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Nuclear translocation of mutagenized forms of human cytomegalovirus glycoprotein B (gpUL55)

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To define structural elements involved in translocation of human cytomegalovirus (HCMV) glycoprotein B (gB) to the inner nuclear membrane (INM) compartment, mutagenized gB derivatives with deletions of the potential membrane anchor domains or of portions of the cytoplasmic tail were stably expressed in human astrocytoma cells. Subcellular localization examined by immunofluorescence and cell fractionation suggested that all gB derivatives reached the INM; however, reduced amounts were found after deletion of the extreme carboxy terminus [amino acids 856–906; gB(Del3)]. Pulse-chase analysis revealed accumulation in nuclear fractions of all gB derivatives during the chase, except for gB(Del3), which exhibited impaired nuclear retention. A carboxy-terminal nucleoplasmin-like signal localized within the respective deletion may thus be involved in nuclear transport and retention of HCMV gB. Immunoprecipitation after ^{32}P-radiolabelling of the gB transfectants verified that the gB molecule is phosphorylated at a carboxy-terminal consensus motif for casein kinase II.

An essential aspect of eukaryotic cellular regulation concerns transport of functional proteins between cellular compartments. In this context, considerable progress has been made in understanding the mechanism of nuclear import of cytosolic proteins by interaction with a chaperon such as importin/karyopherin (Görlich *et al.*, 1995; Moroianu *et al.*, 1995). On the other hand, much less is known about the mode of translocation of transmembrane proteins into the inner nuclear membrane compartment (INM). So far, targeting to the INM of the lamin B receptor (LBR; Smith & Blobel, 1993; Soullam & Worman, 1993), the nuclear pore complex protein gp210 (Wozniak & Blobel, 1992) and herpes simplex virus type 1 glycoprotein B (HSV-1 gB; Raviprakash *et al.*, 1990; Rasile *et al.*, 1993; Gilbert *et al.*, 1994) has been investigated. In the case of gp210 and HSV-1 gB, the transmembrane domains appear to direct nuclear envelope localization. For LBR, controversial data have been reported; on the one hand, the view favoured was that the most carboxy-terminal of eight transmembrane domains served as the signal for INM localization (Smith & Blobel, 1993), while on the other hand, involvement of the amino-terminal nucleoplasmic tail was suggested (Soullam & Worman, 1993).

For human cytomegalovirus (HCMV), the presence of glycoprotein B (gB) in the INM of infected cells was shown in our previous study (Radsak *et al.*, 1990) using cell fractionation as well as immuno-electron microscopy. In order to address the question of nuclear translocation signals of this molecule, experiments were undertaken (i) to examine whether recombinant HCMV gB, which has been shown to be correctly processed in stable transfectants of human astrocytoma cells (U373; Reis *et al.*, 1993), is also transported to the INM in the absence of other viral gene products, and if so (ii) to determine which structural domains are involved in this process.

For this study, stable transfectants of U373 described previously (Reis *et al.*, 1993; Bold *et al.*, 1996; Reschke *et al.*, 1995), expressing either authentic HCMV gB or derivatives thereof with various deletions, were used. Transfectants gB(Mhd1) and gB(Mhd2) expressed gB derivatives with deletions of hydrophobic domains, hd1 and hd2, in the carboxy-terminal portion of the molecule (Reschke *et al.*, 1995); hd2 of HCMV gB has been shown previously to be essential as well as sufficient for membrane anchoring. Five additional transfectants (Bold *et al.*, 1996) were included; these expressed gB derivatives with defined deletions in the cytoplasmic tail, i.e. gB(Del1)–gB(Del5), the respective gB derivatives lacking amino acids (aa) 773–810, 811–855, 856–906, 780–825 or 826–870 (Fig. 1). Deletions 1–3 thus cover in sequential order the entire cytoplasmic tail whereas deletions 4 and 5 overlap deletions 1 and 2, and 2 and 3, respectively.

