were maintained at 37 °C in a 5 %  $\text{CO}_2$  incubator. Neuronal cultures were infected 12 days after plating.

**Antibodies.** Rabbit polyclonal anti-gB, anti-gD and anti-nucleocapsid antibodies (R69, R45 and NC-1, respectively) were generously provided by R. J. Eisenberg and G. H. Cohen (University of Pennsylvania, Philadelphia). Mouse monoclonal antibody directed against the microtubule-associated protein 2 (MAP2) was obtained from Sigma. Mouse monoclonal anti-GFP antibody was purchased from Roche. Rabbit polyclonal antibodies directed against Rab6 (C-19) or against the carboxy terminus of calnexin were obtained from Santa Cruz Biotechnologies and Stressgen Biotechnologies, respectively. Texas red-conjugated donkey anti-rabbit and anti-mouse immunoglobulin G (IgG) were obtained from Jackson ImmunoResearch Laboratories. The goat anti-rabbit IgG peroxidase conjugate and the goat anti-mouse IgG peroxidase conjugate were from Sigma.

**Plasmids.** Plasmid pSR175 containing the UL27-gB coding region of the HSV-1 KOS strain under the control of the cytomegalovirus immediate-early promoter was kindly provided by R. J. Eisenberg and G. H. Cohen. Plasmid pgBct-GFP was constructed by an in-frame insertion of the GFP coding sequence at

the *NheI* site of the UL27-gB gene in pSR175. To this end, the cDNA sequence encoding enhanced GFP was amplified by PCR from plasmid pEGFP-N2 (Clontech) using primers 5'-AAAAAAGCTAGCCGGAGGAGGAGTGAGCAAGGGCGAGGAG-3' and 5'-AAAAATGCTAGCCCACCACCCTTGTACAGCTCGTCCAT-3', which carry an *NheI* restriction site at their 5' ends. The primers were designed to delete the GFP gene initiation and termination codons and to introduce codons for three glycine residues at each extremity of GFP. The amplified and *NheI*-cleaved GFP DNA fragment was then transferred into the *NheI* site of pSR175.

**Recombinant virus.** To construct the KgBct-GFP recombinant virus, subconfluent monolayers of Vero cells were co-transfected with 1 µg K082 genomic DNA and with linearized plasmid pgBct-GFP in a five-fold molar excess (250 ng) using LipofectAMINE according to the manufacturer's recommendations (Gibco-BRL). Plaques were screened 3–5 days after transfection for the presence of spontaneous fluorescence on Vero cells. Isolated KgBct-GFP recombinants were plaque-purified three times on D6 cells. Virus stocks were prepared as for KOS and K082. Viral DNA was analysed by PCR using gB-specific primers bracketing the gB gene *NheI* insertion sites and by Southern blotting as previously described (Potel *et al.*, 2002).

**Complementation.** Complementation assays were performed essentially as initially described (Cai *et al.*, 1988a). Briefly,  $4 \times 10^5$  Vero cells were seeded in 35 mm culture dishes and transfected the following day with 0.25 µg of pSR175 or pgBct-GFP using LipofectAMINE. The cells were infected 24 h later with K082 at an m.o.i. of 0.5 p.f.u. per cell. Extra-cellular virions were washed off 1 h post-infection (p.i.) by treatment with an acid/glycine saline solution, followed by two washes with PBS. Infected cell culture supernatants were harvested 24 h after infection, and titrated on D6 and Vero cells. The titre obtained from K082 virus complemented with native gB was taken as the reference value, and the complementing capacity of the gBct-GFP protein was indicated as a ratio with respect to this reference value.

**Plasmid transfections, virus infections and brefeldin A treatment.** To analyse the expression of proteins, Vero cells were transfected with 1 µg of pgBct-GFP or pSR175 using LipofectAMINE and incubated at 37 °C for 24 h for immunofluorescence studies. To compare *de novo* synthesis of the proteins in the context of virus infection, Vero cells and hippocampal neurons were infected at an m.o.i. of 0.5 p.f.u. per cell and 3 p.f.u. per cell respectively with KgBct-GFP or KGFP-gB for fluorescence studies, and 5 p.f.u. per cell for immunoblot analyses. For brefeldin A (BFA) treatment, infected Vero cells were incubated for 14 h at 37 °C in culture medium containing 0.1 µg brefeldin A ml<sup>-1</sup> (Sigma). Immunofluorescence studies were performed at 16 h p.i. in Vero cells or 24 h p.i. in neurons. Immunoblotting of infected Vero cells was performed at 15 h p.i. To monitor the synthesis of the gB fusion proteins during an entire virus replication cycle, infected Vero and neuronal cells were examined

every hour up to 25 and 30 h p.i., respectively.

**Immunoblot analysis and endoglycosidase treatment.** Cells in 35 mm culture dishes were washed twice with PBS and lysed for 20 min on ice in 50 mM Tris/HCl pH 8, 62.5 mM EDTA, 1 % Nonidet P40, and 0.4 % sodium deoxycholate supplemented with 15  $\mu\text{g}$  antipain dihydrochloride  $\text{ml}^{-1}$ , 2.5  $\mu\text{g}$  aprotinin  $\text{ml}^{-1}$ , 2.5  $\mu\text{g}$  pepstatin  $\text{ml}^{-1}$ , 5  $\mu\text{g}$  chymostatin  $\text{ml}^{-1}$ , 2.5  $\mu\text{g}$  leupeptin  $\text{ml}^{-1}$  and 100  $\mu\text{g}$  Pefabloc  $\text{ml}^{-1}$  (protease inhibitors; Roche). Endoglycosidase H (Endo H) and peptide:*N*-glycosidase F (PNGase F) digestions were performed on about one-twentieth of the cell lysates for 2 h at 37 °C. Samples were run on denaturing SDS-10 % polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were saturated with 5 % non-fat milk in TBST (10 mM Tris/HCl pH 8, 150 mM NaCl, 0.05 % Tween-20) before incubation for 1 h with primary antibody at a dilution of 1:4000 (anti-gB) or 1:1000 (anti-GFP) in TBST/1 % BSA. The secondary antibodies, goat anti-mouse IgG or goat anti-rabbit IgG coupled to horseradish peroxidase, were diluted according to the supplier's instructions. Bound antibodies were detected by enhanced chemiluminescence (Amersham).

