Identification of a Novel Signal Sequence That Targets Transmembrane Proteins to the Nuclear Envelope Inner Membrane*

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Herpesvirus maturation requires translocation of glycoprotein B homologue from the endoplasmic reticulum to the inner nuclear membrane. Glycoprotein B of human cytomegalovirus was used in this context as a model protein. To identify a specific signal sequence within human cytomegalovirus glycoprotein B acting in a modular fashion, coding sequences were recombined with reporter proteins. Immunofluorescence and cell fractionation demonstrated that a short sequence element within the cytoplasmic tail of human cytomegalovirus glycoprotein B was sufficient to translocate the membrane protein CD8 to the inner nuclear membrane. This carboxyl-terminal sequence had no detectable nuclear localization signal activity for soluble β-Galactosidase and could not be substituted by the nuclear localization signal of SV40 T antigen. For glycoprotein B of herpes simplex virus, a carboxyl-terminal element with comparable properties was found. Further experiments showed that the amino acid sequence DRLRHR of human cytomegalovirus B (amino acids 885–890) was sufficient for nuclear envelope translocation. Single residue mutations revealed that the arginine residues in positions 4 and 6 of the DRLRHR sequence were essential for its function. These results support the view that transmembrane protein transport to the inner nuclear membrane is controlled by a mechanism different from that of soluble proteins.

The nuclear envelope consists of three morphologically distinct membrane domains (1, 2): (i) the outer nuclear membrane, which is contiguous with the membranes of the rough endoplasmic reticulum (RER), 1 (ii) the pore membranes, which connect outer and inner nuclear membranes and are associated with the nuclear pore complexes, and (iii) the inner nuclear membrane (INM), which is adjacent to the nuclear lamina, a mesh-work of intermediate filament proteins termed lamins (3). Inner and outer nuclear membranes enclose the perinuclear space that continues into that of the RER. While the outer nuclear membrane shares the membrane proteins of the RER, the INM contains a distinct set of integral proteins (4), including the lamin B receptor (LBR) (5) and the lamina-associated polypeptides 1 and 2 (LAP1 and LAP2) (6, 7).

In cells that are virus-infected, the various cellular membranes are decorated with specific viral proteins in addition. In the case of herpesviruses that undergo a characteristic nuclear phase of maturation, viral transmembrane glycoproteins are also translocated from the site of their synthesis into the INM, e.g., the glycoprotein B (gB) homologues of Epstein-Barr virus (8, 9), herpes simplex virus type 1 (HSV-1) (10), and human cytomegalovirus (HCMV) (11). The specific localization in the INM not only suggests a role of these proteins in herpesvirus envelopment at the inner nuclear membrane (12) but also implies that specific mechanisms mediate their transport from the RER to the INM compartment. It is currently not precisely known how this apparently specific targeting of cellular and viral transmembrane proteins is achieved. For soluble proteins, there is clear evidence that nuclear transport depends on specific sequence motifs, e.g., the nuclear localization signal (NLS) of the SV40 T antigen (13, 14) and the nuclear export signal of HIV Rev protein (15). Such signal sequences have been successfully used for e.g., nuclear targeting of reporter proteins. This observation raised the question of whether comparable signal sequences might exist to target transmembrane proteins to the INM. In previous reports, this aspect was studied for the cellular INM integral proteins LBR and LAP2 as well as the gB homologues of HSV-1 and HCMV. In the case of LBR, two nonoverlapping INM targeting signals have been identified (16–18); i.e., the nuceloplasmic amino-terminal domain as well as the first transmembrane segment appeared to confer nuclear translocation. In the case of LAP2, a type II integral membrane protein, the domain required for targeting to the INM has been restricted to 76 amino-terminal amino acids (19, 20). For HSV-1 gB, it has been suggested that an INM localization signal resides in the membrane anchor domain (21–23). Our previous experiments have shown that in the case of HCMV gB, the nucleoplasmic COOH-terminal 51 amino acids are essential for targeting to the INM (24).