To examine the subcellular distribution of gB-specific antigen in the various transfectants, immunofluorescence with the gB-specific monoclonal antibody (MAb) 27-156 (Spaete *et al.*, 1988) was determined on coverslip cultures after fixation with 4% paraformaldehyde and permeabilization with 0·2% Triton X-100 (Bold *et al.*, 1996), using epifluorescence as well as confocal fittings of a Zeiss microscope (Axioplan) for evalu-

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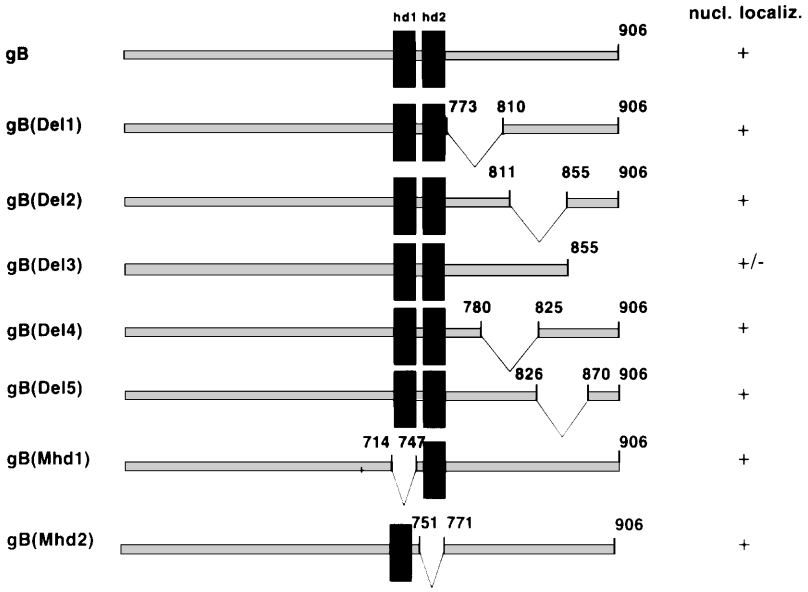

Fig. 1. Schematic presentation of deletions in HCMV gB derivatives expressed by stable transfectants. Nuclear compartmentation (nucl. localiz.) was determined by immunofluorescence (epifluorescence as well as laser scanning confocal fluorescence) and immunoblotting of nuclear fractions using gB-specific MAb 27-156. Corresponding results were obtained with these different techniques (see text).

ation (data not shown). The observations from several experiments are summarized in Fig. 1. Specific staining of the endoplasmic reticulum (ER) and the Golgi as well as nuclear rim staining was the same as that for authentic gB with all the gB derivatives except for gB(Del3), which expresses a gB with a deletion of the extreme carboxy terminus, and where nuclear rim fluorescence appeared somewhat reduced (see Fig. 1, nucl. localiz.). To substantiate these observations by a different experimental approach, parallel cultures (2×10^7 cells each) of the gB transfectants were subjected to fractionation as described previously (Radsak *et al.*, 1990) to obtain Triton X-100-treated nuclei devoid of the outer nuclear membrane (Bogner *et al.*, 1992) and post-nuclear fractions. Immunoblotting was performed (Bogner *et al.*, 1992) with aliquots of fraction extracts, using amounts of nuclear protein approximately five times in excess of post-nuclear fraction protein to account for the difference in cellular gB distribution. When immunostaining was carried out with MAb 1D3, which recognizes protein disulfide isomerase, a resident protein of the ER (Weis *et al.*, 1994), only post-nuclear but not nuclear fractions were stained, indicating that nuclear fractions were not contaminated by remnants of cytoplasmic membranes (data not shown).

Immunoreaction with MAb 27-156, on the other hand, yielded results consistent with the immunofluorescence analysis: gB-specific staining was observed for nuclear as well as post-nuclear fractions regardless of the deletions, except for transfectant gB(Del3), which exhibited reduced staining for the nuclear fraction (data not shown).

