JOURNAL OF VIROLOGY, June 1996, p. 3938–3946 Vol. 70, No. 6
0022-538X/96/$04.00+0

Properties of the Protein Encoded by the U_L32 Open Reading Frame of Herpes Simplex Virus 1

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Received 11 January 1996/Accepted 13 March 1996

The functions previously assigned to the essential herpes simplex virus 1 U_L32 protein were in cleavage and/or packaging of viral DNA and in maturation and/or translocation of viral glycoproteins to the plasma membrane. The amino acid sequence predicts N-linked glycosylation sites and sequences conserved in aspartyl proteases and in zinc-binding proteins. We report the following. (i) The 596-amino-acid U_L32 protein accumulated predominantly in the cytoplasm of infected cells but was not metabolically labeled with glucosamine and did not band with membranes containing a known glycoprotein in flotation sucrose density gradients. The U_L32 protein does not, therefore, have the properties of an intrinsic membrane protein. (ii) Experiments designed to demonstrate aspartyl protease activity in a phage display system failed to reveal proteolytic activity. Moreover, substitution of Asp-110 with Gly in the sequence Asp-Thr-Gly, the hallmark of aspartyl proteases, had no effect on viral replication in Vero and SK-N-SH cell lines or in human foreskin fibroblasts. Therefore, if the U_L32 protein functions as a protease, this function is not required in cells in culture. (iii) Both the native U_L32 protein and a histidine-tagged U_L32 protein made in recombinant baculovirus-infected insect cells bound zinc. The consensus sequence is conserved in the U_L32 homologs from varicella-zoster virus and equine herpesvirus 1. U_L32 protein is therefore a cysteine-rich, zinc-binding essential cytoplasmic protein whose function is not yet clear.

This report concerns the U_L32 open reading frame of herpes simplex virus 1 (HSV-1) predicted to encode a protein of 596 amino acids. The gene belongs to the γ_2 kinetic class inasmuch as its expression requires the synthesis of viral DNA (17, 43). Evidence that the expression of U_L32 is essential for viral replication rests on the isolation of temperature-sensitive (*ts*) mutants mapped in this open reading frame. Our interest in this gene stemmed from the remarkable phenotypes of the mutants ascribed to this gene. Thus HSV-1(KOS)*ts*N20 (8, 45, 50) has been reported to map to a 432-bp *Sal*I-*Eco*RI fragment within the *Eco*RI M fragment as part of the coding domain of U_L32. At the nonpermissive temperature, cells infected with this mutant virus synthesized a comparable amount of "endless" viral DNA (47). The titer of this virus dropped by 4 log units, and the ratio of physical particles to PFU increased by 10^4- to 10^5-fold although electron microscopic studies did not reveal gross aberrations in viral development relative to those seen in wild-type virus-infected cells (44). The U_L32 gene was also assigned as the locus of immune cytolysis resistance by McGeoch et al. (31) on the basis of one of a set of *ts* mutants isolated by Schaffer and colleagues (28, 34). Even though the mutants fell into four complementation groups, they shared resistance to cytolysis mediated by complement and monoclonal antibodies against specific viral glycoproteins at the nonpermissive temperature (28, 34, 35). One of the mutants, HSV-1(KOS)*icrts*149, endowed infected cells with resistance to lysis by complement and antibody against glycoprotein B (gB). Cells infected with this mutant at the nonpermissive temperature made normal levels of gD but reduced levels of gB and gC. The level of all tested viral glycoproteins localized at the cell surface appeared to be normal on the basis of surface ^{125}I labeling (34). These studies suggested that the HSV-1(KOS) gene containing the *icrts*149 mutation was involved in the final steps of glycoprotein maturation, with the consequence that at the nonpermissive temperature viral glycoproteins were transported to the plasma membrane but were either incorrectly processed or improperly inserted in the plasma membrane and therefore were not recognized by antibodies to the glycoproteins.

The HSV-1(KOS) mutation *icrts*149 was mapped to the *Eco*RI O fragment, which contains the 5′-terminal 317 codons of the U_L32 gene, the entire U_L33 gene, and the 5′-terminal 22 codons of the U_L34 gene (34). U_L32 was assumed to play the major role in the mutation, although the involvement of U_L33 or the very N terminus of U_L34, a gene encoding a membrane protein, was not ruled out (31, 41). Although several HSV-1 proteins exhibit multiple and sometimes unrelated functions, even if these two mutations were mapped in two distinct domains, the functions assigned to U_L32 seemed at first glance to be incompatible.

The U_L32 gene is highly conserved among the members of the *Alphaherpesvirinae* subfamily of herpesviruses. An analysis of the predicted amino acid sequence of the U_L32 protein indicates that it is rich in cysteines, that it has a short amino acid sequence remarkably similar to the sequence which serves as the hallmark of retrovirus aspartyl proteases, and that the hydrophobic stretches are compatible with its being a membrane protein. Indeed, the HSV-1(F) U_L32 gene has the highest amino acid sequence homology with gene 28 of equine herpesvirus 1. Gene 28 has been reported to encode the major envelope glycoprotein gp300 (1, 51), although more recently this conclusion has been disputed (49). If amino acid sequence homology was predictive of function, the U_L32 protein could be predicted to be an essential membrane-associated protease. Our results are inconsistent with such a prediction. The results presented in this report show that U_L32 encodes a cytoplasmic protein which binds zinc.

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MATERIALS AND METHODS

Cells and viruses. HSV-1(F) is the prototype HSV-1 used in this laboratory. HSV-1(mP) is the parental virus of HSV-1(mP)*ts*8-22 and of R4947. R7032 is a recombinant virus containing a deletion in the U_S8 gene and as a consequence does not express the Fc-receptor activity associated with gE. BHK, HEp-2, HeLa S3, Vero, and the human neuroblastoma cell line SK-N-SH were obtained from the American Type Culture Collection. The human 143 thymidine kinase minus (143TK$^-$) and the rabbit skin (RS) cell lines were originally obtained from Carlo Croce and John McLaren, respectively. The human foreskin fibroblast (HFF) cell strain was obtained from George Kemble (Aviron Inc., Burlingame, Calif.). All cell lines were grown in Dulbecco's modified Eagle medium supplemented with 5% newborn calf serum (BHK, HEp-2, HeLa, RS, and Vero cells), 5% fetal calf serum and 40 μg of 5′-bromo-2-deoxyuridine per ml of medium (143TK$^-$ cells), or 10% fetal calf serum (SK-N-SH cells and HFF). Infected cells were maintained in medium 199V, consisting of mixture 199 supplemented with 1% calf serum, unless indicated otherwise. Insect SF9 cells were maintained in Grace's medium supplemented with 10% fetal bovine serum.

Reagents and antisera. Restriction endonucleases were from New England Biolabs. T4 DNA ligase was from U. S. Biochemical. U_L10 polyclonal antibody was described elsewhere (2). The monoclonal antibody to gD was purchased from Goodwin Institute (Plantation, Fla.).