We then asked if a specific signal within this domain might function in a modular fashion to mediate localization of a reporter protein to the INM and also what are the specific sequence requirements for such a function. Further experiments were performed to answer the question of signal conservation between the gB homologues of different herpesviruses. Experimental evidence is presented in this study that a short sequence element (amino acids 865–906) within the cytoplasmic tail of HCMV gB exhibits the specific potential to localize the type 1 transmembrane protein CD8 used as a reporter into the INM. This signal sequence had no detectable NLS activity for a soluble reporter protein and could not be replaced by the NLS of SV40 T antigen. A comparable element was identified in HSV-1 gB. The sequence sufficient for INM localization of

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† To whom correspondence should be addressed. Tel.: 49-6421-2864315; Fax: 49-6421-2865482; E-mail: radsak@mailer.uni-marburg.de.
‡ The abbreviations used are: RER, rough endoplasmic reticulum; ER, endoplasmic reticulum; INM, inner nuclear membrane; LBR, lamin B receptor; LAP1 and -2, lamina-associated polypeptide 1 and 2, respectively; gB, glycoprotein B; HSV-1, herpes simplex virus type 1; HCMV, human cytomegalovirus; NLS, nuclear localization signal; PCR, polymerase chain reaction; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s).
Construction of expression plasmid pRC/CMV-gB coding for HCMV gB (25) and pRC/CMV-gB(Del3) coding for HCMV gB lacking residues 865–906, the plasmid pCDNA3.1(−) Myc/His/A (Invitrogen, San Diego, CA, USA) was digested with BamHI and HindIII, and an insert encoding the residues 865–906 of HCMV gB (template for PCR: pRC/CMV-gB) was inserted between these restriction sites. The following pair of oligonucleotides was used: 5′-CGGGATCCACAGATTCTTTGGACGAGCTGAG-3′ and 5′-CCAAACCTTGACTTACGCGTCCG-3′.

Plasmid Encoding β-Galactosidase/HCVMVgB 865–906—For the generation of the plasmid encoding β-galactosidase/HCVMVgB 865–906, the plasmid pCDNA3.1(−) Myc/His/A (Invitrogen, San Diego, CA, USA) was digested with BamHI and HindIII, and an insert encoding the residues 865–906 of HCMV gB (template for PCR: pRC/CMV-gB) was inserted between these restriction sites. The following pair of oligonucleotides was used: 5′-CGGGATCCACAGATTCTTTGGACGAGCTGAG-3′ and 5′-CCAAACCTTGACTTACGCGTCCG-3′.

Plasmid Encoding HCMVgB 885D-A, 886R-A, and 890R-E—To construct the plasmid encoding HCMVgB 885D-A, 886R-A, and 890R-E, the GeneEditor™ in vitro site-directed mutagenesis system (Promega, Madison, WI) was used as recommended by the supplier. The following oligonucleotide was used: 5′-GCTTACGCTAG- TACGCGTTCGACAGCGCGGAAAGG-3′ and 5′-CGGGATCCACAGATTCTTTGGACGAGCTGAG-3′.

Construction of expression plasmid HCMVgB 865–906-NLS SV40 T Antigen—To construct the plasmid encoding HCMVgB 865–906-NLS SV40 T antigen, the plasmid pCDNA3 (Invitrogen) was digested with BamHI and XbaI, an insert generated with the following oligonucleotides was inserted: 5′-CGGGATCCACAGATTCTTTGGACGAGCTGAG-3′ and 5′-CCAAACCTTGACTTACGCGTCCG-3′.

Plasmid Encoding HCMVgB 885D-A, 886R-A, and 890R-E—To construct the plasmid encoding HCMVgB 885D-A, 886R-A, and 890R-E, the GeneEditor™ in vitro site-directed mutagenesis system (Promega, Madison, WI) was used as recommended by the supplier. The following oligonucleotide was used: 5′-GCTTACGCTAG- TACGCGTTCGACAGCGCGGAAAGG-3′ and 5′-CGGGATCCACAGATTCTTTGGACGAGCTGAG-3′.

Cells or isolated nuclei were washed with PBS after each step of fixation and antibody incubation. Transfected cells on coverslips and cell fractions with cells grown in Petri dishes of 145-mm diameter were fixed by cell fractionation with cells grown in Petri dishes of 145-mm diameter. Transfection efficiencies were determined to range between 30 and 40% by immunofluorescence with monoclonal antibodies specific for the expression products.

**Immunofluorescence Microscopy**

Cells or isolated nuclei were washed with PBS after each step of fixation and antibody incubation. Transfected cells on coverslips as well as fixed nuclei on glass slides were fixed with acetone at −20°C for 15 min. Fixed samples were incubated with one of the following primary antibodies: HCMV gB-specific monoclonal antibody (mAb) 27-156 (29), HSV-1 gB-specific mAb 2c (30), CD8-specific mAb OKT8 (31), or β-galactosidase-specific mAb (Promega), followed by a 30-min incubation with rabbit anti-mouse IgG-fluorescein isothiocyanate (1:40 diluted in 3% bovine serum albumin/PBS; Dakopatts, Hamburg, Germany). Preparations were evaluated with a Zeiss fluorescence microscope.
Nuclear Envelope Targeting of Transmembrane Proteins

Radioactive Labeling of Cells

Transfected COS7 cells in 145-mm diameter Petri dishes (3 × 10⁶ cells) were washed with PBS and refed with 10 ml of culture medium lacking methionine for 1 h. Radiolabeling was carried out with 50 μCi of [³⁵S]methionine (specific activity of 1,000 Ci/mmol; Amersham Pharmacia Biotech, Braunschweig, Germany)/ml of methionine-free culture medium for 3 h.

