Herpes Simplex Virus 2 UL45 Is a Type II Membrane Protein

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The genomes of herpes simplex virus types 1 and 2 (HSV-1 and -2) encode at least 11 glycoproteins, of which 10 have transmembrane anchor regions. Several additional open reading frames, such as UL45, encode proteins that have potential membrane-spanning segments but that lack consensus sites for the addition of asparagine-linked carbohydrates (N-CHO) (9). The HSV-1 UL45 protein (UL45-1) has 172 residues and a molecular mass of 18 kDa (11). It is not required for growth of virus in tissue culture (11) but is required for cell-cell fusion caused by a syncytial variant of HSV-1 (6). HSV-2 UL45 (UL45-2) is also a 172-residue protein, with 75% sequence identity to UL45-1. It was first identified as a 20-kDa protein by in vitro translation of transcripts from HSV-2-infected cells (10) and more recently was shown to cross-react weakly with an antibody raised against UL45-1 (12). To further characterize UL45-2, we have raised an antibody against a glutathione S-transferase (GST)–UL45-2 fusion protein and have made a UL45-2 expression vector; these reagents were used to study the time course of UL45-2 synthesis in infected cells, its association with membranes, its orientation in the membrane, and its cellular location.

A UL45-2 expression vector (pAC265) was derived from pGR44 (a gift from Gary Hayward), which contains the UL45-1 open reading frame. The HSV-1 UL45-1 coding sequence was amplified by PCR with primers 5'-TGGATCTCTGGGACTCGTGA-3' and 5'-TGGATCCGGTGACCACTG-3' (BamHI sites underlined); after digestion with BamHI, this fragment was cloned into the BamHI site of pGEX-4T-3 (Pharmacia), in frame with the GST gene, to produce pAC270. Residues 52 to 121 were chosen in order to avoid hydrophobic sequences that might cause the GST–UL45-2 fusion protein to aggregate.

GST–UL45-2 fusion protein was purified from E. coli containing plasmid pAC270, purified from a bacterial lysate with glutathione-Sepharose beads, and used to immunize rabbits (Cocalico Biologics, Inc., Reamstown, Pa.). The rabbits received an initial inoculation of 100 µg, followed by three 50-µg booster injections. Two sera, LSU32 and LSU33, were obtained. Preliminary Western blots showed that both reacted with GST, with GST–UL45-2, and with many proteins in uninfected CV1 and COS7 cells (data not shown). To reduce this background, anti-GST antibodies were removed from LSU32 by passage through a column of GST cross-linked to glutathione-Sepharose beads by dimethyl pimelimidate-HCl (2). Dot blot analysis showed that LSU32 then reacted with GST–UL45-2 but not with GST. To test its reactivity with bona fide UL45-2, cytoplasmic extracts were prepared from CV1 cells infected with HSV-2 and from COS7 cells transfected with pAC265, using Nonidet P-40 (NP-40) and sodium deoxycholate detergents for cell lysis (7). The extracts were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (5), transferred to nitrocellulose, and probed with LSU32 followed by 125I-protein A (Fig. 1). A band of 20 kDa was present in the infected extract (lane 1) and the transfected extract (lane 3), but not in mock-infected (lane 2) or mock-transfected (lane 4) extracts, confirming the reactivity of LSU32 with UL45-2. A second band, of approximately 100 kDa, was found in all extracts and

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FIG. 1. Recognition of UL45-2 by LSU32 antiserum. CV1 cells were infected with HSV-2 strain 333 at an MOI of 1 PFU per cell (lane 1) or mock infected (lane 2). COS7 cells were transfected with pAC265 by the calcium phosphate procedure (lane 3) or mock transfected (lane 4). Cytoplasmic extracts were prepared at 18 h p.i. or 40 h posttransfection, electrophoresed on a 12.5% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with LSU32 and then 125I-protein A. Molecular weights (in thousands [K]) are noted at the right.
therefore represents a cellular protein with which LSU32 cross-reacts.

UL45-1 has been shown to be required for cell-cell fusion (6); however, the protein was not detected in infected cells until 9 h postinfection (p.i.) (12) whereas fusion has been observed as early as 5 h p.i. (8). Many factors may be responsible for this difference, such as the use of different cell types, virus strains, and multiplicities of infection (MOI). Therefore, the time course of UL45-2 synthesis in infected cells was compared to that of the viral glycoproteins gB-2 and gD-2, which by analogy with their HSV-1 counterparts are probably essential for cell-cell fusion, and also to that of gC-2. Cytoplasmic extracts of CV1 cells infected with HSV-2 at an MOI of 1 PFU per cell were prepared at 0 to 12 h p.i., and Western blots of SDS-polyacrylamide gels were probed with the polyclonal antibodies LSU32 (UL45-2), R90 (gB-2), R46 (gC-2), and R7 (gD-2) (Fig. 2). UL45-2 was first detected at 4 h p.i., as was gC-2; gB-2 and gD-2 were easily detectable at 4 h and barely detectable at 2 h. The appearance of UL45-2 at a time comparable to the time of appearance of these glycoproteins is consistent with a role in membrane fusion.