Induction and purification of GST-U_L32 fusion protein. A 527-bp *Sac*I-*Sph*I fragment encoding the U_L32 codons 50 to 224 was cloned into the *Sac*I-*Hin*dIII sites of a glutathione *S*-transferase (GST) fusion protein expression vector pGEX-KG (16). *Escherichia coli* cells transformed with this plasmid were induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h after 2 h of log-phase growth. The cell pellet was sonicated briefly and solubilized in lysis buffer consisting of phosphate-buffered saline, 1% Triton X-100, 0.1 mM tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), 0.1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM phenylmethylsulfonyl fluoride, and 50 mM EDTA. Because the GST-U_L32 fusion protein was insoluble, the pellet was solubilized in disruption buffer (50 mM Tris [pH 7.0], 2% sodium dodecyl sulfate [SDS], 0.7 M β-mercaptoethanol, 2.75% sucrose) and electrophoretically separated in denaturing polyacrylamide gel. The fusion protein was visualized by staining in cold 0.1 M KCl and electroeluted from a gel slice into a buffer containing 50 mM NH_4HCO_3 and 0.1% SDS. Eluted proteins were vacuum dried, resuspended in water, and precipitated with acetone. The pellet was resuspended in water and precipitated with acetone twice to remove excess SDS. The final suspension was mixed with Freund's adjuvant and was used to immunize rabbits according to standard procedures.

Generation of recombinant baculovirus expressing U_L32 protein. A 2.5-kb *Nco*I-*Kpn*I fragment from the *Eco*RV E fragment of the HSV-1(F) genome including the entire U_L32 coding sequence was cloned into the *Stu*I-*Kpn*I site of a baculovirus expression vector, pAcSG-HisNT-C (Pharmingen), and the U_L32 gene was expressed as a histidine-tagged fusion protein with 37 extra amino acid residues at the N terminus of U_L32. The recombinant virus (BV-32) was plaque purified and amplified to a high titer. The purification of $(His)_6$-tagged U_L32 fusion protein was performed as described by the manufacturer (Qiagen), with slight modification. BV-32-infected SF9 cells were pelleted and resuspended in binding buffer (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl, 10 mM β-mercaptoethanol, 5 mM imidazole, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM TPCK, 0.1 mM TLCK). The sample was briefly sonicated, cleared by high-speed centrifugation, loaded on a nickel-Sepharose column (Ni-NTA resin; Qiagen), and eluted with a 50 to 250 mM gradient of imidazole in washing buffer (same as binding buffer except for 50 mM sodium phosphate buffer, pH 6.0). Fractions containing the eluted U_L32 fusion protein were pooled.

Immunoblotting. Infected cells were harvested directly in disruption buffer, briefly sonicated, stored at room temperature for 15 min unless indicated otherwise, electrophoretically separated in denaturing polyacrylamide gel cross-linked with *N*,*N*′-diallyltartardiamide, electrically transferred to a nitrocellulose sheet, blocked with 5% nonfat milk in PBS(A) (phosphate-buffered saline), and incubated with primary antibodies. After extensive rinsing in blocking solution the nitrocellulose sheets were reacted with goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Bio-Rad). The substrate for the color-developing reaction and molecular weight standard were obtained from Promega.

Intracellular localization of U_L32 protein. 143TK$^-$ cells were seeded on four-well slides. When the monolayer cultures became 80% confluent, the cells were either mock infected or exposed to 20 PFU of R7032 virus per cell. At 17 h after infection, the slides were rinsed with PBS(A), fixed in methanol at −20°C for 20 min, and reacted with the polyclonal antibody to U_L32 and subsequently to anti-rabbit immunoglobulin G (IgG) conjugated to Texas red (Molecular Probes). For surface immunostaining, cells were grown on coverslips and exposed to 5 PFU of R7032 virus per cell. At 15 h after infection the coverslips were fixed in 3% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature for 20 min. Coverslips were rinsed in PBS(A) and blocked in 1% bovine serum albumin (BSA) in PBS(A) at room temperature for 20 min. The immunostaining procedure was as previously described (6). The primary antibody used was a mouse monoclonal antibody to gD or the U_L32 polyclonal antibody described above. The secondary antibody was goat anti-mouse or anti-rabbit IgG conjugated with fluorescein (Sigma). After extensive rinsing with PBS(A), the slides were mounted in buffered glycerol and examined with a fluorescence microscope.

Isolation of membranes from infected cells. The procedures used in this study were adapted from those described by Balch et al. (3). Specifically, six 150-cm^2 flasks of RS cells were exposed to 5 PFU of HSV-1(F) per cell. At 13 to 15 h after infection, the cells in one culture were labeled in 1 ml of medium 199V lacking methionine but supplemented with 100 μCi of [^{35}S]cysteine (>1,000 mCi/μmol; Amersham) and 50 μCi of [^{35}S]methionine (>1,000 mCi/μmol; Amersham). At 18 h after infection, monolayers were washed once with PBS(A), scraped, pelleted by centrifugation, resuspended in 1 mM phosphate buffer (pH 7.4), and disrupted by 20 strokes of Dounce homogenization. The sample was adjusted to 0.25 M sucrose and subjected to low-speed centrifugation for 10 min to spin down the nuclei. The supernatant fluid was adjusted to 12 ml with a final sucrose concentration of 1.4 M and transferred to a 34-ml centrifugation tube. A 14-ml layer of 1.2 M sucrose solution and an 8-ml layer of 0.8 M sucrose solution, both in 1 mM phosphate buffer (pH 7.4), were layered successively on top. The step gradient was allowed to equilibrate in the cold for 3 h and then was centrifuged at 25,000 rpm in a Beckman SW27 rotor for 2.5 h. Fractions (2 ml each) were collected from the top of the gradient, diluted with at least 10 volumes of phosphate buffer, and centrifuged at 25,000 rpm for 2 h in a Beckman SW27 rotor to collect proteins or protein complexes associated with large cellular macromolecular structures. In a separate experiment, total proteins from each fraction were precipitated with acetone. The pellets obtained from each fraction were solubilized in disruption buffer and subjected to electrophoresis in polyacrylamide gels.

Immunoprecipitation. RS cells were either mock infected or infected with 10 PFU of R7032 per cell and were labeled with 50 μCi of [^{35}S]methionine (see above) in medium 199V with 5% of its original methionine content or 5 μCi of [^{14}C]glucosamine (>200 mCi/mmol; Amersham) at 6 h after infection. Cells were harvested at 18 h postinfection and lysed in lysis buffer [PBS(A) with 1% Nonidet P-40, 1% deoxycholate, 0.01 mM TPCK, and 0.01 mM TLCK]. Samples were subjected to brief sonication and then centrifuged at 14,000 × *g* for 5 min. The supernatant fluid was incubated with 3 μl of U_L32 polyclonal antibodies at 4°C for 1 h with constant rotation. Protein A-agarose beads (50% in lysis buffer) were added, and the reaction was allowed to proceed for another hour. The beads were washed in 1 ml of wash buffer (lysis buffer with 0.1% SDS) four times, and the bound proteins were solubilized in disruption buffer and subjected to electrophoresis in denaturing gels.