Cell Fractionation, Preparation of Extracts, and Immunoprecipitation

All buffers used for cell fractionation contained 100 units of aprotinin/ml and 0.3 mM phenylmethylsulfonyl fluoride. Cell cultures were washed twice with cold PBS, harvested by scraping into cold PBS, and collected by sedimentation (1,000 × g for 5 min). Cell pellets were resedimented in hypotonic buffer (TKM; 25 mM Tris-Cl, pH 7.4, 5 mM KCl, and 1 mM MgCl₂) and resuspended in 1 ml of the same buffer at 4 °C for 5 min prior to the addition of TKM containing 1% Nonidet P-40 (23). The mixture was incubated with occasional vortexing for 5 min on ice. After incubation, an aliquot of 200 μl was saved (homogenate fraction). Nuclei were sedimented by centrifugation at 1,000 × g for 5 min, and the supernatant fraction was used as the postnuclear fraction. The nuclear pellet was resuspended in TKM containing 0.5% Nonidet P-40 and 100 mM NaCl and resedimented at 2,100 × g for 15 min through a 5-ml cushion of 1.62 M sucrose in TKM. The pelleted nuclei were washed with TKM containing 0.5% Nonidet P-40 and 100 mM NaCl. The quality of the nuclear fraction was examined by phase-contrast microscopy.

In some instances, cell fractionation was carried out by an alternative procedure exploiting density properties for separation of nuclear membrane fractions as described by Graham (32). In brief, following harvest in PBS, cells (5 × 10⁸) were resuspended in 3 ml of TKM and broken with approximately 10 strokes of a type S pestle of a Dounce homogenizer. Subsequently two volumes of 2.3 M sucrose were added to bring sucrose concentration to 1.62 M, and the mixture was layered over a 3-ml cushion of 1.62 M sucrose in TKM. By ultracentrifugation at 130,000 × g for 30 min in a SW41 rotor of a Beckman centrifuge, nuclei were sedimented through the cushion to the bottom of the tube, whereas membranes remained at the interface. Nuclei were consecutively washed in PBS or PBS containing 0.5% Triton X-100 prior to a final washing step in PBS.

For immunofluorescent staining of isolated nuclei, an aliquot of the nuclear fraction was air-dried on glass slides. For immunoprecipitation, the nuclear pellet was lysed in nuclear lysis buffer (25 mM Tris-Cl, pH 7.4, 300 mM NaCl, 0.5% Nonidet P-40, and 0.5% deoxycholate). Postnuclear and homogenate fractions were adjusted to the identical concentrations of salt and detergents of the lysis buffer. All fractions were centrifuged at 125,000 × g for 30 min prior to use.

Homogenate fraction, nuclear fraction, and 1/4 of the postnuclear fraction, respectively, were precleared by incubation with protein A-Sepharose CL4B beads (Sigma) prior to incubation overnight at 4 °C with mAb 27-156, mAb 2c, or mAb OKT8. Immunocomplexes were adsorbed for 2 h at 4 °C onto protein A-Sepharose CL4B beads coated with rabbit anti-mouse IgG (Dakopatts). Following six cycles of washing with detergent buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.02% bovine serum albumin, 100 units of trypsin/ml, and 0.3 mM phenylmethylsulfonyl fluoride) and one washing cycle with TN buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 100 units of trypsin/ml, and 0.3 mM phenylmethylsulfonyl fluoride), the precipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (33) and consecutive fixation and fluorography (34) of the dried gels.

PRELIMINARY EXPERIMENTS

Preliminary experiments were carried out in order to substantiate that the experimental approach of cell fractionation and consecutive immunoprecipitation reliably separated RER-resident proteins from those in the INM (4, 23). For this purpose, parallel cultures of COS7 cells were transfected with expression plasmids pRC/CMV-gB encoding INM-bound authentic HCMV gB (25) or with pCMUV-CD8/E19 encoding an ER-resident CD8 chimera (27) (Fig. 1A). Transfected cultures were radiolabeled at 48 h posttransfection and subjected to analysis by the combined procedures of cell fractionation and immunoprecipitation as described above. The results shown in Fig. 1B (a) demonstrate that HCMV gB was recovered from nuclear as well as postnuclear fractions (lanes N and PN), whereas CD8/E19 was precipitated from postnuclear but not from nuclear fractions (Fig. 1B (b), lanes PN and N). This observation strongly suggested that the separation procedure used adequately served the experimental purpose.