**Immunofluorescence assays.** Transfected or infected Vero cells and neurons on glass coverslips were fixed with 4 % (w/v) paraformaldehyde in PBS for 20 min at room temperature and then permeabilized with 0.1 % Triton X-100 for 2 min for staining of internal antigens, or directly processed for surface staining. Cells were washed three times with PBS and then incubated with primary antibodies for 1 h at 37 °C. The gB, gD, calnexin and Rab6 antisera were diluted 1:300, the anti-nucleocapsid antibody was diluted 1:500, and the anti-MAP2 antibody was diluted 1:100 in PBS containing 10 % donkey serum. Coverslips were extensively washed with PBS, before labelling with the secondary anti-rabbit IgG antibody at a dilution of 1:300, or with the secondary anti-mouse IgG antibody at a dilution of 1:200 in PBS containing 10 % donkey serum for 1 h. After three rinses with PBS and one with water, coverslips were mounted onto glass slides and then analysed with a conventional Zeiss Axiophot fluorescence microscope. Spontaneous fluorescence was visualized using standard fluorescein isothiocyanate excitation–emission filter sets, and Texas red signals were visualized using narrow-band Texas red filters. Images obtained with a Hamamatsu digital camera were processed using the Starwise fluostar program (version 5.9.2s).

## RESULTS

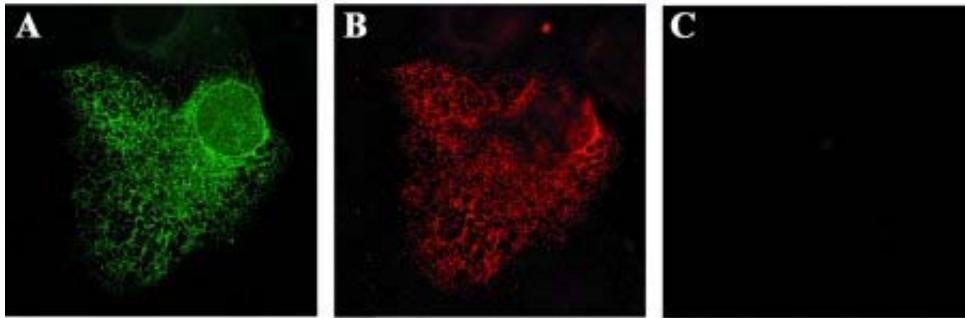
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### **Construction of a recombinant HSV-1 containing GFP inserted within the carboxy-terminal domain of gB**

To analyse the intra-cellular sorting of HSV-1 gB in infected cells, we constructed recombinant viruses containing GFP fused to gB. The site of incorporation of GFP may influence the functionality and the emission of fluorescence of the resulting protein. Here, we inserted GFP into the cytosolic domain of gB. To this end, the GFP coding sequence was inserted in-frame at the *NheI* site of the UL27-gB gene in pSR175, giving rise to plasmid pgBct-GFP, which encodes a fusion protein containing GFP at position 841 of gB within the carboxy-terminal domain of the glycoprotein. In this construct, three glycine residues were added at both ends of the GFP open reading frame (Cubitt *et al.*, 1995; Moriyoshi *et al.*, 1996). Vero cells were co-transfected with the gB-null K082 viral DNA (Cai *et al.*, 1987) and the plasmid pgBct-GFP. Progeny viruses isolated 4–5 days after transfection were plated on Vero cells and recombinants were screened for their ability to form spontaneously fluorescent plaques. However, mixed populations of fluorescent and non-fluorescent plaques were obtained even after repeated passages. To purify the desired recombinant, progeny viruses were plated onto gB-complementing D6 cells and plaque-purified three times. When Vero cells were infected with the resulting KgBct-GFP, sparse fluorescent cells were observed, but no plaques were detected, and essentially no infectious virus was released into the Vero culture supernatant. The KgBct-GFP genome contained the expected recombinant gB-GFP gene in the *BamHI*-G genome fragment which contains the UL27 gene, as verified by PCR and Southern blot (data not shown). To further ensure that the defect in infectivity of KgBct-GFP was due to the fusion protein, the complementation capacity of gBct-GFP was compared to that of native gB, using a previously reported assay (Cai *et al.*, 1987; Potel *et al.*, 2002). Our results showed that gBct-GFP was unable to restore infectivity of the gB-null K082 virus, since titres of complemented virus approximated 0.3 %, compared to the 100 % reference value obtained with native gB.