Substrate phage screening. The phage display system was a gift of James A. Wells of Genentech Inc. and was described previously by Wells et al. (29, 30). Briefly, a Costar 24-well dish was coated with human growth hormone-binding protein by exposing the wells to a concentration of 2 μg of protein per ml in 50 mM sodium carbonate buffer, pH 9.0, overnight at 4°C. The next day, the plate was blocked with 0.5% BSA (protease free) in PBS(A) at room temperature for 1 h. The blocking solution was removed, and substrate phage (10^{11} ampicillin-resistant CFU) was added and allowed to adsorb onto the plate at room temperature for 2 h. Unbound phage was removed by washing the plate extensively with 0.01% Tween 20 in PBS(A). Each well was equilibrated with reaction buffer (0.1 M citrate buffer, pH 5.0) at 37°C for 10 min, and this was followed by the addition of the U_L32 fusion protein. The reaction was continued at 37°C for 2 h with gentle shaking. At the end of the incubation, the phage released into the supernatant fluid encoded peptide sequences sensitive to protease cleavage and was collected. The phage resistant to protease cleavage was stripped off the dish with 0.1 M acetate buffer, pH 2.0, to disrupt the binding of substrate phage to human growth hormone-binding protein. Both populations of phage were assayed by measuring ampicillin-resistant CFU in *E. coli*. The pentapeptide sequence presented on the released phage was then analyzed by sequencing of the phagemid DNA, and the consensus cleavage site was derived.

Construction of U_L32 Asp-110-to-Gly mutant virus. The coding sequence of U_L32 was changed at amino acid 110 from aspartic acid to glycine, and as a consequence, a unique restriction site (*Kpn*I) was introduced at the same position. Mutagenized U_L32 coding sequence was synthesized by PCR with two sets of primers: DG1 and DG2 (5′GGAACTGCAGCCATGGCAACTTCGCC3′ and 5′GAAAAGGGCCCGGTACCCAAAAGCCC3′, respectively) and DG3 and DG4 (5′GAGGTACCGGGCCCTTTTCCGCG3′ and 5′CGTCTAGATG GGGGTCGGTGTCATAC3′, respectively). The PCR products of DG1 with DG2 and DG3 with DG4 were digested with *Kpn*I and ligated with T4 ligase. The ligated products were gel purified and digested with *Pst*I and *Xba*I prior to cloning into vector pGEM3Zf+. The mutation was confirmed by DNA sequencing. The resulting plasmid, pRB4946, was ligated with a 500-bp *Nco*I fragment containing the entire U_L33 coding sequence to generate pRB4947. To construct the D-110-to-G mutant virus, a U_L33 *ts* mutant virus, HSV-1(mP)*ts*8-22, isolated by this laboratory (39) was rescued with the HSV-1 DNA sequences contained in pRB4947. The general procedure of constructing recombinant virus was described elsewhere (40). The progeny viruses were selected by plating the progeny of the transfection at 39.5°C. The introduction of the mutation in U_L32 was verified by hybridization of electrophoretically separated restriction digests of progeny viral DNA.

Southern blot analysis. Cytoplasmic viral DNA was extracted as described elsewhere (38). Purified DNA was digested with *Kpn*I, electrophoretically separated, and transferred to a nylon membrane (Bio-Rad). The plasmid pRB4947,

