Open Reading Frame E3-10.9K of Subspecies B1 Human Adenoviruses Encodes a Family of Late Orthologous Proteins That Vary in Their Predicted Structural Features and Subcellular Localization

Kathryn M. Frietze, 1 Samuel K. Campos, 2 and Adriana E. Kajon 1*

Infectious Disease Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive SE, Albuquerque, New Mexico 87108, 1 and BIOS Institute, University of Arizona, 1657 E. Helen St., Tucson, Arizona 85721 2

Received 8 March 2010/Accepted 14 August 2010

Subspecies B1 human adenoviruses (HAdV-B1s) are important causative agents of acute respiratory disease, but the molecular bases of their distinct pathobiology are still poorly understood. Marked differences in genetic content between HAdV-B1s and the well-characterized HAdV-Cs that may contribute to distinct pathogenic properties map to the E3 region. Between the highly conserved E3-19K and E3-10.4K/RIDα open reading frames (ORFs), and in the same location as the HAdV-C ADP/E3-11.6K ORF, HAdV-B1s carry ORFs E3-20.1K and E3-20.5K and a polymorphic third ORF, designated E3-10.9K, that varies in the size of its predicted product among HAdV-B1 serotypes and genomic variants. As an initial effort to define the function of the E3-10.9K ORF, we carried out a biochemical characterization of this E3-10.9K-encoded orthologous proteins and investigated their expression in infected cells. Sequence-based predictions suggested that E3-10.9K orthologs with a hydrophobic domain are integral membrane proteins. Ectopically expressed, C-terminally tagged (with enhanced green fluorescent protein [EGFP]) E3-10.9K and E3-9K localized primarily to the plasma membrane, while E3-7.7K localized primarily to a juxtanuclear compartment that could not be identified. EGFP fusion proteins with a hydrophobic domain were N and O glycosylated. EGFP-tagged E3-4.8K, which lacked the hydrophobic domain, displayed diffuse cellular localization similar to that of the EGFP control. E3-10.9K transcripts from the major late promoter were detected at late time points postinfection. A C-terminally hemagglutinin-tagged version of E3-9K was detected by immunoprecipitation at late times postinfection in the membrane fraction of mutant virus-infected cells. These data suggest a role for ORF E3-10.9K-encoded proteins at late stages of HAdV-B1 replication, with potentially important functional implications for the documented ORF polymorphism.

The species β human adenoviruses (HAdV-Bs), in particular the serotypes clustered within subspecies B1 (HAdV-3, HAdV-7, HAdV-16, HAdV-21, and HAdV-50), are frequent causative agents of acute respiratory disease in both children and young adults (15, 25, 35, 53). Infections by HAdV-3, HAdV-7, and HAdV-21 have documented associations with severe clinical manifestations and fatal pneumonia worldwide (1, 20, 30, 43).