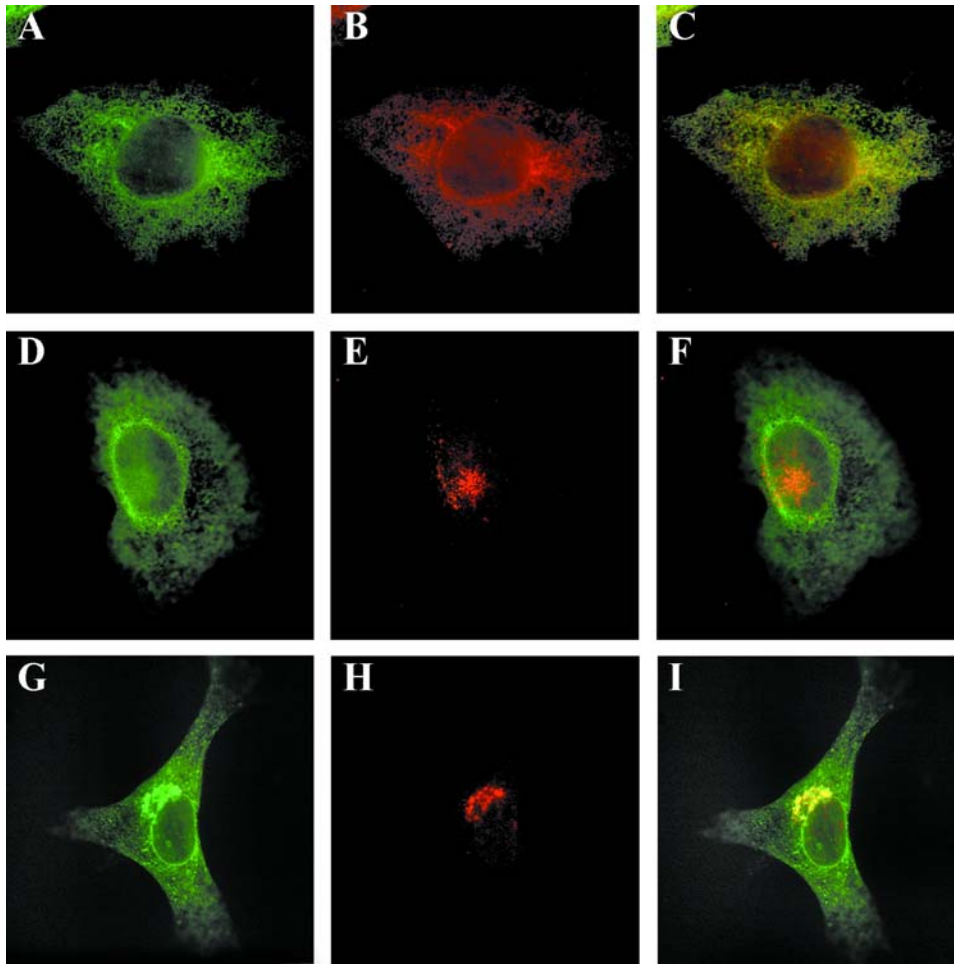
### **Subcellular localization of GFP-tagged gB in transfected and infected Vero cells**

To further investigate the functional defect in gBct-GFP, the subcellular localization of the protein was visualized in transfected Vero cells, using both the spontaneously arising GFP fluorescence and the Texas-red coupled anti-gB staining. As controls, images were compared with those obtained with native gB and with the previously reported GFP-gB, which contains GFP fused to the ectodomain of gB. In permeabilized cells, gB and GFP-gB accumulate in the peri-nuclear region (Gilbert *et al.*, 1994; Potel *et al.*, 2002), whereas gBct-GFP was visualized exclusively as a thin rim around the nuclear membrane and in a reticular structure of the cytoplasm (Fig. 1A, B). In addition, in non-permeabilized cells, native gB and GFP-gB exhibit a punctate pattern at the cell surface (Potel *et al.*, 2002), whereas gBct-GFP was not detectable at the surface on any cell (Fig. 1C).



**Fig. 1.** Subcellular localization of gBct-GFP in transfected cells. Vero cells were transfected with plasmid pgBct-GFP. Images show spontaneous GFP fluorescence (A) or indirect immunofluorescence from an anti-gB antibody and Texas red-labelled secondary antibodies on permeabilized (B) and non-permeabilized (C) cells. Fluorescence was visualized with a Zeiss Axiophot fluorescence microscope.