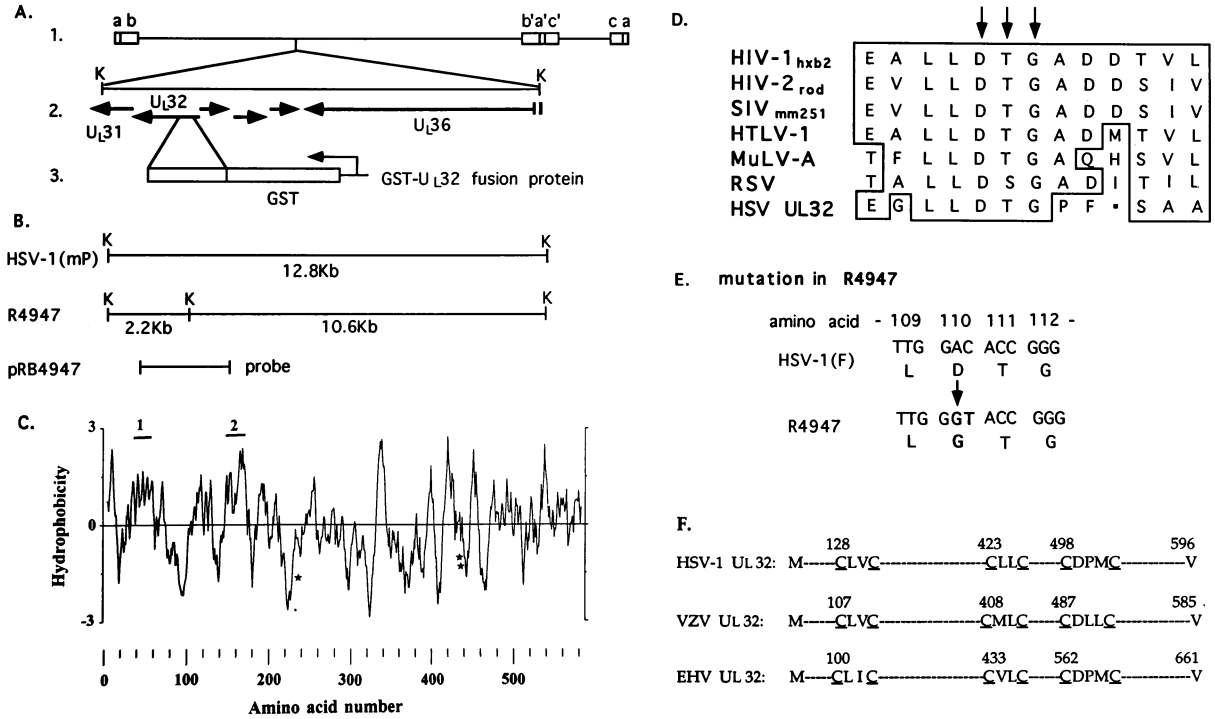

FIG. 1. (A) Diagrammatic representation of relative arrangements of genes U_L31 to U_L36 in the *Kpn*I B fragment and of the structure of the GST-U_L32 chimeric gene encoding the corresponding fusion protein. Open rectangles on line 1 represent the inverted repeat sequences *ab* and *b′a′* flanking the unique long sequence and *a′c′* and *ca* sequences flanking the unique short sequence. The expanded figure shows the *Kpn*I fragment containing all or portions of the domains of genes U_L31 to U_L36 as illustrated in line 2. The arrows below line 2 indicate the open reading frames. The positions and directions of the arrows show the locations and directions of the coding sequences of U_L31 through U_L36. The open rectangles on line 3 represent the coding sequences of the chimeric gene whose product consists of the N terminus of the U_L32 protein from amino acids 50 to 224 fused to the C terminus of GST. (B) Representation of the new *Kpn*I site introduced into R4947. The top line indicates the 12.8-kb *Kpn*I B fragment. The second line indicates the new *Kpn*I site in R4947; therefore, the fragment is broken down to two fragments of 2.2 and 10.6 kb. The third line indicates the probe used in the Southern blot analyses. (C) Kyte-Doolittle hydrophobicity profile of U_L32 coding sequence. Amino acid numbers are indicated at the bottom. The long stretches of hydrophobic amino acid residues which could form long uninterrupted α-helical structures are indicated by the bars above the plot and span from amino acids 31 to 50 (domain 1) and 151 to 180 (domain 2). The asterisks indicate the consensus glycosylation sites at position 245, 451, and 452. (D) Comparison of the conserved domains of retroviral proteases with a similar domain present in HSV-1 U_L32. Amino acid sequence alignment of the active site of retroviral proteases with the HSV-1 U_L32 amino acids 106 to 117. Arrows indicate the hallmark sequence of aspartyl proteases. HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; HTLV, human T-cell leukemia virus; MuLV-A, Abelson murine leukemia virus; RSV, Rous sarcoma virus. (E) Nucleotide substitution and corresponding amino acid change introduced into recombinant virus R4947 to inactivate putative proteolytic activity of U_L32 protein. Asp-110 was mutated to Gly in R4947 to knock out the putative protease activity. (F) Conservation of cysteine residues in U_L32 homologs of three members of the *Alphaherpesvirinae* subfamily of herpesviruses. Conserved cysteine residues with either the CXXC or CXXXC motif are underlined. VZV, varicella-zoster virus; EHV, equine herpes virus.

containing the U_L32 and U_L33 coding sequences, was labeled with the DuPont nick translation kit and used as a probe for hybridization with the membrane.

Zinc blot assay. Zinc blot assays were done as described elsewhere (4, 32, 46).

RESULTS

Synthesis of GST-U_L32 fusion protein. The GST-U_L32 fusion protein was made in *E. coli* transformed with a plasmid (Fig. 1A). The sequence inserted into pGEX-KG encodes U_L32 gene amino acids 50 to 224. The correct induced fusion protein would have a molecular weight of 46,000. The electrophoretically separated lysates of the induced bacteria in the denaturing gels are shown in Fig. 2. The lysate of induced cells (Fig. 2, lane 3) expressed a protein migrating with an apparent M_r of approximately 46,000; this protein was absent from the uninduced cell lysate (Fig. 2, lane 2). The purified protein (Fig. 2, lane 4) was used as antigen to immunize rabbits to generate polyclonal antibody. The procedures for the preparation of the antigen for rabbit immunization are described in Materials and Methods.

Specificity of U_L32 polyclonal antiserum. To test the U_L32 antiserum, BHK, RS, HeLa, HEp-2, 143TK$^-$, or SK-N-SH cell cultures were either mock infected or exposed to 10 PFU of HSV-1(F) per cell. The cells were harvested at 18 h after infection, proteins were solubilized in disruption buffer, electrophoretically separated in a denaturing gel, transferred to a nitrocellulose sheet, and reacted with the anti-U_L32 antiserum. As shown in Fig. 3A, the antiserum reacted with only a single band in lysates of HSV-1(F)-infected cells and not with those of mock-infected cells. The protein band recognized by the antibody migrated with an electrophoretic mobility of 67,000 (M_r), which is close to the predicted M_r of 64,000 for the U_L32 protein (31).

The reactivity of the U_L32 polyclonal antibody with the lysate of BV-32-infected SF9 cells further attests to the specificity of the antibody. As shown in Fig. 3B, the antibody reacted only with proteins in electrophoretically separated lysates of insect cells infected with the recombinant baculovirus. The multiple bands (Fig. 3B, lane 3) were due to proteolytic degradation from the amino terminus of the protein, since in other assays, only the most slowly migrating species shown in this lane bound to the nickel-Sepharose column (data not shown). The full-length histidine-tagged U_L32 fusion protein migrated slightly more slowly in denaturing gels than did the U_L32 protein in the HSV-1(F)-infected cell lysate (Fig. 3B, lanes 3 and 4) because of the extra 37 amino acids derived from

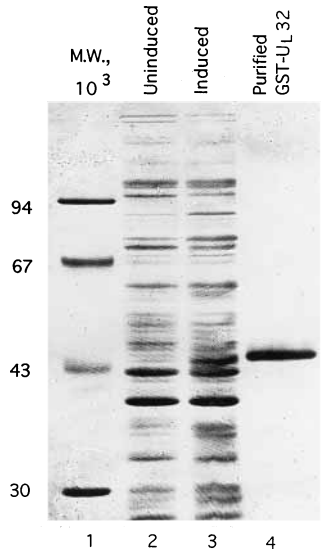

FIG. 2. Photograph of Coomassie brilliant blue-stained gel containing electrophoretically separated proteins of *E. coli* before induction (lane 2) and after induction (lane 3) and purified GST-U_L32 fusion protein (lane 4). Lane 1 contains molecular weight markers (Promega). The induced GST-U_L32 protein had an apparent M_r of 46,000.

the baculovirus expression vector sequence added to the N terminus of U_L32.

The level of expression of the U_L32 protein varied from one cell line to another. For equal numbers of cells infected at the same multiplicity of infection, the yields from RS and BHK cells were higher than those obtained from the other cell lines tested. Therefore, these two cell lines were used in most of the studies described below.

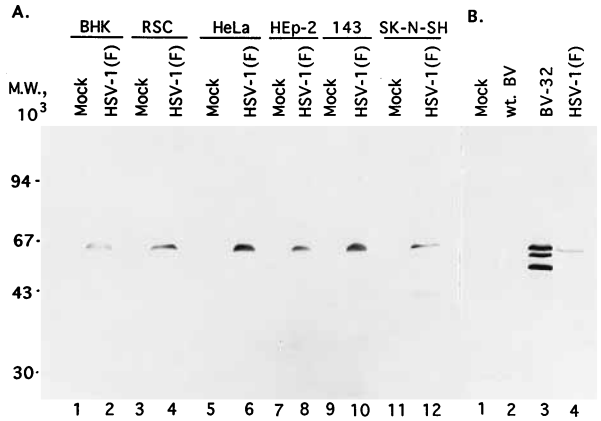

FIG. 3. (A) Photograph of immunoblot of electrophoretically separated lysates of mock-infected or infected cells reacted with U_L32 polyclonal antibody. Various cell lines were either mock infected or infected with 10 PFU of HSV-1(F) per cell. The cells were harvested at 18 h after infection and solubilized in disruption buffer, and the lysates were electrophoretically separated in denaturing gel, transferred to a nitrocellulose sheet, and reacted with U_L32 antibody. Lanes 1, 3, 5, 7, 9, and 11 contain lysates of mock-infected cells; lanes 2, 4, 6, 8, 10, and 12 contain lysates of HSV-1(F)-infected cells. The cell lines are identified above the gel. (B) Photograph of an immunoblot of electrophoretically separated lysates of insect cells infected with a recombinant baculovirus and of HSV-1(F)-infected RS cells reacted with U_L32 polyclonal antibody. Lysates of mock-infected (lane 1), wild-type baculovirus-infected (lane 2), or recombinant baculovirus (BV-32)-infected SF9 cells were solubilized in disruption buffer, separated in a denaturing gel, transferred to a nitrocellulose membrane, and reacted with U_L32 antiserum. HSV-1(F)-infected RS cells were subjected to the same procedure (lane 4) and served as a marker to identify the position of native U_L32 protein. The positions of the molecular weight markers are shown at the left.