RESULTS

The Carboxyl-terminal Domain of the Cytoplasmic Tail of HCMV gB Is Sufficient to Confer Targeting to the INM—Our previous experiments have shown that translocation of recombinant HCMV gB to the INM was dependent on the 51 carboxyl-terminal amino acids (24). In order to address the question of whether the putative signal within this domain was transferable, chimeras of defined segments of HCMV gB and the plasma membrane protein CD8 as a reporter were constructed (Fig. 2A). Following transfection of respective expression plasmids into COS7 cells, subcellular localization of the chimeric proteins was examined by immunofluorescence (Fig. 2B). In order to distinguish between localization of the chimeras in the RER or INM (see “Experimental Procedures”) immunoprecipitations from nuclear and postnuclear fractions of radioactively labeled transfected cells (Fig. 2C) were carried out as well. For this purpose, nuclear fractions were prepared by treatment with nonionic detergent which removes outer nuclear and RER membranes and does not affect the integrity of the INM (23, 4).

Immunofluorescence of the transfected cells with the appropriate monoclonal antibodies yielded consistent images for each expression product, as depicted in Fig. 2A. For wild-type gB (Fig. 2A, a) characteristic staining was obtained of the RER and the nuclear envelope (Fig. 2B, a), which corresponded to...
Intracellular localization of transmembrane CD8/HCMV gB chimeras. A, a–f, plasmids were constructed encoding authentic HCMV gB or CD8 or CD8/HCMV gB chimeras. Filled and open boxes in the diagrams shown represent HCMV gB- and CD8-specific protein segments; predicted membrane anchor domains are enlarged. Numbers above and below the diagrams indicate positions of residues of the amino acid sequence of HCMV gB and CD8, respectively; I indicates the amino terminus. The gap sign (e) marks the position of a deletion. CT, cytoplasmic domain; TM, transmembrane domain. B, a–f, for intracellular localization of the recombinant products, subconfluent coverslip cultures of COS7 cells were transfected with the various constructs depicted in A for 48 h prior to fixation with cold acetone and indirect immunofluorescence staining using HCMV gB-specific mAb 27-156 or mAb OKT8. C, a–f, to examine nuclear compartmentalization of the recombinant products, transfected COS7 cells (3 × 10⁶ cells) were radiolabeled with [³⁵S]methionine as described under “Experimental Procedures” prior to cell fractionation, immunoprecipitation of fraction extracts with HCMV gB-specific mAb 27-156 or mAb OKT8, and analysis of precipitates by SDS-PAGE and fluorography. H, homogenate fraction; N, nuclear fraction; PN, postnuclear fraction.

that in infected or stably transfected cells described previously (24). Immunoprecipitations confirmed the presence of HCMV gB in solubilized nuclear fractions representing INM as well as postnuclear fractions representing solubilized cytoplasmic membranes including RER and plasma membranes (Fig. 2C, a). These results suggested that a significant portion of authentic HCMV gB was localized in the INM of transiently transfected COS7 cells. Transfections with authentic CD8 (Fig. 2A, b), on the other hand, resulted predominantly in cell surface and Golgi staining (Fig. 2B, b). As expected, CD8 was not detected in nuclear fractions by immunoprecipitation (Fig. 2C, b). Distribution of chimeric CD8 with the cytoplasmic tail of HCMV gB (Fig. 2A, c), however, again showed localization in the RER and at the nuclear rim (Fig. 2B, c), and immunoprecipitation demonstrated its presence in the nuclear fraction (Fig. 2C, c). Identical results were obtained when chimeras were examined consisting of the amino-terminal portion of CD8 including the transmembrane domain fused to carboxy-terminal residues 865-906 of HCMV gB (Fig. 2, A–C, d). As expected, the CD8 chimera with the cytoplasmic tail of HCMV gB lacking amino acids 865-906 was not localized in the INM (Fig. 2, A–C, e). Also, replacement of the membrane anchor of CD8 by that of HCMV gB (Fig. 2A, f) resulted in neither nuclear rim staining (Fig. 2B, f) nor immunoprecipitation from the nuclear fractions (2C, f).

These data suggest that the carboxyl-terminal 42 amino acids of HCMV gB are sufficient to translocate a heterologous transmembrane reporter protein to the INM.