Kyte and Doolittle hydropathy plots of the UL45-1 and UL45-2 amino acid sequences reveal a potential membrane-spanning sequence between residues 24 and 48. Solubilization of UL45-1 from HSV-1 virions with NP-40 detergent provides further evidence for its association with membranes (12). To determine if UL45-2 is a membrane protein, CV1 cells were infected with HSV-2 at an MOI of 1 PFU per cell and fractionated at 18 h p.i. The cells were scraped into phosphate-buffered saline, pelleted by low-speed centrifugation, and resuspended in 10 mM Tris-HCl (pH 7.5)-10 mM NaCl-3 mM MgCl₂ (reticulocyte standard buffer [RSB]). After cell lysis by one freeze-thaw cycle and sonication, the lysate was centrifuged at 70,000 × g for 30 min at 4°C to produce a pellet and a soluble fraction. The pellet was resuspended either in 0.1 M Na₂CO₃ (pH 11.0) (to solubilize peripheral membrane proteins) or in RSB containing NP-40 and sodium deoxycholate (to solubilize peripheral and integral membrane proteins) and then centrifuged again at 70,000 × g for 30 min. The supernatants contained high-pH-soluble or detergent-soluble proteins, respectively. The pellets containing high-pH-insoluble or de-

![FIG. 2. Time course of UL45-2 synthesis, compared to those of gB-2, gC-2, and gD-2. CV1 cells were infected with HSV-2 at an MOI of 1 PFU per cell. Cytoplasmic extracts were prepared at 2-h intervals from 0 to 12 h p.i., electrophoresed on denaturing polyacrylamide gels, transferred to nitrocellulose, and probed with LSU32 (UL45-2), R90 (gB-2), R46 (gC-2), or R7 (gD-2) and then with 125I-protein A.

![FIG. 3. Membrane association of UL45-2. CV1 cells were infected with HSV-2 for 18 h and then fractionated as described in the text. All fractions were electrophoresed on a denaturing gel, transferred to nitrocellulose, and probed with LSU32 and then with 125I-protein A.](image-url)
tergent-insoluble proteins were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer. As a control, a cytoplasmic extract of unfraccionated cells was prepared by detergent lysis. All fractions were electrophoresed, transferred to nitrocellulose, and probed with LSU32. Molecular weights (in thousands [K]) are noted at the right.

FIG. 4. Membrane orientation of UL45-2. COS7 cells were transfected with pAC265 (wild-type UL45-2, lanes 1 and 2), pMM336 (R4N mutant, lanes 3 and 4), or pMM337 (E111N mutant, lanes 5 and 6). Cytoplasmic extracts were prepared at 40 h posttransfection, incubated for 2 h at 37°C in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of endo F, electrophoresed on a denaturing gel, transferred to nitrocellulose, and probed with LSU32 and then with 125I-protein A. The cellular location of UL45-2 was examined by immunofluorescence analysis of COS7 cells transfected with plasmid pAC265. The cells were fixed with 3% paraformaldehyde–2% sucrose in phosphate-buffered saline; for staining of internal antigens, they were subsequently permeabilized by incubation in Dulbecco modified Eagle medium (DMEM)–5% fetal bovine serum (FBS) containing 0.5% Triton X-100 detergent and 10% sucrose. Incubation with the primary antibody (LSU32) was for 30 min at room temperature in DMEM–5% FBS. Incubation with the secondary antibody (goat anti-rabbit immunoglobulin G [IgG]–fluorescein isothiocyanate [FITC]) [Southern Biotechnologies] was for 30 min at room temperature in DMEM–1% FBS. UL45-2 was detected within the cell lumen of the endoplasmic reticulum (ER), defining UL45-2 as a type II membrane protein.

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if it was present at the cell surface. The intracellular localization was further investigated by cotransfection of COS7 cells with pAC265 and pXM-BiP-Tag (7a), a plasmid that expresses an epitope-tagged form of the ER-resident protein BiP. After fixation and permeabilization, the cells were stained with LSU32 and a mouse monoclonal antibody that recognizes the epitope tag (12CA5; Boehringer Mannheim), followed by goat anti-rabbit IgG-FITC and goat anti-mouse IgG-tetramethyl rhodamine isothiocyanate (TRITC). As shown in Fig. 5, the patterns of staining for UL45-2 (Fig. 5A) and BiP (Fig. 5B) were very similar, indicating that UL45-2 is largely if not entirely contained within the ER in transfected cells. Clearly, it would be interesting to know if UL45-2 is also retained within the ER in infected cells. Unfortunately, after blocking the viral Fc receptor with an excess of nonspecific rabbit IgG, we have not been able to detect UL45-2 in infected cells using biotinylated LSU32 and then streptavidin-FITC.

In summary, the work described in this paper is a characterization of important properties of the HSV-2 UL45 protein. A polyclonal antibody against UL45-2 recognized the protein in infected cells and in cells transfected with an expression plasmid. UL45-2 was shown to be an integral membrane protein and was synthesized sufficiently early during infection to be involved in cell-cell fusion. Furthermore, it was found to have a type II membrane orientation, the first HSV membrane protein known to have this orientation. In transfected cells, UL45-2 is retained within the ER, but in infected cells, its location is not yet known. Future studies will address the issues of whether UL45-2 is required for HSV-2 entry into cells or for cell-cell fusion in various syncytial backgrounds and, if so, how it participates in these processes.

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