To further examine the time-course of subcellular transport of the mutant proteins, parallel cultures (2×10^7 cells each) of the transfectants were biosynthetically labelled with [^{35}S]methionine (70 μCi/ml; specific activity 1000 Ci/mmol) for 2 h and subsequently chased with excess unlabelled methionine for 4 h. The transfectant expressing authentic gB and HCMV strain AD169-infected U373 cultures at 72 h post-infection served as controls. Cell fractionation into nuclear and post-nuclear fractions as described above was carried out after the pulse as well as after chase intervals prior to immunoprecipitation of extracts with MAb 27-156, separation of the precipitates by SDS–PAGE under reducing conditions and fluorography (Radsak *et al.*, 1990; Fig. 2). The aliquots of nuclear- and post-nuclear fraction extracts used were again adjusted to account for the difference in cellular gB distribution (see above). In the case of post-nuclear fractions of infected cells, gB precursor of approximately 150 kDa was precipitated from pulse samples, and from chase samples cleavage products of about 100 kDa and 55 kDa were recovered in addition (Fig. 2, U373 AD169, post., lanes pulse and chase; Britt & Vugler, 1989); precipitates from nuclear fractions of infected U373 contained only uncleaved precursor (Radsak *et al.*, 1990), predominantly in the chase sample (Fig. 2, U373 AD169, nuc., lanes pulse and chase). One likely interpretation for the observed distribution of gB-specific label in pulse and chase samples from infected cells is that radiolabel in the post-nuclear pulse sample represents biosynthesis of gB in the ER, and that in the chase samples results from subsequent accumulation of gB in the nuclear compartment and cellular transport of gB along the exocytic pathway, respectively. As for the transfectants, this experimental approach revealed the presence of gB precursor in pulse as well as chase samples of post-nuclear fractions (Fig. 2, gB transfectants, post., lanes pulse and chase). The results shown for gB(Del1) and gB(Del5) are representative of those for gB(Del2) and gB(del4) also (not shown). It has been shown previously that proteolytic processing of the gB derivatives is not efficient in these transfectants, whereas correct exocytic transport to the surface membrane is unimpaired (Bold *et al.*, 1996). In nuclear fractions