Despite their important role in the etiology of human disease, little is known about the molecular biology and pathobiology of the HAdV-Bs. At the genomic level, the most striking differences between HAdV-Bs and the well-characterized HAdV-Cs map to the E3 region, a cassette of genes involved in modulation of host responses to infection (5, 23, 34). Several of the open reading frames (ORFs) unique to the HAdV-B2s encode products of unknown function that may contribute to the distinct pathogenic properties of this group of HAdVs. A few of the HAdV-B-specific ORFs have been characterized only biochemically (17, 19). Figure 1 shows a comparative map of the E3 regions of HAdV-C and HAdV-B1. The genomes of all known HAdV-Bs contain ORFs E3-20.1K and E3-20.5K, between the highly conserved ORFs E3-19K and E3-10.4K/RIDα. In HAdV-Cs, this genomic region encodes the adenovirus death protein (ADP)/E3-11.6K (60), which is expressed from the major late promoter (MLP) at late times of infection and has been shown to facilitate viral progeny egress and spread (41, 48, 52). Immediately upstream of ORF E3-10.3K/RIDα, HAdV-B1s carry a third ORF, which varies in the size of its predicted protein product, between 4.8 kDa and 10.9 kDa, among HAdV-B1 serotypes and genomic variants (27, 28, 42) and which we designated E3-10.9K, after the longest predicted polypeptide. E3-10.9K is also the candidate ancestral gene from which this family of orthologs derived by speciation. Extensive sequence variation in this particular gene exists among HAdV-B1 isolates (28; A. E. Kajon, unpublished observations). Interestingly, some genomic variants (also referred to as genome types) of HAdV-7 and HAdV-3, such as HAdV-7h and HAdV-3a, appear to be natural ORF E3-10.9K knockout mutants due to mutated AUG start codons (28). Although the exact mechanism generating this diversity is unknown, the presence of polypyrimidine and polypurine runs, direct and inverted repeats, and palindromic motifs suggests a role for illegitimate recombination (28). The intraserotypic genetic variability is particularly extensive for HAdV-3 and HAdV-7, but the significance of this variation, which has not previously been described for any other E3-encoded protein, will remain unclear until the function encoded by this ORF is elucidated.
As an initial effort to define the role of ORF E3-10.9K in the HAdV-B1 life cycle and to begin to elucidate the potential implications of the documented polymorphism for HAdV-B1 fitness and virulence, we carried out a biochemical characterization of selected E3-10.9K orthologs representing the naturally occurring diversity documented for this coding region and investigated their expression in infected cells. Sequence-based structural predictions suggested that some of the E3-10.9K orthologs are integral membrane proteins. Due to the inadequately performed analysis of polyclonal rabbit sera raised against keyhole limpet hemocyanin (KLH)-conjugated synthetic peptides, subcellular localization and glycosylation studies were carried out using ectopically expressed C-terminal enhanced green fluorescent protein (EGFP)-tagged fusion in HeLa cells by use of a tetracycline-regulated expression system. Reverse transcription-PCR (RT-PCR) was used to examine the temporal expression of ORF E3-10.9K transcripts in HAdV-B1-infected A549 cells. In addition, an HAdV-3p mutant virus encoding a C-termally hemagglutinin (HA)-tagged version of the 9-kDa ortholog, E3-9K, was generated to examine the temporal expression of the protein during infection of A549 cells.

**MATERIALS AND METHODS**

**Cell lines, viruses, and infections.** A549 cells (ATCC CCL-185) were grown in 8% (vol/vol) newborn calf serum (NBCS)-supplemented Eagle minimum essential medium (8% EMEM). Infected cells were maintained in 2% (vol/vol) NBCS-supplemented Eagle minimum essential medium (8% EMEM). HeLa T-Rex cells (Invitrogen, Carlsbad, CA) were grown according to the manufacturer's instructions. Viruses used for this study included the prototype strains of HAdV-3 (strain GB) (39), HAdV-7 (strain Gomen) (3), and HAdV-16 (strain Ch.79) (36) and the HAdV-7 field strains KCH4 (55), Argentina 87-922 (25), and NHRC 611 (courtesy of Department of Respiratory Diseases Research, Naval Health Research Center). These viruses represent genome types 3p, 7p, 16p, 7b, 7h, and 7d2, respectively, and carry the following orthologous variants (or sequence profiles) of ORF E3-10.9K, as previously described (28): E3-9K, E3-7.7K, E3-10.9K, E3-4.8K, null, and E3-4.8K, respectively. The origin and genomic characteristics of the viruses are described in Table 1.

**Viruses** were grown in A549 cells, harvested by three freeze-thaw cycles, and clarified of cell debris by centrifugation. Infectious virus titers were determined by standard plaque assay on A549 cell monolayers under an agarose-medium overlay. Adenovirus infections of A549 cell monolayers were carried out at a multiplicity of infection (MOI) of 5 or 10 PFU per cell. Cell medium was aspirated, viral inoculum was added, and flasks were incubated at 37°C with 5% CO2 for 1 h with periodic rocking. For 1-β-d-arabinofuranosylcytosine (araC) experiments, medium was replenished with 2% EMEM containing 40 μg/ml araC (Sigma, St. Louis, MO). Inhibition of viral DNA replication was maintained by supplementing the infection medium with an additional 40 μg/ml of araC at 8, 24, and 36 h postinfection (p.i.).

**Antibodies.** Antibodies used for immunofluorescence microscopy, Western blot analysis, and immunoprecipitations were mouse anti-HA (MMS-101R; Covance, Princeton, NJ), mouse anti-EGFP (JL-8; Clontech, Mountain View, CA), mouse anti-calnexin (Ab2798; Abcam, Cambridge, MA), mouse anti-Golgin 97 (Ab2684; Abcam, Cambridge, MA), mouse anti-58K-Golgi protein (A21270; Molecular Probes, Carlsbad, CA), mouse anti-vimentin (sc-6260; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GADPH) (14C10; Cell Signaling, Danvers, MA), rabbit anti-HA-conjugated agarose beads (Ab27029; Abcam, Cambridge, MA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (115-035-003; Jackson ImmunoResearch, West Grove, PA). Cy3-conjugated AffiniPure donkey anti-mouse IgG(H+L) (715-175-070; Jackson ImmunoResearch, West Grove, PA) was used as a secondary antibody for immunofluorescence staining. The antibody used for immunofluorescence microscopy was diluted in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (PBS-T) and 1% (vol/vol) goat serum.