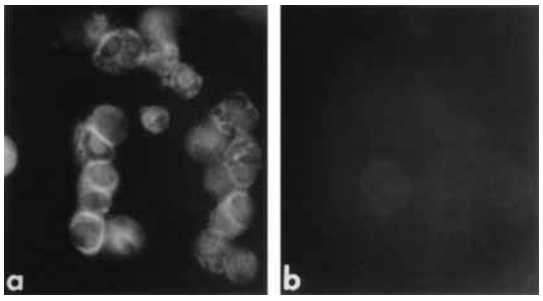

FIG. 4. Photomicrographs of immunofluorescent patterns of mock-infected (b) or infected (a) 143TK$^-$ cells reacted with the U_L32 antiserum and then with goat-anti rabbit IgG conjugated to Texas red. The cells grown on slides were either mock infected or exposed to 20 PFU of R7032 per cell. At 17 h after infection the cells were fixed in cold methanol and reacted with U_L32 antibody as described in Materials and Methods. Note that at 17 h after infection the cells round up and tend to clump; the cytoplasm formed a fluorescent shell around the nucleus. U_L32 protein was detected in nuclei late in infection.

The U_L32 protein is localized predominantly in the cytoplasm. In this series of experiments, slide cultures of 143TK$^-$ cells were either mock infected or exposed to 20 PFU of R7032 per cell. At 17 h after infection, cells were fixed in cold methanol and reacted with the U_L32 antiserum and then with goat anti-rabbit IgG conjugated to Texas red as described in Materials and Methods. The U_L32 antibody reacted with antigen present predominantly in the cytoplasm of infected cells but not with uninfected cells (Fig. 4a and b).

The U_L32 protein is not associated with cytoplasmic membranes. The impetus for the experiments described in this section was based on two observations. The first observation is that the predicted amino acid sequence of the U_L32 protein is compatible with that of a membrane-associated protein. As shown in Fig. 1C, the hydrophobicity profile indicated that a substantial portion of the protein is hydrophobic. Of these, one domain (amino acids 31 through 50 [domain 1 in Fig. 1C]) is predicted by both Chou and Fasman (7) and Garnier et al. (13) to have an α-helical structure whereas the second domain (amino acids 151 to 180 [domain 2 in Fig. 1C]) has an α-helical structure according to Garnier et al. but not according to Chou and Fasman. The second observation is that the U_L32 open reading frame sequence is well conserved among the *Alphaherpesvirinae* (Fig. 5) and that at least one potential homolog, that of equine herpesvirus 1, was reported to be a membrane protein (51). To test the hypothesis that U_L32 protein is associated with cytoplasmic membranes, three series of experiments were done.

In the first series unfixed infected cells were reacted with either U_L32 antiserum or with monoclonal antibody to gD, a viral glycoprotein known to be present on the surface of infected cells. The experiment was done as described in Materials and Methods, and surface fluorescence was observed with the antibody to gD but not with the antibody to U_L32 protein (data not shown).

The experiments described above did not exclude the possibility that the epitopes were inaccessible to the U_L32 polyclonal antibody, and therefore in a second series of experiments RS cells exposed to 5 PFU of HSV-1(F) per cell were labeled with [^{35}S]methionine (>1,000 mCi/μmol; Amersham) for 2 h at 13 h after infection. The infected cells were harvested and lysed by Dounce homogenization, and the cytoplasmic extract was subjected to flotation centrifugation as described in

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           1                                                        50
EHV        ........ma AscnldVigE dggCaltgG. .WqpGAFERp YmgFDarLLs
VZV        mdrveseepM dgfespVFsE ntss..nsG. .WcsdAFsds YIAYnPaLLl
HSV-1      .........M AtsppgVLas vavCeespGs sWkaGAFERa YVAFDPsLLa
Consensus  ---------- ------V--- --------G- -W---AF--- Y------LL-

           51                                                       100
EHV        tNssLCSELi FsaHLmqIsP ..Tpqpreqv DVcedPd... ..........
VZV        kNDlLfSELL FASHLInVPr ..aiEnnVty EassAvg... ..........
HSV-1      lNEaLCaELL tASHvIgVPP vgTlDedVaa DVvtAPsrar ggagdgggsg
Consensus  -N--L--EL- ---H------ ---------- ---------- ----------

           101                                                      150
EHV        ......NdPP EPScaqFVD. .....AVaDs LALDklCLIC RTIDLYrRqF
VZV        .....vdnem tsStteFIE. .....eIgDv LALDRaCLVC RTlDLYKRkF
HSV-1      grggprNpPP DPcgegLlDt gpfsaAaiDt FALDRpCLVC RTIELYKqtY
Consensus  ---------- ---------- --------D- -ALD--CL-C RT--LY----

           151                                                      200
EHV        GLSPQWIADY AMLCtKtLAA PPCAVaTvVA AFEFVYLMDk HYLRRgKtTL
VZV        GLtPeWVADY AMLCmKsLAs PPCAVvTFsA AFEFVYLMDR yYLcRynvTL
HSV-1      rLSPQWVADY AfLCaKcLgA PhCAasiFVA AFEFVYvMDR HFLRtkKaTL
Consensus  -L-P-W-ADY A-LC-K-L-- P-CA-----A AFEFVY-MD- --L-----TL

           201                                                      250
EHV        VGaFARRvLT LvDIQRHFFL HVCFRTDGGV PrcaAsgtaP aatamaGlgM
VZV        VGSFARRtLs LlDIQRHFFL HVCFRTDGGl .........P girPpPGkeM
HSV-1      VGSFARfaLT inDIhRHFFL HcCFRTDGGV PgrhA.qkqP kpsPsPG...
Consensus  VG-FAR--L- --DI-RHFFL H-CFRTDGG- ---------P ------G---

           251                                                      300
EHV        AdKVQYSNYS FLVQSsTRAM LLTVAdvpsg DdGalqavph grhGagrpad
VZV        AnKVrYSNYS FFVQavvRAa LLsIstsrlD Etetrksfyf nqdG......
HSV-1      AaKVQYSNYS FLaQSaTRAL igTlAsggeE gaGsaag... ..........
Consensus  A-KV-YSNYS F--Q---RA- ---------- ---------- ----------