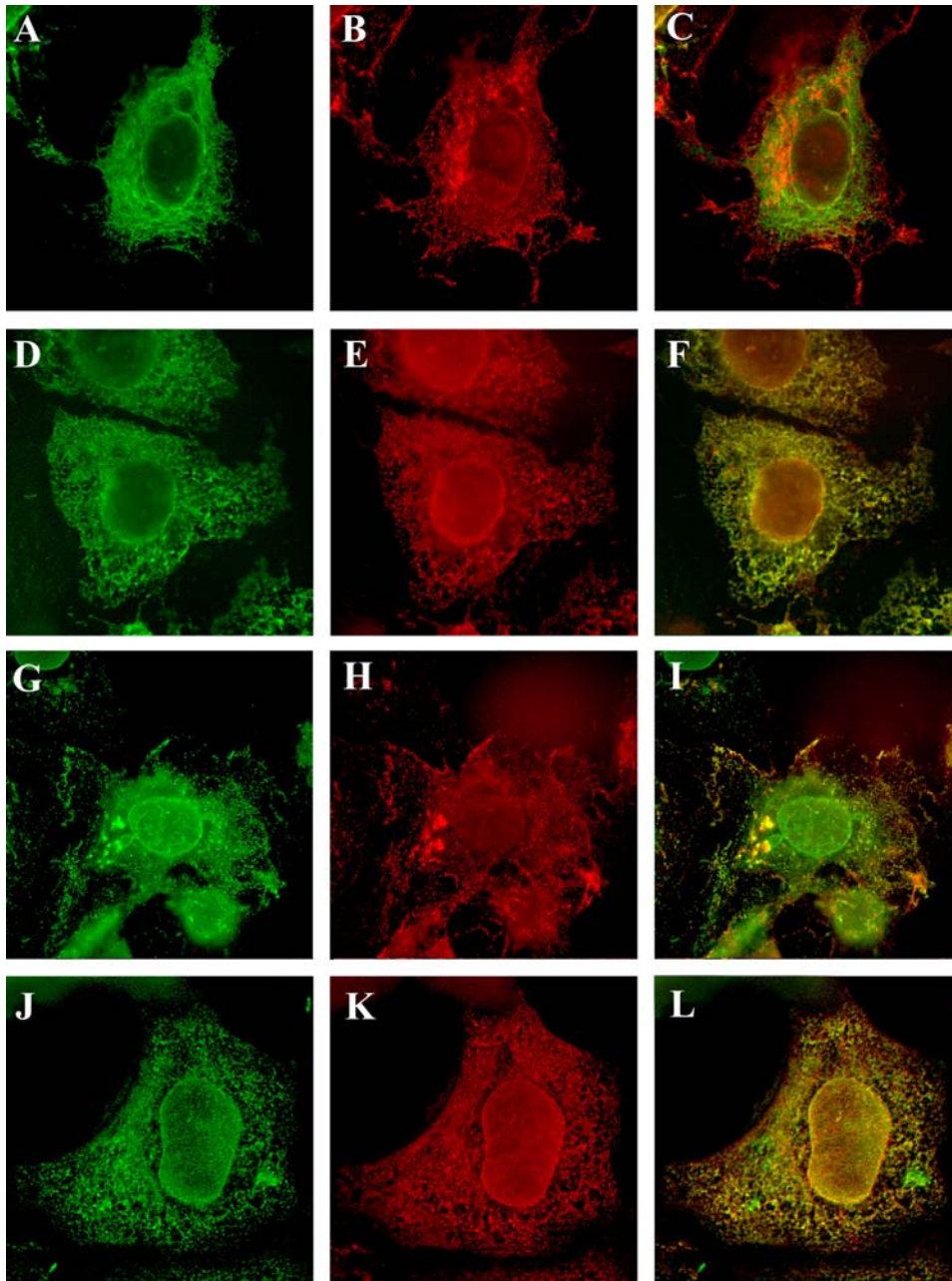
To analyse the precise subcellular localization of the fusion proteins in the context of virus infection, infected Vero cells were labelled with cell compartment-specific antibodies. Calnexin is a chaperone molecule located in the ER, which associates with precursor forms of several HSV glycoproteins including gB, following partial trimming of *N*-linked oligosaccharides (Yamashita *et al.*, 1996). Rab6, a member of the family of guanosine triphosphatases which regulate vesicular transport and membrane traffic in eukaryotic cells, is localized to the Golgi complex (Echard *et al.*, 1998). In cells infected with the defective KgBct-GFP virus, staining with the calnexin antibody confirmed that gBct-GFP localized to the ER (Fig. 2A, B, C), whereas gBct-GFP did not co-localize with Rab6 (Fig. 2D, E, F). This result indicates that the molecule was retained in the ER and did not gain access to the Golgi. In contrast, in KGFP-gB-infected cells, the fusion protein GFP-gB was localized in the ER and also accumulated in a compartment that was identified as belonging to the Golgi complex by the Rab6 marker (Fig. 2G, H, I).



**Fig. 2.** Subcellular localization of gBct-GFP and GFP-gB in infected cells. Vero cells were infected with KgBct-GFP (A–F) or KGFP-gB (G–I) at an m.o.i. of 0.5, fixed and permeabilized 16 h p.i. Images show spontaneous GFP fluorescence (A, D, G) or indirect immunofluorescence from an anti-calnexin (B) or anti-Rab6 (E, H) antibody and Texas red-labelled secondary antibodies. (C), (F) and (I) correspond to the respective merged images.

To investigate whether the defect of gBct-GFP modified the cellular localization of another component of the viral envelope, infected Vero cells were labelled with an anti-glycoprotein D (gD) antibody. In cells infected with the defective KgBct-GFP virus, as observed in transfection studies, gBct-GFP did not reach the cell surface and was retained in the ER, whereas gD was detected at the cell membrane, and accumulated in a perinuclear compartment (compare Fig. 3A, B and C). BFA treatment dispersed gD to the cytosol and suppressed its Golgi-like compartmentalization, whereas the localization of gBct-GFP was not modified (compare Fig. 3A, B and C with D, E and F). These results contrasted sharply with those obtained in cells infected with the functional KGFP-gB virus, where high concentrations and accumulations of both envelope proteins gD and GFP-gB were visible in the Golgi and at the cell surface (Fig. 3G, H). The co-localization of both fluorescent markers at these sites in superimposition images demonstrated the physical proximity of both molecules, suggesting these are sites where virion particles assemble before budding (Fig. 3I). As expected, both molecules were redistributed in the cytosol after BFA treatment (compare Fig. 3G, H with K). Remarkably, images of native gD were comparable in cells infected with either virus, thus showing that the presence of the defective gBct-GFP did not alter the

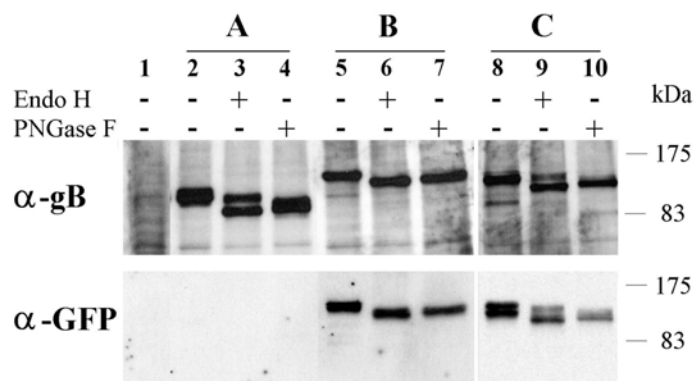
steady state cellular localization of gD (compare Fig. 3B with H and C with I).



**Fig. 3.** Comparative distribution of gBct-GFP, GFP-gB and gD in infected cells. Vero cells were infected with KgBct-GFP (A–F) and KGFP-gB (G–L) at an m.o.i. of 0.5, fixed and permeabilized 15 h p.i. In (D–F) and (J–L), cells were treated with BFA 1 h p.i. Images show spontaneous GFP fluorescence (A, D, G, J) or indirect immunofluorescence from an anti-gD antibody (B, E, H, K) and Texas red-labelled secondary antibodies. (C), (F), (I) and (L) correspond to the respective merged images.