**TABLE 1. Characteristics of subspecies B1 viruses used in this study, representing naturally occurring mutants of ORF E3-10.9K**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Genome typea</th>
<th>ORF E3-10.9K ortholog</th>
<th>Molecular mass (kDa) of predicted polypeptide</th>
<th>Predicted features of the encoded polypeptidec</th>
<th>No. of predicted glycosylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAdV-16</td>
<td>Ch.79</td>
<td>16p</td>
<td>E3-10.9K</td>
<td>10.9</td>
<td>+ + +</td>
<td>2</td>
</tr>
<tr>
<td>HAdV-3</td>
<td>GB</td>
<td>3p</td>
<td>E3-9K</td>
<td>11.6</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>HAdV-7</td>
<td>Gomen</td>
<td>7p</td>
<td>E3-7.7K</td>
<td>7.7</td>
<td>+ + +</td>
<td>2</td>
</tr>
<tr>
<td>KCH4</td>
<td>7b</td>
<td>E3-4.8K</td>
<td>4.8</td>
<td>+ + +</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NHRC611</td>
<td>7d2</td>
<td>E3-4.8K</td>
<td>4.8</td>
<td>+ + +</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>87-922</td>
<td>Null</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

a Determined by restriction enzyme analysis.
b Due to a 51-nucleotide in-frame deletion, as previously described (28).
c Predicted tyrosine-based sorting motif (YSPM) (4).

**FIG. 1. Comparison of the coding capacities of the E3 regions of species C and subspecies B1 HAdVs.**

![Graphical representation of the coding capacities of the E3 regions of species C and subspecies B1 HAdVs.](http://jvi.asm.org/Downloaded from http://jvi.asm.org on October 24, 2017 by guest)
Sequence-based structural predictions. Hydrophobicity plots were generated using the Kyte-Doolittle algorithm implemented in Protein (Lasergene, version 7.0.0, DNASTar, Inc., Madison, WI). N-glycosylation and O-glycosylation sites were predicted using the Web-based programs NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOglyc 3.1 (http://www.cbs.dtu.dk/services/NetOglyc/) (24). Protein topology was predicted using the Web-based prediction program TopPred (http://mobile pacteur.fr/cgi-bin/portal.py?form=toppred) (8, 54). CSS-Palm 2.0 (38) was used for prediction of palmitoylation sites.

Generation of constructs for ectopic expression of C-terminal EGFP fusion proteins. Due to the inadequate performance of polyclonal rabbit sera raised against KLH-conjugated synthetic peptides, subcellular localization and glycosylation studies were carried out using ectopically expressed EGFP fusion proteins in HeLa cells by use of a tetracycline-regulated expression system. Viral DNA was isolated from infected monolayers of A549 cells as previously described (26) and was used as a template for PCR-based cloning of the E3:10.9K ORF. ORF E3:10.9K ortholog genes were first amplified from genomic viral DNA by use of a high-fidelity polymerase (iProof; Bio-Rad, Hercules, CA) and primers whose sequences incorporated convenient restriction sites and mutations of the stop codon and then were cloned into the pEOPF-Ni cloning vector (Chontech, Mountain View, CA). For tetracycline-inducible expression, ORF E3:10.9K–EGFP fusions were then subcloned into the pCDNA 4/TO vector (Invitrogen, Carlsbad, CA), using HindIII and NotI sites. Plasmids generated were designated pCDNA 4/TO 4.8 K-EGFP, pCDNA 4/TO 7.7 K-EGFP, pCDNA 4/TO 9 K-EGFP, and pCDNA 4/TO 10.9 K-EGFP. All constructs were checked for accuracy by sequencing.

Transfections. HeLa T-REX cells (Invitrogen, Carlsbad, CA) were seeded on coverslips or 100-mm dishes and transfected the following day with 0.3 µg or 2 µg of plasmid DNA, respectively, using Effectene reagent (Qiagen, Valencia, CA) following the manufacturer’s recommended protocols. Tetracycline (1 µg/ml) was added to cells at 24 h posttransfection to induce expression of EGFP-tagged E3:10.9K ortholog or control EGFP. At 24 h postinduction, cells were either fixed for immunofluorescence microscopy or harvested for protein analysis as described below.