           301                                                      350
EHV        ggggvFgpkQ QStvAALMsW KECAkMIDCS gSERRrpGat mTCCERArAD
VZV        .....LtGgp QpLaAALaNW KDCARMVDCS sSEhRtsG.m iTCaERAlkE
HSV-1      ......sGtQ pSLttALMNW KDCARLlDCt egrR...Ggg dsCCtRAaAr
Consensus  ---------- -----AL--W K-CA---DC- -------G-- --C--RA---

           351                                                      400
EHV        DdEYErqLls ..tEntYlgs Adnqaeggnd thlkWGYADL tLLLLSq.ss
VZV        DiEFEdILiD klkksSYvea A......... ....WGYADL aLLLLSG.vA
HSV-1      ngEFEtVagD repEeS.... .......... .pdtWaYADL vLLLLaGtpA
Consensus  --E-E----- ---------- ---------- ----W-YADL -LLLL-----

           401                                                      450
EHV        TWEasEkTsl AsqsRRACVe eYWasHRtvl aRDtAPRFAr FvDaDAvPDt
VZV        TWnvdErTnc AiEtRvgCVk sYWqAnRien sRDVpkqFsk FTseDAcPEv
HSV-1      vWEsgpqlra AaEaRRAtVr qsWeAHRgar tRDVAPRFAq FTEpDAqPDl
Consensus  -W-------- A---R---V- --W---R--- -RD----F-- F---DA-P--

           451                                                      500
EHV        AtGPVLATTL KHvRsRGRTc aECvLCNLiL tRehWLALRR FKRDVIsYSs
VZV        AFGPILlTTL KnakcRGRTn tECMLCcLLt IghYWiALRq FKRDIlaYSa
HSV-1      dLGPlMATvL KHgRgRGRTg gECLLCNLLL VRaYWLALRR LrasVVrYSe
Consensus  --GP---T-L K----RGRT- -EC-LC-L-- ----W-ALR- -------YS-

           501                                                      550
EHV        NNanLFDCIs PVlsALSdaN sepLagdcgv ggggTcPeDs GRFleLMhAA
VZV        NNTSLFDCIe PVInAwSlDN pikLkfpf.. .......nDe GRFItivkAA
HSV-1      NNTSLFDCIv PVVdqLeaD. .......... ..peTqPgDg GRFVsLLrAA
Consensus  NN--LFDCI- PV-------- ---------- --------D- GRF-----AA

           551                                                      600
EHV        GtEAIYKHLF CDPMCALvEL QTNPsVLFsp igPppEPDEi ELqKArLAse
VZV        GsEAVYKHLF CDlLCALsEL QTNPkILFaH .PttaDkEvL ELyKAqLAaq
HSV-1      GpEAIFKHMF CDPMCAitEM evdPwVLFgH .PPathPDEL lLhKAkLAcg
Consensus  G-EA--KH-F CD--CA--E- ---P--LF-- ---------- -L-KA-LA--

           601                                                      650
EHV        NWFsGRVCAG LWALAFTFKT YQIFtPKPTA cAAFIkDAGL LLRRHnlpLI
VZV        NRFEGRVCAG LWtLAYaFKa YQIFprKPTA nAAFIRDgGL MLRRHaISLV
HSV-1      NeFEGRVCia LRALiYTFKT YQVFvPKPTA lAtFVREAGa LLRRHsISLl
Consensus  N-F-GRVC-- L--L---FK- YQ-F--KPTA -A-F----G- -LRRH---L-