## Post-translational processing of GFP-tagged gB

To compare biochemically the processing of both fusion proteins, their transport through the exocytic pathway was examined by studying their electrophoretic mobility after endoglycosidase treatment. Infected Vero cell lysates were treated with Endo H or PNGase F and subsequently analysed by immunoblotting with anti-gB and anti-GFP antibodies. In untreated KOS-infected cell lysates, gB is detected as a doublet migrating at about 110–120 kDa, corresponding to the high mobility immature form and the low mobility mature form of the protein which exhibit high sensitivity and partial resistance to Endo H, respectively, while both forms are completely sensitive to PNGase F (Fig. 4A, lanes 2, 3 and 4). In contrast, in KgBct-GFP-infected cell lysates, this profile was replaced with a unique band migrating at about 140 kDa, corresponding to the expected size of the fusion molecule, which exhibited complete sensitivity to Endo H digestion (Fig. 4B, lanes 5, 6 and 7). This result indicates that gBct-GFP corresponds to an immature form of the fusion protein, which has not undergone the late steps of glycosylation and remains associated with the ER. In contrast, in KGFP-gB-infected cell lysates, GFP-gB was detected with either antibody (albeit more clearly with the anti-GFP antibody) as a doublet of about 140 kDa (Fig. 4C, lane 8). In addition to the Endo H-sensitive immature form, a slower-migrating molecule was visible that showed partial resistance to Endo H treatment (Fig. 4C, compare lanes 8, 9 and 10), indicating that GFP-gB is processed and glycosylated in the Golgi.

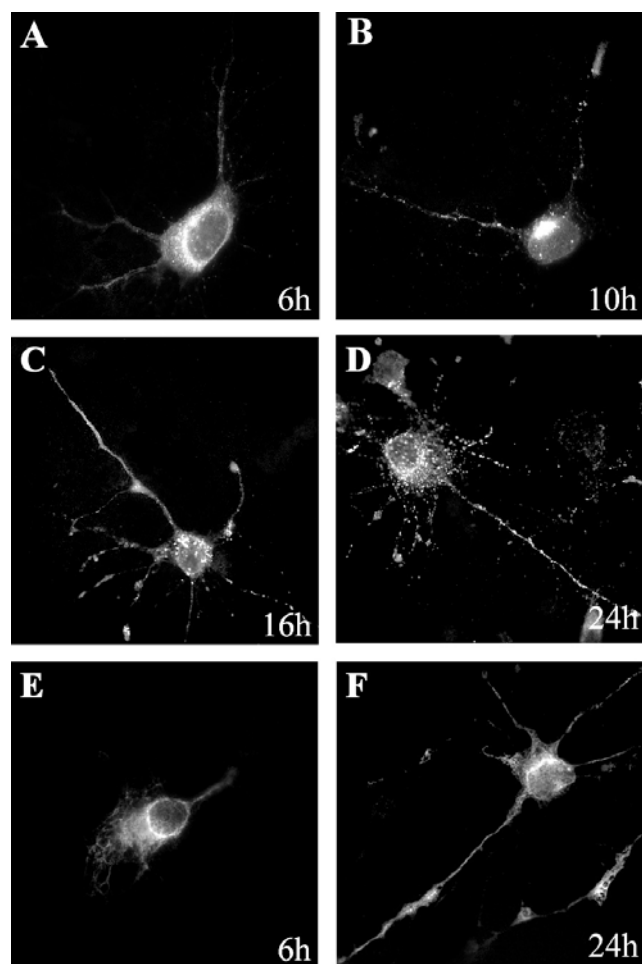


**Fig. 4.** Immunoblot analysis. Vero cells were either mock-infected (lane 1) or infected with KOS (A), KgBct-GFP (B) and KGFP-gB (C) at an m.o.i. of 5 and harvested 8 h p.i. Lysates were treated with Endo H or PNGase F as indicated above each lane and subjected to denaturing SDS-PAGE. Immunoblots were prepared and probed with anti-gB ( $\alpha$ -gB) and anti-GFP ( $\alpha$ -GFP) antibodies.