The Carboxyl-terminal Signal of HCMV gB Is Functionally Distinct from the NLS of Soluble Proteins—Experiments were performed to examine functional analogies between a conventional NLS of soluble proteins and the signal residing at the carboxyl terminus of HCMV gB. For this purpose, the NLS of SV40 T antigen or amino acids 865-906 of HCMV gB were fused to soluble β-galactosidase (Fig. 3A, a), and subcellular localization of the transiently expressed chimeric proteins was examined by immunofluorescence. This approach resulted in the exclusive intranuclear detection of the HCMV gB chimera (3B, b), whereas for the β-galactosidase/HCMV gB chimera the cytoplasmic staining comparable with that of authentic β-galactosidase was observed (Fig. 3B, c and a). This observation suggested that the domain of HCMV gB was not a functional substitute for a conventional NLS for soluble proteins.

Vice versa, the effect of replacement of the carboxyl-terminal domain of HCMV gB by the conventional NLS on subcellular distribution was examined (Fig. 4, A–C, c). Authentic HCMV gB and HCMV gB with a deletion of the carboxyl-terminal domain served as controls (Fig. 4, A–C, a and b). The immunofluorescence pattern obtained for this chimera (Fig. 4B, c) equaled that of HCMV gB with the carboxyl-terminal deletion (Fig. 4B, b). Correspondingly, no significant amounts of the protein were recovered from nuclear extracts by immunoprecipitation (Fig. 4C, c). From these experiments it was concluded that the carboxyl-terminal domain of HCMV gB is functionally distinct from the NLS of soluble proteins.

Amino Acid Residues 832–867 of the Cytoplasmic Tail of HSV-1 gB Are Sufficient to Confer Targeting to the INM—In previous reports it has been suggested that transport of HSV-1 gB to the INM depends on the transmembrane domain (21–23). In order to reexamine this notion, defined portions of HSV-1 gB were fused to a portion of the CD8 protein (Fig. 5A, a–h).
Subcellular localization of the transiently expressed chimeric proteins was again determined by immunofluorescence (Fig. 5B) and confirmed by immunoprecipitation of the expression products from fraction extracts of radioactively labeled transfected cells (Fig. 5C). Cells expressing wild-type HSV-1 gB exhibited nuclear rim as well as RER staining (Fig. 5B, a), and, correspondingly, gB was specifically precipitated from nuclear and postnuclear fractions (Fig. 5C, a), which was in expected contrast to subcellular distribution of the authentic plasma membrane protein CD8 (Fig. 5, A–C, b). Replacement of the transmembrane domain of CD8 by that of HSV-1 gB (Fig. 5A, c) neither detectably altered the staining pattern (Fig. 5B, c) nor redirected this chimeric protein to the INM (Fig. 5C, c). In contrast, the CD8 chimera with the cytoplasmic tail of HSV-1 gB (Fig. 5A, d) was efficiently translocated to the INM (Fig. 5, B–C, d). Thus, under the conditions used here, the cytoplasmic tail but not the transmembrane domain of HSV-1 gB was sufficient to mediate transport to the INM. To further define the domain of the HSV-1 gB cytoplasmic tail more precisely, CD8 chimeras were created replacing the authentic cytoplasmic tail with various portions of the HSV-1 gB cytoplasmic tail (Fig. 5A, e–h). This approach revealed that, contrary to HCMV gB, the putative signal of HSV-1 gB does not reside in the extreme carboxyl terminus of the molecule (Fig. 5, A–C, e) but within the middle portion of the cytoplasmic tail (Fig. 5, A–C, f and h).

**Signal Domain Sequences of HCMV and HSV-1 gB Exhibit a Limited Homology**—To identify common features of the putative transport signals of HCMV and HSV-1 gB, deduced amino acid sequences of the domains in question, aa 865–906 of the HCMV and aa 832–867 of the HSV-1 gB sequence, were aligned using the program BESTFIT (Genetics Computer Group, Inc. (Madison, WI). As shown in Fig. 6, alignment revealed only limited homology between the two domains (similarity, 46.43%; identity, 10.7%). Using the PHD program (profile fed neural networks system, Heidelberg) in the case of HCMV gB a helical conformation was predicted for a segment including aa 882–890 following a proline residue in position 881 which is generally assumed to introduce a conformational turn. For HSV-1 gB, on the other hand, helical conformation was predicted for a longer segment extending from aa 837 to 865. For further identification of the putative HCMV gB signal, positively charged conserved residues (Lys877 HCMV gB versus Arg844 HSV-1 gB; Arg866 HCMV gB versus Arg858 HSV-1 gB; His869 HCMV gB versus His861 HSV-1 gB; Arg890 HCMV gB versus Lys862 HSV-1 gB) with predicted conformational exposure were chosen.