           651    660
EHV        SLEHTLCnYV
VZV        SLEHTLskYV
HSV-1      SLEHTLCtYV
Consensus  SLEHTL--YV
```

FIG. 5. Amino acid sequence alignment of the U_L32 homologs in equine herpes virus (EHV) (51), varicella-zoster virus (VZV) (11), and HSV-1 (31). The consensus line shows residues shared among the three homologs.

Materials and Methods. After centrifugation, proteins contained in macromolecular complexes in each fraction were pelleted, solubilized in disruption buffer, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, subjected to autoradiography, and probed with antibody to the U_L32 protein and with the rabbit polyclonal antibody to gM encoded by the U_L10 gene (2). As could be expected, gM localized in fractions 3 to 9, spanning the fractions where an opaque membrane band was visualized (fractions 4 to 5). The U_L32 protein was not pelleted under the conditions tested and could not be detected by anti-U_L32 antibody (data not shown). To determine the localization of the U_L32 protein, the total protein from each fraction was precipitated with acetone, solubilized in disruption buffer, electrophoretically separated in denaturing gels, electrically transferred to a nitrocellulose sheet, and reacted with the antibody to U_L32. Under these conditions, the U_L32 protein was detected in fractions 10 to 17, that is, in fractions clearly distinct from those containing the majority of gM (fractions 3 to 9) (Fig. 6). From this experiment, we conclude that U_L32 protein does not associate with cellular membranes.

The third series of experiments was based on the report that the homolog of U_L32 in equine herpes virus 1, the product of gene 28, is highly glycosylated (51). To test whether the HSV-1 U_L32 protein is also a glycoprotein, infected RS cells were metabolically labeled with either [^{35}S]methionine or [^{14}C]glucosamine. U_L32 polyclonal antibody specifically immunoprecipitated a [^{35}S]methionine-labeled protein which migrated with the same mobility as a band on an immunoblot reacting with the U_L32 antibody. The signal was absent in mock-infected cells or in infected cell lysates reacted with preimmune serum. The examination of [^{14}C]glucosamine-labeled proteins by the same procedure failed to show labeled protein species in the precipitate, even though the U_L32 protein was clearly precipitated by this procedure (data not shown). From this experiment, we conclude that U_L32 protein is not glycosylated to a detectable degree.

U_L32 has some of the hallmarks of a protease, but it does not appear to express a proteolytic activity essential for viral replication. Although the overall sequence of the U_L32 protein is well conserved, the N-terminal domain of the predicted amino acid sequence, especially amino acids 56 to 119, varies considerably from one herpesvirus to the other (Fig. 5). During the course of these studies, our attention was drawn to the observation that amino acids 106 to 117 of HSV-1 U_L32 have a limited but intriguing homology to the active sites of several retroviral aspartyl proteases (Fig. 1D). To test the hypothesis that U_L32 is an essential protease, two series of experiments were done.

In the first series of experiments, we adapted the substrate phage display system to investigate the putative protease activity of the U_L32 protein. This system, established by Wells et al., has been tested successfully in selecting good cleavage sites

FIG. 6. Photograph of an immunoblot of fractions harvested from a flotation sucrose density gradient. HSV-1(F)-infected RS cells were lysed by Dounce homogenization, and the cytoplasmic portion was subjected to flotation by centrifugation in a sucrose density gradient. After centrifugation the proteins contained in 2-ml fractions were precipitated with cold acetone, resuspended in disruption buffer, electrophoretically separated in a denaturing gel, transferred to a nitrocellulose sheet, and reacted with U_L10 and U_L32 antibodies. The procedures were performed as described in Materials and Methods. The fraction numbers are indicated above each lane. The protein bands reacting with U_L10 or U_L32 antibody are marked on the right.

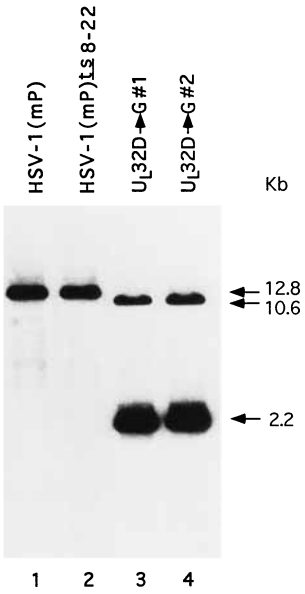

FIG. 7. Autoradiogram of electrophoretically separated DNA hybridized with labeled probe. Cytoplasmic viral DNA was digested with *Kpn*I, electrophoretically separated in agarose gel, transferred to a nylon membrane, and hybridized with labeled pRB4947. Lanes 1 and 2, DNA from cells infected with the indicated HSV-1 strains; lanes 3 and 4, DNA from cells infected with the indicated U_L32 D-110-to-G mutant virus isolates. Isolate 1 was designated R4947 and used in subsequent studies.

for several serine proteases (29, 30). Each substrate phage in the library carries phagemid sequence encoding a fusion protein consisting of truncated M13 phage gene III protein and a random pentapeptide fused to human growth hormone. This fusion protein is synthesized and anchored on the surface of phage particle in the presence of helper phage. This phage library was first immobilized on a solid substrate coated with human growth hormone-binding protein. Because of the strong affinity between human growth hormone and its binding protein, immobilized phage could be released only by protease cleavage at the random peptide sequence. Therefore, it provided a stringent selection for good protease cleavage sequences. After multiple rounds of screening followed by amplification, substrate phage presenting good target peptide sequences would be selected and enriched. The pentapeptide sequence presented on the released phage was then analyzed by sequencing of the phagemid DNA, and the consensus cleavage site was derived. The experiment was done as described in Materials and Methods, using purified U_L32 fusion protein expressed from recombinant baculovirus as a source of the putative protease and the phage display library kindly sent to us by Genentech Inc. After six rounds of successive screening, the titers of released phage did not increase significantly compared with those after the initial screening. Furthermore, at the last passage, the amount of phage released by U_L32 protein increased less than twofold relative to the mock-treated sample. We conclude from these assays either that the sequence recognized by the putative protease was longer than the variable pentameric sequence contained in the phage tail or that U_L32 protein does not function as a protease. Consistent with this conclusion was the observation that the sequence of several phagemids derived from the final round of screening did not yield a detectable consensus sequence (data not shown).

The second series of experiments was based on the observation that the amino acid sequence Asp-Thr-Gly in the context of the sequences listed in Fig. 1D is the hallmark of aspartyl proteases (10, 12, 20). The aspartic acid residue is crucial inasmuch as substitutions of this residue have been shown to abolish protease activity (22, 27, 33). To test the effect of such a mutation, we took advantage of the mutant HSV-1(mP)*ts*8-22 carrying a *ts* mutation in U_L33 (39) to introduce a mutation resulting in the replacement of Asp-110 with Gly. Specifically, as described in Materials and Methods, we constructed a fragment containing the wild-type U_L33 gene and a U_L32 gene in which the codon 110 sequence GAC was replaced with GGT (Fig. 1E), and simultaneously, a diagnostic *Kpn*I site was introduced at the site of the amino acid substitution. The mutation was introduced into the U_L32 coding sequence while the *ts* lesion of U_L33 was also repaired. Progeny viruses were selected for recombinants that could grow at the nonpermissive temperature (39.5°C). Hybridization of labeled DNA probes to electrophoretically separated DNA fragments verified the presence of the diagnostic *Kpn*I site as delineated in Fig. 1B. The presence of a new *Kpn*I site reduced the *Kpn*I B fragment, 12.8 kb in HSV-1(mP) and HSV-1 (mP)*ts*8-22 (Fig. 7, lanes 1 and 2), to two fragments of 10.6 and 2.2 kb in the recombinant virus (Fig. 7, lanes 3 and 4). The purified isolate 1 was designated R4947. The plating efficiency of R4947 at the nonpermissive temperature was comparable to that of HSV-1(mP) (data not shown).

To test the ability of the mutant virus to replicate, Vero or SK-N-SH cells or HFFs were exposed to 1 PFU of virus per cell and incubated at 34°C for 24 h. The titer of the yield of virus from each of the cultures was then determined on Vero cells. As shown in Table 1, R4947 did not differ from its parent viruses with respect to its ability to replicate in the three types of cells. These results are not consistent with the hypothesis that the U_L32 protein specifies an essential aspartyl protease.