## Subcellular localization of GFP-tagged gB in infected hippocampal neurons

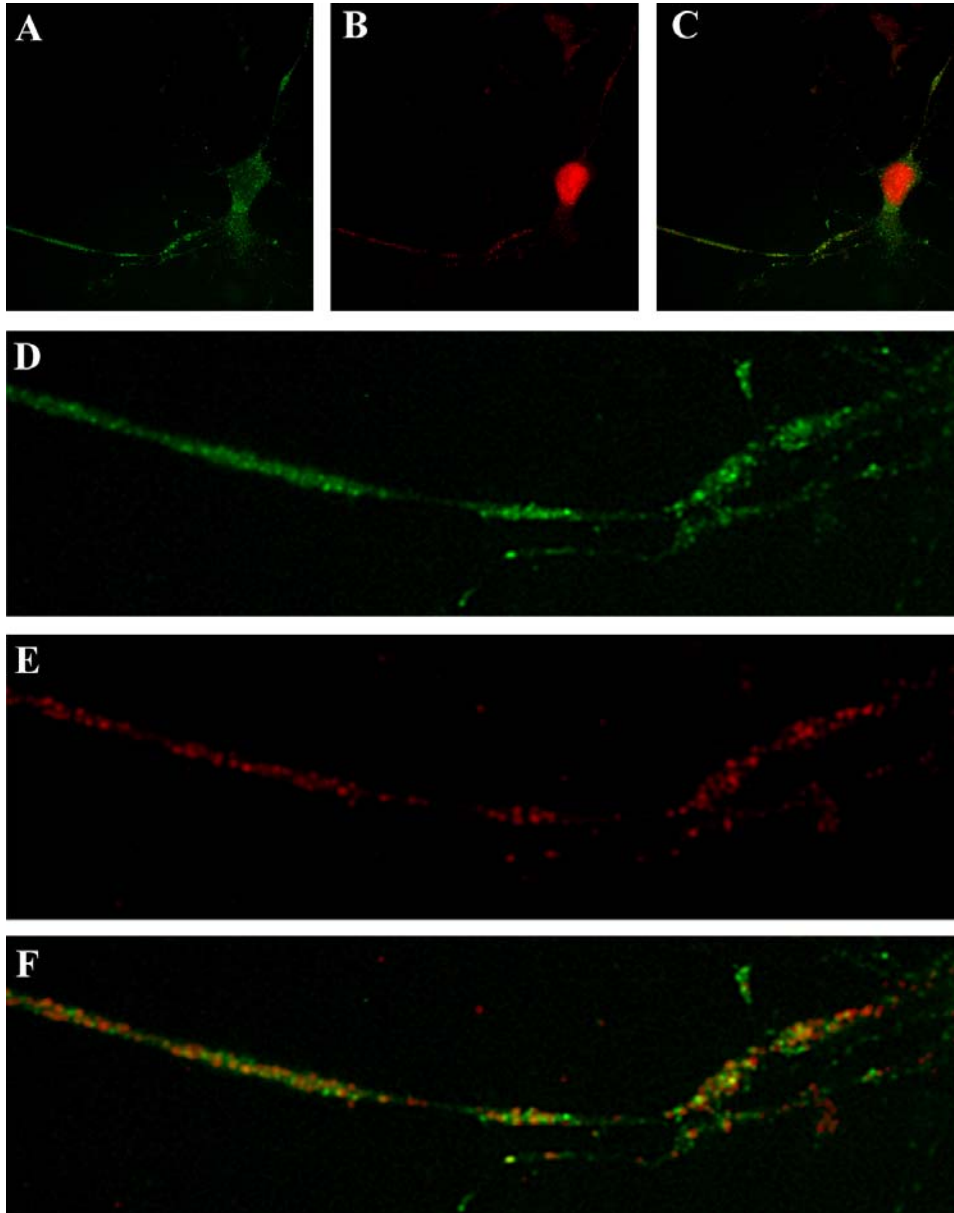
To analyse the fusion proteins in the context of virus infection in neuronal cells, well-differentiated foetal hippocampal neurons were infected 12 days after plating with either virus, and GFP fluorescence was analysed every 2 h up to 24 h p.i. In neurons infected with the KgBct-GFP virus, the fluorescence emitted by the defective fusion protein was visible from 6 h as a thin rim around the nucleus, and was detected diffusely in the cytoplasm and in the proximal parts of neuritic extensions. At later stages, up to 24 h p.i., a reticular aspect of the fluorescence was also visible all along thick neurites (Fig. 5E, F). This aspect was different from that obtained in KGFP-gB infected neurons, where the functional fusion protein

accumulated from 10 h in perinuclear patches (Fig. 5B–D), and was detected mostly as small and bright puncta in neurites, suggesting that GFP-gB had left the ER and was transported in vesicles.



**Fig. 5.** Temporal expression of gBct-GFP and GFP-gB in infected hippocampal neurons. Neurons were infected with KGFP-gB (A–D) or KgBct-GFP (E–F) at an m.o.i. of 1 and GFP fluorescence was analysed on fixed cells every hour during an entire replication cycle. Images were taken at the times p.i. indicated in each panel.

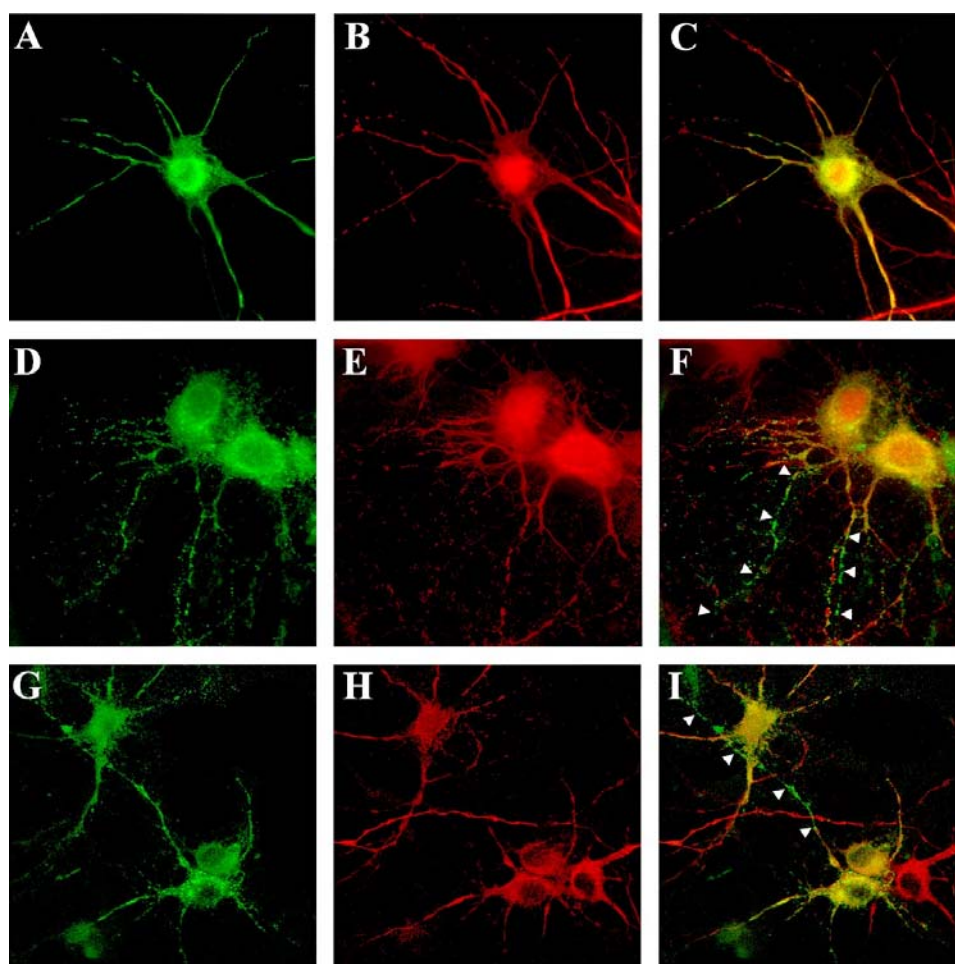
To further investigate where the functional GFP-gB might assemble with capsids, infected neurons were stained with a nucleocapsid antibody detected by Texas red staining. The nucleocapsid staining, which mainly recognizes the capsid protein VP5, was observed in the neurites as numerous distinct small puncta. The homogeneous aspect and size of these puncta suggested that they represent individual nucleocapsids, although some of them might be VP5 proteins transported in vesicles (Fig. 6B, C). When compared, the fluorescence from the two markers was not superimposed in the processes of neurons (Fig. 6A, B, C), which supports the argument that nucleocapsids and GFP-gB are transported separately in neurites. In particular, in these cultured neurons we did not observe images comparable to those previously observed in Vero cells (Potel *et al.*, 2002), which suggested that assembly of the virion particle occurs in proximity to the nucleus or at the cell surface. Unfortunately process termini could not be distinguished in the cultures.