Protein extraction and glycosidase digestion. At the time of harvest, transfected cells in 100-mm culture dishes were washed twice with ice-cold PBS and incubated on ice with RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland) for 5 min. Cell lysates were collected and clarified by centrifugation at 16,000 × g for 15 min to remove cellular debris. Supernatants were analyzed for total protein content by a standard Bradford assay.

Aliquots of transfected cell lysates containing 40 µg of total protein or the digestion control feticin were digested using reagents included in an enzymatic protein deglycosylation kit (Sigma, St. Louis, MO). All digestions were carried out at 37°C. An undigested protein sample extracted from each infected cell lysate incubated at 37°C in the absence of glycosidases was included as a control for determining the acylating incubation.

For digestion with peptide:N-glycosidase (PNGase), 1 µl of enzyme was added to the reaction mixture as indicated in the manufacturer’s instructions and then incubated for 3 h. For digestion with O-glycosidase and neuraminidase, 1 µl of neuraminidase was first added to the reaction mixture and incubated for 3 h, and then O-glycosidase was added and incubated for an additional 3 h. For digestion with all glycosidases, the sequence of enzyme addition was PNGase, neuraminidase, and then O-glycosidase. After addition of each enzyme, reaction mixtures were incubated for 3 h. Samples (10.4 µl) were separated under reducing conditions in morpholinopropanesulfonic acid (MOPS)-SDS running buffer in NuPAGE Novex 12% polyacrylamide Bis-Tris gels (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and subsequently were either transfected electrophoretically to polyvinylidene difluoride (PVDF) membranes as described below or stained with SimplyBlue Safe stain (Invitrogen, Carlsbad, CA).

Tunicamycin inhibition of N-glycosylation. Transfected cells in 100-mm culture dishes were treated with 10 µg/ml tunicamycin-supplemented growth medium to inhibit N-glycosylation. One hour later, 1 µg/ml tetracycline was added to induce protein expression. Cells were washed and harvested at 24 h postinduction as indicated in “Protein extraction and glycosidase digestion.” Samples (10 µl) were separated under reducing conditions in MOPS-SDS running buffer in NuPAGE Novex 12% polyacrylamide Bis-Tris gels (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and then were transferred electrophoretically to PVDF membranes for Western blot analysis as described below.

Immunoprecipitations. Rabbit anti-HA-conjugated beads (15 µl) (Abcam, Cambridge, MA) were added to an aliquot of cell lysate containing 1 mg of total protein, further diluted to 500 µl with RIPA buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland), and incubated overnight at 4°C with rotation. Beads were collected at 500 × g for 30 s and washed three times for 5 min in cold RIPA buffer with protease inhibitor cocktail (Roche, Basel, Switzerland). Bound protein was then eluted in 60 µl of sample buffer with reducing agent by being heated at 70°C for 10 min. Samples (15 µl) were separated under reducing conditions in morpholineethanesulfonic acid (MES)-SDS running buffer in NuPAGE Novex 12% polyacrylamide Bis-Tris gels (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and then were transferred electrophoretically to PVDF membranes for analysis as described below.

Membrane fractionation. A549 cells were infected at an MOI of 10 PFU/ml. At 48 h p.i., cells were washed twice in 1× PBS, and 1 ml hypotonic buffer (40 mM HEPES, 4 mM EDTA, 4 mM EGTA, 10 mM dithiothreitol [DTT], 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Complete Mini protease inhibitor cocktail [Roche, Basel, Switzerland] was added. Cells were scraped from the dish, transferred to a 1.5-ml tube, and incubated on ice for 30 min. After incubation, samples were sonicated three times with 2-s pulses and centrifuged at 100,000 × g for 1 h at 4°C in a swinging-bucket-rotor ultracentrifuge. After centrifugation, the supernatant (cytosolic fraction) was collected for Western blot analysis. The pellet was resuspended in 1 ml of 1× lysis buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 1 mM sodium vanadate, 50 mM NaF, Complete Mini protease inhibitor cocktail [Roche, Basel, Switzerland]), and residual debris was removed by centrifugation at 15 min at 16,000 × g. The supernatant (membrane fraction) was collected. Samples were analyzed for total protein content by a standard Bradford assay. Samples (10 µg of total protein) were separated under reducing conditions in MES-SDS running buffer in NuPAGE Novex 12% polyacrylamide Bis-Tris gels (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions and then were transferred electrophoretically to PVDF membranes for 45 min at 100 V in transfer buffer containing 50 mM Tris base, 380 mM glycine, 0.1% SDS, and 20% methanol.