**Substitution of Amino Acids 885, 886, 889, and 890 in the HCMV gB Signal Domain Prevents Transport to the INM**—In order to analyze the functional role of the conserved charged residues (Asp885 → Ala, Arg886 → Ala, His889 → Ghr, and Arg890 → Ghr), corresponding substitutions were introduced in
the authentic molecule as well as in the chimera CD8/HCMV gB 865–906 (Fig. 7A, c and d). Subcellular distribution of the transiently expressed proteins was again examined by immunofluorescence (Fig. 1B, c and d) and immunoprecipitations as described above (Fig. 7C, c and d) using wild-type HCMV gB as well as wild-type CD8 as controls (Fig. 7, A–C, a and b). These specific simultaneous substitutions of residues 885, 886, 889, and 890 prevented transport of HCMV gB as well as that of the chimera CD8/HCMV gB 865–906 to the INM (Fig. 7, B and C, c and d).

This observation suggested a functional role of conserved amino acid residues 885, 886, 889, and 890 or at least a subset thereof for translocation of HCMV gB to the INM.

**Amino Acid Residues DRLRHR of HCMV gB Represent a Signal for Transport to the INM**—Subsequent experiments served to examine whether the short conserved amino acid hexamer 885–890 of HCMV gB was sufficient to localize a reporter protein to the INM. The amino acid sequence DRLRHR was therefore fused to CD8 (Fig. 8A), and subcellular localization of the transiently expressed chimeric protein was analyzed by immunofluorescence. Nuclear rim staining was obtained for the chimera (Fig. 8B, c) but not for native CD8 (Fig. 8B, b). This intracellular distribution was confirmed by immunoprecipitation of isolated transfected nuclei (Fig. 8C, a–c). This experimental approach was used in place of immunoprecipitation because chimeric proteins resisted sufficient solubilization.

To further substantiate this observation, fractionation of cells transfected with the chimeric constructs depicted in Fig. 9A, was carried out by a procedure described by Graham (32) that exploits defined density characteristics for purification of nuclei (see “Experimental Procedures”). Following isolation, purified nuclei were subjected to treatment with nonionic detergent or left untreated prior to immunofluorescence with appropriate specific antibodies (Fig. 9B). Untreated transfected nuclei exhibited specific staining as expected under the conditions used, indicating intactness of the entire nuclear envelope including the outer nuclear membrane, which is contiguous with the ER (Fig. 9B, Triton X-100 , −). After detergent treatment, which removes the outer nuclear membrane (i.e. ER-resident products), specific fluorescence signals were absent in the case of CD8- or CD8/E19-transfected nuclei but still persisted in those transfected with the constructs coding for HCMV gB or CD8/DRLRHR (Fig. 9B, Triton X-100 , +). This observation verified that the latter products were indeed also localized in the detergent-resistant inner lamella of the nuclear envelope.

These findings strongly supported the concept of a novel short and modular signal sequence that mediates transport to the INM.

**Arginine Residues in Positions 4 and 6 of the Novel Signal Sequence DRLRHR Are Essential for Its Function**—Mutational analysis was performed to further determine the functional relevance of single amino acid residues within the novel signal sequence. For this purpose, each residue of the sequence element was substituted individually by alanine in the CD8/DRL-
RHR chimera (Fig. 10A). Examination of subcellular distribution of the transiently expressed chimeric proteins by immunofluorescence (Fig. 10B) allowed the following conclusions. Substitutions of residues aa 885, 886, and 887 of HCMV gB did not detectably influence the intracellular distribution of chima; i.e. nuclear rim staining was still observed (Fig. 10B, c, d, and e), similarly to that exhibited by the positive control (Fig. 10B, b). On the other hand, substitution of residues 888 and 890 prevented perinuclear staining of the respective CD8/DRLRHR chimeras (Fig. 10B, f and h). The chimera with a substitution of residue 889 exhibited plasma membrane and Golgi staining in most cells; however, nuclear rim staining was still occasionally observed (roughly 10% of transfected cells). Intracellular distribution was again confirmed by immunofluorescent staining of isolated transfected nuclei (Fig. 10C, a–h).

These observations indicated that arginine residues in positions 4 and 6 of the sequence DRLRHR are essential for its function as an INM transport signal.

**DISCUSSION**

The results presented show that the amino acid sequence DRLRHR of HCMV gB (aa 885–890) was sufficient to mediate transport of a reporter type I transmembrane protein to the INM. A comparable signal was identified in the cytoplasmic tail of HSV-1 gB. Arginine residues in positions 4 and 6 of the HCMV gB signal sequence appeared to be essential for its function as a transport signal. Our experiments also imply that the underlying transport machinery for transmembrane proteins is distinct from the importin/karyopherin system responsible for nuclear import of soluble proteins. We thus propose to term this novel signal sequence as “localization signal for inner nuclear membrane” (LSINM).