**Fig. 6.** Distribution of GFP-gB and capsids in infected hippocampal neurons. Neurons were infected with KGFP-gB at an m.o.i. of 0.5, fixed and permeabilized 15 h p.i. Images show spontaneous GFP fluorescence (A, D) or indirect immunofluorescence from an anti-nucleocapsid antibody (B, E) and Texas red-labelled secondary antibodies. (C) and (F) correspond to the respective merged images. (D–F) Higher magnifications of a neuronal process seen in images (A–C).

We then examined the polarized transport of gB in neurons in more detail. After 12 days in culture, hippocampal neurons are at a stage where axons and dendrites can be distinguished because of their characteristic morphology and the polarized segregation of molecular markers to neuronal compartments. MAP-2 staining is strictly excluded from the axon in our cultures, so that axons can be identified as processes lacking this marker (West *et al.*, 1997). Therefore we compared the gB-associated GFP

fluorescence and the Texas-red-coupled anti-MAP2 staining in neurons infected with either recombinant virus. As shown by fluorescence imaging, in neurons infected with the defective KgBct-GFP virus, the diffuse fluorescence generated by gBct-GFP was observed in the soma and in the thick MAP2-positive dendrites of infected cells (Fig. 7A, B and C). These results were different from those observed in neurons infected with KGFP-gB. As above, the fluorescence of the functional GFP-gB was punctate, and was visualized in the soma of cells and in the MAP2-stained dendrite network (Fig. 7, compare D with E, and G with H). In addition, GFP fluorescence was clearly detected in thinner and longer neurites, which were identified as axons by the absence of MAP2 staining (Fig. 7F and I). Altogether, these results indicate that whereas the functional GFP-gB gained access to the dendritic tree and the axonal compartment of hippocampal neurons, insertion of GFP into the cytosolic domain of gB altered the axonal sorting of the resulting defective protein.



**Fig. 7.** Comparative polarized distribution of gBct-GFP and GFP-gB in infected hippocampal neurons. Neurons were infected with KgBct-GFP (A–C) or KGFP-gB (D–I) at an m.o.i. of 1, fixed and permeabilized 24 h p.i. Images show either spontaneous GFP fluorescence (A, D, G) or indirect immunofluorescence from an anti-MAP2 antibody (B, E, H) and Texas red-labelled secondary antibodies. (C), (F) and (I) correspond to the respective merged images.

## DISCUSSION

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Understanding how structural components of HSV are transported along neuronal cells is the first step in deciphering the mechanisms responsible for virus neuroinvasion through complex anatomic pathways. *In vivo*, the virus is primarily transported from its cutaneous portal of entry to the peripheral nervous system, and is mostly restricted to neurons of the sensory ganglia from where it can move back to the skin and mucosa. Most *in vitro* models of neuronal infection with alphaherpesviruses have therefore been established using sensory dorsal root or sympathetic ganglia neurons (Tomishima *et al.*, 2001). However, occasionally *in vivo* the virus may reach the central nervous system, where the hippocampus is a preferential and highly susceptible target of infection from where the virus may reactivate (Whitley & Gnann, 2002). Hippocampal cultures therefore constitute a relevant model of infection. Here, we used two recombinant HSV-1, containing GFP inserted at two different sites of the essential envelope protein gB, to study the subcellular localization and processing of the glycoprotein in cells of epithelial lineage and in primary differentiated and polarized hippocampal neurons. Our results provide support to the hypothesis of separate transport of capsids and envelope proteins in the processes of neurons, and argue in favour of the role of the cytosolic domain of gB in protein maturation necessary for axonal transport.