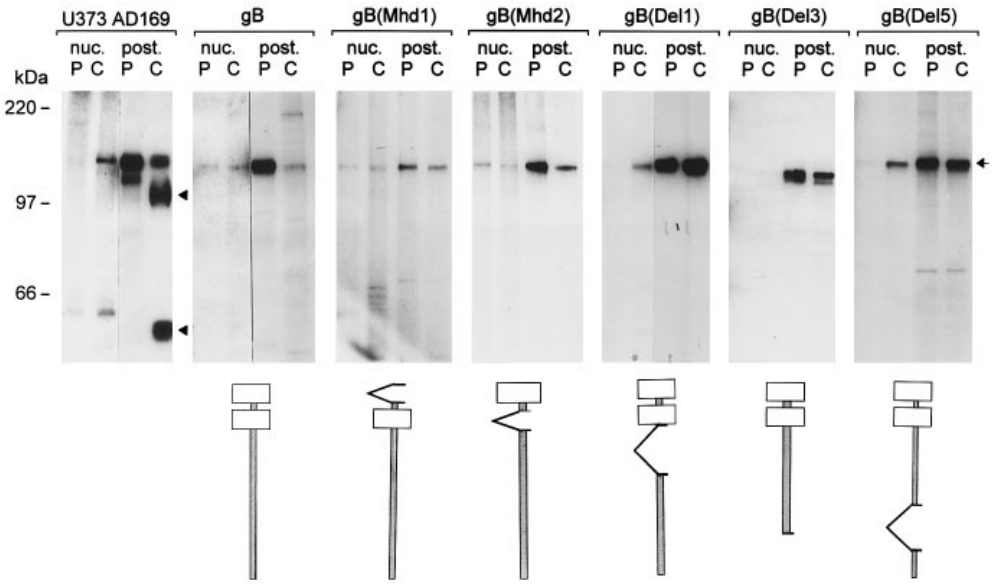

Fig. 2. Analysis under pulse-chase conditions of HCMV strain AD169-infected U373 (U373 AD169) and transfectants expressing authentic gB (gB), derivatives with deletions of hydrophobic domain 1 or hydrophobic domain 2 [gB(Mhd1) and gB(Mhd2)] or derivatives with deletions in the cytoplasmic tail [gB(Del1) (gB-Del3) and (gB-Del5)]. Parallel infected (m.o.i. 3; 72 h p.i.) or transfectant cultures (2×10^7 cells each) were biosynthetically radiolabelled with [^{35}S]methionine (70 μCi/ml; specific activity 1000 Ci/mmol) for 2 h and a consecutive chase with excess unlabelled methionine for 4 h. After the pulse as well as after chase intervals cells were fractionated into nuclear fractions including INM (nuc.) and post-nuclear fractions (post.). Aliquots of fraction extracts were immunoprecipitated with monoclonal antibody MAb 27-156. To account for the difference in subcellular gB distribution the amounts of corresponding nuclear extracts used were five times in excess of the amounts used of cytoplasmic extracts. The precipitates from pulse (P) and chase (C) samples were separated by SDS–PAGE (8% polyacrylamide) under reducing conditions and analysed by fluorography. On the left the positions of the molecular mass markers (M) myosin (220 kDa), phosphorylase b (97 kDa) and bovine serum albumin (66 kDa) are indicated; the arrow on the right of panel gB(Del5) indicates the position of gB precursor, and the triangles on the right of panel (U373 Ad169) indicate the position of gB-specific proteolytic cleavage products of about 100 kDa and 55 kDa.

of most transfectants gB-specific radiolabel was again consistently prominent in chase samples, as observed also for nuclei from infected cells except those expressing gB derivatives with hd deletions, where significant amounts of gB were present already in pulse samples (Fig. 2, gB transfectants, nuc., lanes pulse and chase). The most obvious exception, however, was gB(Del3) where little if any gB-specific radiolabel was precipitated from the chase samples [Fig. 2, gB(Del3), nuc., lanes pulse and chase]. This result was in line with the finding of reduced amounts of nuclear gB in the case of gB(Del3), as described above (Fig. 1), and furthermore implied that deletion of the extreme carboxy terminus significantly interfered with accumulation of the gB molecule in the nuclear compartment. Given that the gB derivative in gB(Del5), which has an overlapping deletion (see Fig. 1), exhibited unimpaired nuclear transport, the result for gB(Del3) suggested that a relevant signal for nuclear translocation may be located within the 36 carboxy-terminal amino acids of the HCMV gB molecule (see below).

It has been reported recently that authentic HCMV gB expressed in U373 is phosphorylated, whereas a truncated gB form is not, suggesting that at least one of several potential phosphorylation sites in the cytoplasmic tail is utilized (Tugizov *et al.*, 1995). To map the phosphorylation site more precisely, parallel cultures ($1{\cdot}5 \times 10^6$ cells each) of our transfectants were biosynthetically labelled with [^{32}P]orthophosphate (400 μCi/ml; specific activity 3000 Ci/mmol) for 6 h. Immunoprecipitation of extracts with MAb 27-156, as described above, revealed gB-specific radiolabel for authentic gB as well as for mutagenized forms, except for the gB derivative lacking the extreme carboxy terminus (Fig. 3, lane gB-Del3). This finding suggested (i) that gB in our stable human transfectants was most likely phosphorylated exclusively at the carboxy-terminal cellular casein kinase II consensus motif (SXXE) at amino acid position 900–903, and (ii) that likely conformational alterations resulting from deletions 1, 2, 4 and 5 did not interfere with phosphorylation. This notion extends a recent report demonstrating that soluble recombinant HCMV gB is phosphorylated at the serine residue at position −7 from the carboxy terminus (Norais *et al.*, 1996).

The results presented here extend previous observations on processing, transport and function of recombinant HCMV gB (Reis *et al.*, 1993; Bold *et al.*, 1996; Reschke *et al.*, 1995) by showing that this molecule is also correctly translocated to the INM in the absence of other viral products. Regarding the structural domains of the molecule involved in nuclear compartmentation, data described here are in line with a recent report on the gB homologue of Epstein–Barr virus (EBV),