Intracellular localization of the expressed chimeric proteins was analyzed by immunofluorescence staining of transfected cells. Nuclear rim staining was interpreted as indicative for INM localization; however, the observations by fluorescence microscopy needed to be confirmed by appropriate cell fractionation (23, 35) in order to definitely distinguish between outer and inner nuclear membranes prior to immunorecognition. In the fractionation protocols, Nonidet P-40 or Triton X-100 was used to obtain nuclei devoid of outer nuclear membrane com-

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**Fig. 6.** Amino acid sequence alignment of defined segments of the cytoplasmic tails of HCMV gB and HSV-1 gB. Numbers indicate positions of residues of the amino acid sequences of HCMV gB and HSV-1 gB, respectively; I indicates the amino terminus. Predicted membrane anchor domains of gB schemes are enlarged. Vertical lines and points between individual residues of the aligned sequences indicate identical or similar amino acids. Residues of the HCMV gB sequence in boldface type were substituted by the residues indicated above.

**Fig. 7.** Intracellular localization of HCMV gB and the chimera CD8/HCMV gB 865–906 with substitutions of amino acids 885, 886, 889, and 890. A, a–d, plasmids were constructed encoding authentic HCMV gB or CD8 or CD8/HCMV gB chimeras. Filled and open boxes in the diagrams shown represent HCMV gB- and CD8-specific protein segments; predicted membrane anchor domains are enlarged. Numbers above and below the diagrams indicate positions of residues of the amino acid sequence of HCMV gB and CD8, respectively; I indicates the amino terminus; white lines at the carboxyl terminus indicate substitutions of residues. B, a–d, for intracellular localization of the recombinant products, subconfluent coverslip cultures of COS7 cells were transfected with the various constructs depicted in A for 48 h prior to fixation with cold acetone and indirect immunofluorescence staining using HCMV gB-specific mAb 27-156 or mAb OKT8. C, a–d, to examine nuclear compartmentalization of the recombinant products, transfected COS7 cells (3 × 10⁶ cells) were radio-labeled with [³⁵S]methionine as described under “Experimental Procedures” prior to cell fractionation, immunoprecipitation of fraction extracts with HCMV gB-specific mAb 27-156 or mAb OKT8, and analysis of precipitates by SDS-PAGE and fluorography. H, homogenate fraction; N, nuclear fraction; PN, postnuclear fraction.
ponents (4, 23) prior to the specific immunorecognition procedures described. Under these conditions, the authentic plasma membrane protein CD8 as well as chimeric CD8 carrying the retention signal of the ER-resident adenoviral transmembrane protein E3/19K were not associated with nuclear fractions. These controls showed that nuclear fractions were not contaminated by remnants of cytoplasmic membranes and thus indicated that the fractionation procedures used were adequate. Further confirmation of the intracellular localization of the proteins examined by immunoelectron microscopy failed due to insufficient antibody binding to cryosections.

Regarding HSV-1 gB, our findings are in contrast with previous reports suggesting that the transmembrane domain is an essential transport signal for INM translocation (21–23). This discrepancy may reflect the different experimental approach taken by these authors, who used vesicular stomatitis virus G protein as a reporter. A problem with this approach is that vesicular stomatitis virus G is known to be constitutively trans-

FIG. 8. Intracellular localization of CD8 with a carboxyl-terminal extension by amino acid residues 885–890 of HCMV gB. A, a–c, plasmids were constructed encoding authentic HCMV gB or CD8 or CD8 extended by aa residues 885–890 of HCMV gB. Filled and open boxes in the diagrams shown represent HCMV gB- and CD8-specific protein segments; predicted membrane anchor domains are enlarged. Numbers above and below the diagrams indicate positions of residues of the amino acid sequence of HCMV gB and CD8, respectively; 1 indicates the amino terminus. B, a–c, for intracellular localization of the recombinant products, subconfluent coverslip cultures of COS7 cells were transfected with the various constructs depicted in A for 48 h prior to fixation with cold acetone and indirect immunofluorescence staining using HCMV gB-specific mAb 27-156 or mAb OKT8. C, a–f, to examine nuclear compartmentalization of the recombinant products, transfected COS7 cells (3 × 10^6 cells) were subjected to cell fractionation prior to indirect immunofluorescence of isolated transfected nuclei with HCMV gB-specific mAb 27-156 or mAb OKT8.