U_L32 protein binds to zinc in a zinc blot assay. The CXXC domain (Fig. 1F) is conserved among all *Alphaherpesvirinae* U_L32 homologs and is reminiscent of the known zinc-binding motif (4, 9, 32). The putative zinc-binding activity of the U_L32 protein was investigated in a zinc blot assay. In this series of experiments U_L32 fusion protein expressed by recombinant baculovirus in insect cells or R7032-infected RS cell lysates were immunoprecipitated with U_L32 polyclonal antiserum, electrophoretically separated in an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and renatured in renaturing buffer (100 mM Tris [pH 6.8], 50 mM NaCl, 10 mM dithiothreitol) at room temperature for 1 h with three changes of buffer. The nitrocellulose membrane was then rinsed several times in labeling buffer (100 mM Tris [pH 6.8], 50 mM NaCl) and then reacted with $^{65}ZnCl_2$ (Amersham) at a concentration of 15 μM in labeling buffer at room temperature for 30 min, rinsed extensively in 100 mM Tris (pH 6.8)–50 mM NaCl–1 mM dithiothreitol for 1 h, air dried, and subjected to autoradiography. The results were as follows.

(i) One protein species in the immunoprecipitate from

TABLE 1. Titers of wild-type and mutant viruses in infected Vero and SK-N-SH cells and in HFFs[a]

Virus	Titer in:		
	Vero cells	SK-N-SH cells	HFFs
HSV-1(mP)	2.7×10^7	4.7×10^7	3.3×10^7
HSV-1(mP)*ts*8-22	1.2×10^7	2.3×10^7	1.4×10^7
R4947	1.4×10^7	2.3×10^7	1.5×10^7

[a] The cells were infected with virus at 1 PFU per cell at 34°C and harvested at 24 h after infection. Virus titers were determined in Vero cells.

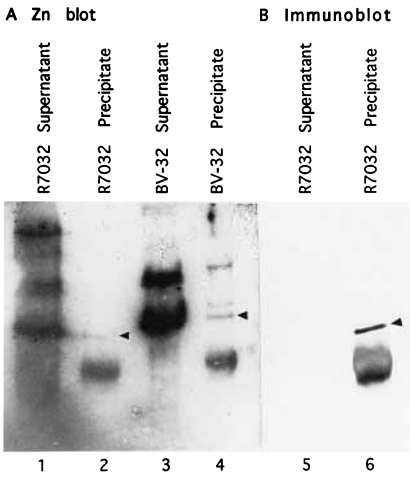

FIG. 8. Autoradiographic images and photograph of the immunoblot of electrophoretically separated proteins in denaturing gel. U_L32 protein from R7032-infected RS cell lysates or partially purified baculovirus-expressed U_L32 fusion protein was immunoprecipitated with U_L32 antibody. The supernatant fluid and immunoprecipitate were boiled in disruption buffer, separated in denaturing gel, transferred to nitrocellulose membrane, renatured, and reacted with $^{65}ZnCl_2$ as described in Results. A duplicate sample containing supernatant fluid and immunoprecipitate of the R7032-infected cell lysate was electrophoretically separated as described above but reacted with antibody to U_L32 protein. (A) Autoradiogram of $^{65}ZnCl_2$ blot assay. Lanes 1 and 2, supernatant fluid and immunoprecipitate of R7032-infected cell lysate, respectively; lanes 3 and 4, supernatant fluid and immunoprecipitate of partially purified His-tagged U_L32 protein with U_L32 antibody, respectively. (B) Lanes 5 and 6, immunoblot of samples as in lanes 1 and 2. Arrowheads indicate the positions of the U_L32 protein.

R7032-infected lysate (arrowhead in Fig. 8, lane 2) comigrating with the signal in a duplicate lane probed with U_L32 antibody (arrowhead in Fig. 8, lane 6) was shown to bind zinc.

(ii) In the immunoprecipitate from partially purified baculovirus-expressed U_L32 fusion protein, there was also a band with slightly lower mobility which interacted with zinc (arrowhead in Fig. 8, lane 4). Since the U_L32 fusion protein contained additional amino acids, it migrated more slowly than the protein band from R7032-infected cell lysate reacting with the anti-U_L32 antibody (data not shown).

(iii) The identities of the other positive bands in the immunoprecipitate from the partially purified baculovirus lysate are unknown. It is noteworthy that the samples in lanes 3 and 4 were first affinity purified through a nickel-Sepharose column and therefore may be enriched for proteins exhibiting metal-binding activity.

(iv) The supernatant fraction obtained from immunoprecipitation of the R7032-infected lysate contained a large number of proteins. However, only a limited number of proteins were also labeled with $^{65}ZnCl_2$ (Fig. 8, lane 1), suggesting the specificity of this assay.

(v) The broad band of zinc-binding protein migrating more quickly than the U_L32 protein was seen repeatedly in the immunoprecipitate and corresponds to the band containing the immunoglobulin G heavy chain. Zinc finger-like motifs are present in the constant region of the IgG heavy chain, although direct proof of zinc-binding activity in the heavy chain has not been reported (42).

We conclude from this series of experiments that the U_L32 protein expressed either from HSV-1-infected cells or from recombinant baculovirus as a fusion protein displays zinc-binding activity.

DISCUSSION

The salient features of the results reported here are as follows.

(i) The predicted amino acid sequence of the U_L32 protein has four potentially significant features: long hydrophobic domains and three N-linked glycosylation sites compatible with association with membranes, numerous conserved cysteines, a sequence compatible with a zinc-binding domain, and a sequence resembling the highly conserved hallmark of retrovirus aspartyl proteases.

(ii) The impetus to investigate the association of U_L32 protein with membranes stemmed from the analyses of the predicted amino acid sequence which suggested the presence of long hydrophobic α-helical domains and of the report that equine herpesvirus 1 gp300 is a homolog of U_L32. Although our studies indicate that U_L32 protein accumulates predominantly in the cytoplasm, we were unable to label the protein with glucosamine, demonstrate its association with cellular membranes in sucrose density gradient flotation studies, or detect the presence of the protein on the surface of infected cells.

(iii) The investigation of the potential function of the U_L32 protein as a protease stemmed from the amino acid sequence shown in Fig. 1D, which bears resemblance to the conserved amino acid sequence of retrovirus aspartyl proteases. Although earlier studies have shown that the HSV-1 U_L26 open reading frame encodes a protease, the function of this protease is restricted to cleavage of a small set of proteins involved in maturation of the capsid (23–26). We could predict that a protease is active in infected cell membranes since at least one glycoprotein (gG-2) is processed by posttranslational cleavage (48). In HSV-1, gB has also been reported to be proteolytically processed in a specific cell line (Vero cell) (36, 37).

We have attempted to demonstrate proteolytic activity in a phage display system. The phage library we have obtained has been used successfully to identify the cleavage site of the protease furin (29). The disadvantage of this library is the limitation of presenting a protease cleavage sequence longer than the pentameric amino acid segment. As a consequence, proteases requiring longer recognition sequences, the aspartyl proteases of retroviruses (5), for instance, may not cleave in this system. Therefore, no specific phage would be released and amplified, and therefore no consensus sequence could be derived. Our experiments were not successful, indicating either that the U_L32 protein is not a protease or that the recognition sequence for cleavage is longer than the variable pentameric sequence. The second experiment, involving the replacement of U_L32 Asp-110 with Gly, was expected to inactivate the protease, since Asp in the context of Asp-Thr-Gly is essential for proteolytic activity. The mutant virus, however, could not be differentiated with respect to growth in two cell lines and in a cell strain (HFF). We may conclude from these studies that either U_L32 protein does not have proteolytic activity or that this activity is not essential either in continuous (Vero and SK-N-SH) cell lines or in a primary cell strain (HFF).

As a general rule, proteolytic cleavages are essential for viral replication and many viruses encode their own protease. The exceptions do abound. Although the maturation of influenza viruses requires the cleavage of hemagglutinin, this function is supplied by the host cell (19, 21). HSV encodes proteins whose functions duplicate functions expressed by proteins expressed in dividing cells (e.g., thymidine kinase, ribonucleotide reductase, etc.) (14, 15, 18). It is conceivable that the virus encodes an additional protease which is not essential in dividing cells.

(iv) Of the 22 cysteines predicted in U_L32 protein, 14 are

conserved in *Alphaherpesvirinae* homologs. Equally significant, as shown in Fig. 1F, the amino acid sequences from residues 128 to 131 and 423 to 426 bear homology to zinc-binding domains, and these sequences appear to be conserved in varicella-zoster virus and in equine herpesvirus 1. Our studies reported here indicate that U_L32 protein binds zinc. We have demonstrated the binding of zinc both to the U_L32 protein made in infected cells and to the fusion protein made in recombinant baculovirus-infected insect cells.

In summary, we have demonstrated that the product of the U_L32 gene is a predominantly cytoplasmic protein which binds zinc and does not associate with membranes and that, if the gene product expresses proteolytic activity, the former function is not critical in infected cells in culture. Site-specific mutagenesis experiments now in progress may shed more light on the function of this gene.

ACKNOWLEDGMENTS

We thank James A. Wells of Genentech Inc. for making available to us the phage display system and Robert Garry of Tulane University for drawing our attention to the aspartyl protease active site sequence.

These studies were aided by grants from the National Cancer Institute (CA47451) and the National Institute for Allergy and Infectious Diseases (AI124009) of the United States Public Health Service.

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