The long ectodomain of gB tolerates insertion of GFP, while preserving the functionality of the resulting protein and virus infectivity (Potel *et al.*, 2002). Here, we showed that GFP-gB was biochemically processed similarly as native gB. In infected Vero cells, GFP-gB accumulated in the Golgi complex and at the cell surface. Its physical proximity to capsids as well as to another envelope glycoprotein, gD, strongly suggests that virus particle assembly occurs at these sites. In infected hippocampal neurons, however, GFP-gB puncta resembling vesicles were distinct from nucleocapsids and nucleocapsid proteins in the neuronal body as well as along the processes of neurons, and no obvious superimposition of nucleocapsid and gB markers could be detected by immunofluorescence staining. These observations are in agreement with the hypothesis first proposed by Penfold *et al.* (1994), that capsids are not enveloped in the cell body but rather move separately from other viral components, in order to acquire their final envelope near the axon terminal, thus permitting assembly of infectious particles at the site where the virus is to be delivered. Similar results were reported in a model of pseudorabies virus infection of sensory dorsal root neurons (Smith *et al.*, 2001). Exactly how and where assembly and egress of mature virions occur from these cells remains to be investigated.

The cytosolic domain of gB is one of the longest among herpesviruses glycoproteins, and has been extensively studied (Baghian *et al.*, 1993; Cai *et al.*, 1987, 1988a, b; Gilbert *et al.*, 1994; Rasile *et al.*, 1993; Raviprakash *et al.*, 1990). With the aim to further investigate the function of this domain, we constructed a virus where GFP was inserted into the carboxy-terminal domain of the protein. Previous

analyses of the post-translational processing of gB polypeptides in transfected cells showed that neither reduction of the cytoplasmic domain of gB by up to 43 residues (Butcher *et al.*, 1990) nor insertion of a few amino acids at position 45 of the cytoplasmic domain (Navarro *et al.*, 1991) prevented the intracellular transport of the glycoprotein. In this study however, insertion of GFP in-frame within the carboxy-terminal domain of gB resulted in a virus which was unable to propagate in non-complementing cells. This was due to gBct-GFP, as shown in a complementation assay where gBct-GFP provided *in trans* was unable to restore infectivity of the gB-null virus K082. Insertion of GFP might have changed a structural feature of gB, leading to misfolding of the fusion protein which would prevent its dissociation from ER chaperones, since mutated misfolded forms of gB are retained in the ER (Laquerre *et al.*, 1998; Navarro *et al.*, 1991; Zheng *et al.*, 1996). Retention of gBct-GFP in the ER might also be due to disruption of a targeting signal localized in the carboxy-terminal tail of gB, similar to those found in herpesviruses gB homologues (Heineman & Hall, 2002; Heineman *et al.*, 2000; Meyer & Radsak, 2000). Current studies are under way to further investigate this hypothesis. Whatever the mechanism underlying the block in gBct-GFP transport, the molecule was retained in the ER and did not undergo the final steps of glycosylation. However, the defect in gBct-GFP did not prevent the maturation and sorting of another envelope protein, gD, in cells infected with KgBct-GFP.

Embryonic hippocampal neuron cultures were used to analyse the transport of gB during HSV-1 infection. As observed in epithelial cells, the punctate pattern of the functional GFP-gB in neurons suggested that the molecule exited from the ER, but whether this aspect is related to transport to the cell surface, endocytosis or elements located at the cell surface is not known. Local sites of brighter fluorescence observed in the long processes of neurons could be related to local enlargements due to attachment to the substratum, or to sites of contact with the processes of other neurons. The reason for using embryonic hippocampal neurons is that these cells can be stably polarized and well-differentiated under certain conditions (Dotti *et al.*, 1988), and they have provided the support for studies focused on the sorting of viral membrane proteins. For example, using a temperature-sensitive mutant of vesicular stomatitis virus, glycoprotein G appeared to be polarized to the somato-dendritic domain, whereas haemagglutinin of a wild-type influenza virus was polarized to the axon. These studies suggested that similar mechanisms may control sorting in epithelial cells and in neurons, and showed that many apical and basolateral proteins are axonal and somato-dendritic respectively, although some proteins contradict this hypothesis (Dotti *et al.*, 1993; Dotti & Simons, 1990; Jareb & Banker, 1998; Ledesma *et al.*, 1998). In this study, by performing infection at low virus titres so that infected neurons are separated from each other, and by using a dendrite-specific marker, we could distinguish between the somato-dendritic and the axonal distribution of HSV-1 gB. In KGFP-gB infected cells, GFP-gB was sorted to the axon as well as the dendrites of hippocampal neurons. In contrast, in KgBct-GFP-infected cultures, gBct-GFP, which was retained in the ER, was restricted to the somato-dendritic compartment of neurons. Various axonal and

dendritic sorting signals have been identified in membrane proteins and many of these are located in the cytosolic domains (Bradke & Dotti, 1998). Herpesviruses homologues of gB have been shown to contain targeting sequences contained within their cytosolic domain. For example, an acidic cluster in the cytosolic domain of human cytomegalovirus gB is a key determinant for targeting gB to apical membranes in epithelial cells (Tugizov *et al.*, 1996). The role of such motifs in neuronal sorting has not been investigated. Axonal sorting of gB could be related to specific interactions with other viral proteins during infection. Recently, a model for pseudorabies virus sorting in sympathetic cervical neurons has been proposed, where membrane proteins such as gB would traffic in axons via an interaction with another viral protein, US9, yet still interact via their cytoplasmic tail with transport vesicles to traffic in other regions of the neuron (Tomishima *et al.*, 2001). KgBct-GFP, which contains a selective defect in gB maturation and subsequently axonal sorting, should prove useful in the analysis of the role of protein interactions in the sorting of viral components and subsequent virus maturation.

In summary, we showed that in infected hippocampal neurons, GFP-gB was transported separately from nucleocapsids and nucleocapsid proteins in the neurites, and was sorted to the axon. In contrast, the ER-retained gBct-GFP was restricted to the soma and dendrites of differentiated infected neurons. Future comparative studies of infection performed with the defective KgBct-GFP or the functional KGFP-gB should constitute a valuable model to study host and viral factors involved in the polarized transport of gB and in assembly of HSV-1 in neurons.

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