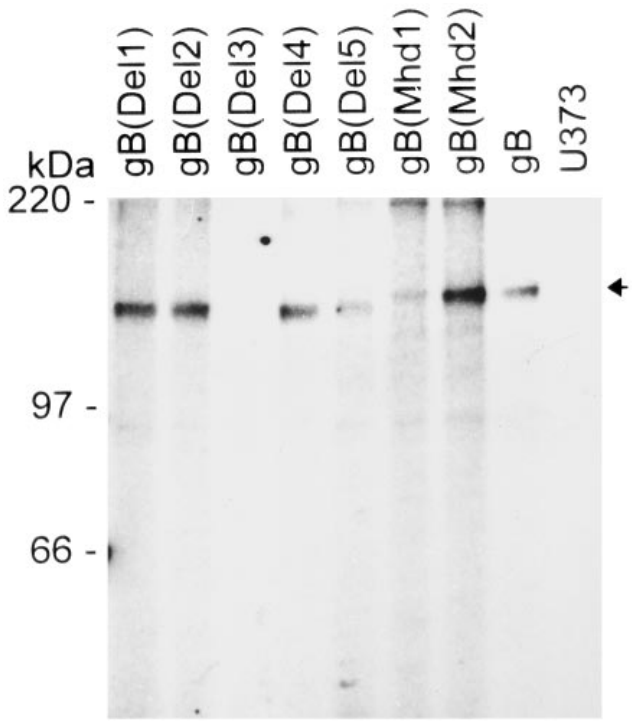

Fig. 3. Mapping of the phosphorylation domain of HCMV gB. Parallel cultures ($1{\cdot}5 \times 10^6$ cells each) of transfectants [gB(Del1)–(Del5), gB(Mhd1), gB(Mhd2)] and U373 cells (U373) were biosynthetically radiolabelled with [^{32}P]orthophosphate (400 μCi/ml; specific activity 3000 Ci/mmol) for 6 h prior to extraction, immunoprecipitation with MAb 27-156, separation of the precipitates by SDS–PAGE (8% polyacrylamide) and fluorography. The molecular mass markers indicated on the left were identical to those in Fig. 2; the arrow on the right indicates the position of gB precursor.

which appears to have a nuclear localization signal in the cytoplasmic tail (Lee & Longnecker, 1996). Interestingly, the segment of 23 amino acids (aa 818–840 of EBV gB) shown to contain the signal is located near the carboxy terminus and has an obvious cluster of basic residues (aa 823–839; see below). On the other hand, for the HSV-1 gB homologue it has been suggested that the membrane anchor domain alone directs INM localization and that the cytoplasmic tail is not involved (Raviprakash *et al.*, 1990; Gilbert *et al.*, 1994). This conclusion was based on quantification of the subcellular distribution of gB derivatives with shortened cytoplasmic tails, or of various gB–vesicular stomatitis (VSV) G protein chimeras. In our opinion, the data reported for HSV-1 gB do not entirely exclude a functional role of the cytoplasmic tail because several cytoplasmic tail mutants were found in the nuclear compartment in reduced amounts, an observation that remained unexplained. Pulse-chase analysis, as described here, which should reflect the dynamic process of nuclear transport more precisely, was not done.

Regarding the present observations on herpesvirus gB homologues, we favour the view that both the membrane anchor domain as well as the cytoplasmic tail may be involved in nuclear localization, possibly serving different functions: i.e. translocation to and retention in the INM. In principle, for constitutive nuclear translocation, e.g. by lateral diffusion, the anchor domain should be needed. Our finding that HCMV gB was translocated to the nuclear compartment (Figs 1 and 2), in spite of the deletion of hd2 which has been shown to be essential for membrane anchoring (Reschke *et al.*, 1995), does not apparently fit with this notion; however, one likely interpretation for this observation may be that for a portion of the mutagenized molecules hd1 may substitute for hd2 as a membrane anchor (Rasile *et al.*, 1993). It is noteworthy in the context of nuclear translocation that the carboxy terminus of HCMV gB has an accumulation of basic amino acids (**K**GQ**K**PNLLD**R**L**R**H**RK**, aa 877–892) that recalls the sequence of the nucleoplasmin nuclear localization motif (Robbins *et al.*, 1991), i.e. a cluster of basic amino acids flanked by polar or hydrophobic residues. It may be speculated that such a signal domain is needed for interaction with a cytosolic chaperon to support efficient translocation to the INM. Nuclear retention, on the other hand, may be achieved by binding of gB to a cellular factor in the nucleus. In the case of LBR, an authentic cellular transmembrane glycoprotein of the INM, a chromatin binding protein has been discussed as a likely ligand (Worman *et al.*, 1990). It remains unclear at present whether carboxy-terminal phosphorylation of the gB molecule plays a role in this context. Experiments are underway to study the mechanism of nuclear translocation and retention of HCMV gB in more detail.

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