FIG. 9. Differentiation between ER or INM localization of recombinant products using extensively purified nuclei. A, a–d, plasmids encoding authentic HCMV gB, CD8, CD8/E19, or CD8/DRLRHR were used for transfection. Filled and open boxes in the diagrams shown represent HCMV gB- and CD8-specific protein segments; predicted membrane anchor domains are enlarged. Numbers above and below the diagrams indicate positions of residues of the amino acid sequence of HCMV gB and CD8, respectively; 1 indicates the amino terminus. B, a–d, to examine nuclear compartmentalization of the recombinant products, transfected COS7 cells (3 × 10^6 cells) were subjected to cell fractionation according to Graham (32) prior to indirect immunofluorescence of untreated (Triton X-100 −) or detergent-treated purified transfected nuclei (Triton X-100 +) with HCMV gB-specific mAb 27-156 or mAb OKT8.
ported into the INM (36). Based on our results, we suggest that amino acid residues 832–867 in the carboxyl-terminal portion of HSV-1 gB are the critical determinants of its transport to the INM. The variant positioning of the nuclear translocation motifs within the cytoplasmic tails of gB homologs of HCMV and HSV may indicate differences in their functional conformations.

Inner nuclear membrane targeting has been extensively studied for LBR and LAP2. In the case of LBR, the amino-terminal domain as well as the first transmembrane segment of LBR were reported to mediate nuclear localization (16–18). Interestingly, using the BESTFIT program (Genetics Computer Group), a signal motif comparable with that described here for HCMV gB was identified in the nucleoplasmic tail of LBR (aa 56–61 of the LBR sequence). It would be of interest in this context to examine whether this putative LBR signal is sufficient for vectorial reporter protein transport. For LAP2, which is also a type II transmembrane protein, translocation to the INM has been shown to be promoted by amino acid residues 298–370 in the nucleoplasmic amino terminus of the molecule (19, 20). Alignment did not reveal similarities to the HCMV gB signal sequence. In any case, vice versa substitutions should be carried out to possibly identify additional relevant signal motifs.

The results of the present study show that a structural motif different from those sufficient to target soluble proteins into the nucleus is needed to target a membrane protein to the INM. This strongly suggests that the two protein classes, transmembrane and soluble proteins, are transported to the nuclear compartment via distinct mechanisms.

It may be speculated that HCMV gB is transported to the INM by lateral diffusion along the interconnecting membranes of the RER/outer nuclear membrane and the nuclear pores/INM. This pathway was proposed for directed transport of the LBR (16–18, 37), the LAP2 (19, 20), and the pore membrane protein gp210 (38). The pore complex could potentially represent a topological barrier; however, structural studies have proposed that 10-nm diameter channels exist at the periphery of the pore complex (39, 40). Therefore, INM proteins should meet specific structural prerequisites. Recent studies have shown that elongation of the amino-terminal tail of the LBR indeed prevents accumulation of the protein in the INM (18). This observation is consistent with the proposed pathway.

Such lateral diffusion could be followed by trapping of proteins at the INM by binding interactions with nuclear proteins. The finding that LBR, LAP1, and LAP2 interact with lamins (7, 41) suggests that lamin binding in particular may function to retain proteins in the INM. Interestingly, the major nuclear envelope targeting domain of LAP2 appears to be identical with its lamin binding region (19).

Experiments are in progress to examine this notion for HCMV gB. Furthermore, the novel signal sequence of HCMV gB may be exploited as an appropriate experimental tool to

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**FIG. 10. Intracellular localization of the CD8/DRLRHR chimeras following individual substitutions in the sequence element DRLRHR by alanine.** 

A, a–h, plasmids were constructed encoding authentic CD8 or CD8 extended by DRLRHR or by DRLRHR with individual substitutions. Open boxes in the diagrams shown represent the CD8 protein; predicted membrane anchor domains are enlarged. Filled boxes represent the DRLRHR or mutagenized (white lines) DRLRHR extensions. Numbers below the diagrams indicate positions of residues of the amino acid sequence of CD8; those above indicate positions of amino acids of the HCMV gB signal. 1 indicates the amino terminus. B, a–h, for intracellular localization of the recombinant products, subconfluent coverslip cultures of COS7 cells were transfected with the various constructs depicted in A for 48 h prior to fixation with cold acetone and indirect immunofluorescence staining using mAb OKT8. C, a–h, to examine nuclear compartmentalization of the recombinant products, transfected COS7 cells (3 × 10⁶ cells) were subjected to cell fractionation prior to indirect immunofluorescence of isolated transfected nuclei with HCMV gB-specific mAb 27-156 or CD8-specific mAb OKT8.

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identify chaperone(s) representing a putative distinct machinery for translocation of transmembrane proteins to the INM.

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