Western blotting. Membranes were blocked with 5% (wt/vol) nonfat dry milk in 0.1% (vol/vol) Tween 20–Tris-buffered saline (TBS-T; blocking buffer) for 1 h at room temperature. The primary antibody, diluted 1:1,000 in blocking buffer, was incubated with the membrane overnight at 4°C with rocking. After washes in 0.1% (vol/vol) TBS-T, the membrane was incubated with peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson Immunoresearch) diluted 1:100,000 in blocking buffer for 1 h at room temperature. After being washed, the membrane was treated with Western Lightning Plus chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA) according to the manufacturer’s instructions and then exposed to blue X-ray film. For Western blot analysis of membrane fractionation experiments, the membrane was treated with SuperSignal West Femto maximum-sensitivity substrate (Thermo Scientific, Rockford, IL).

Immunofluorescence microscopy. Transfected cells on coverslips were washed three times in PBS and fixed in 4% (wt/vol) paraformaldehyde in PBS for 15 min at room temperature 24 h after induction with tetracycline. Fixed cells were rinsed twice in PBS, and then washed cells were permeabilized by treatment with 0.2% (vol/vol) Triton X-100 in PBS for 2 min at room temperature. Coverslips were washed three times in PBS and blocked for 1 h with 10% (vol/vol) goat or donkey serum in PBS-T at room temperature with rocking. Coverslips were also washed three times in PBS-T after being blocked and after incubation with primary and secondary antibodies. The Cy5-conjugated donkey anti-mouse IgG described above was used as a secondary antibody for all immunofluorescence staining in this study. After secondary antibody incubation in the dark, coverslips were washed three times in PBS-T and three times in PBS. Coverslips were briefly submerged in double-distilled water to remove excess salt and were mounted in Slowfade Gold with DAPI (Molecular Probes, Carlsbad, CA) on glass slides. For staining with wheat germ agglutinin-Alexa Fluor 647 conjugate (WGA-647) (Molecular Probes, Carlsbad, CA), transfected cells on coverslips were rinsed three times in PBS and incubated for 10 min at 37°C with 200 µl of 5-µg/ml WGA-647 in PBS. Cells were then fixed, quenched, and mounted as indicated above.

Images were generated with a Zeiss Axioplan epifluorescence microscope with a Hamamatsu digital camera controlled with SlideBook image analysis software, version 4.0 (Intelligent Imaging Innovations, Denver, CO). Images were deconvolved using the nearest neighbor algorithm contained within the SlideBook software, and figures were prepared using Adobe Photoshop CS3, version 10.0.1 Adobe Systems Inc., San Jose, CA.

RNA isolation. A549 cells were infected at an MOI of 5 PFU/cell. At 8 h and 24 h p.i., total RNA was extracted using RNAqueous (Ambion, Austin, TX) following the manufacturer’s instructions. RNA was treated with a Turbo DNA-free kit (Ambion, Austin, TX) to remove contaminating DNA. Elimination of
DNA was confirmed by the absence of visible bands after PCR with DNase-treated total RNA as the template (no-RT control).

RT-PCR. RNA (1 μg) and random decamers were used to generate cDNA by use of a RETROscript kit (Ambion, Austin, TX) in a 20-μl total volume following the manufacturer’s recommended protocol. The cDNA product was amplified by PCR (1 μl) in a total volume of 50 μl of PCR mix, including 1× GoTaq buffer, 1.25 units of GoTaq polymerase, a 0.2 mM concentration of each deoxyribonucleoside triphosphate, 1.5 mM MgCl2, and 25 pmol of each primer (Promega, Madison, WI). The primers used for RT-PCR are described in Table 2. The reaction mixtures were heated to 95°C for 5 min for the initial denaturation step, followed by 34 cycles of 94°C for 30 s, the annealing temperature for 30 s, and 72°C for 30 s. The annealing temperature used for each primer pair was 2°C lower than the lowest melting temperature for the primer pair. Cycling was followed by a final extension at 72°C for 7 min, at the end of which the reaction mix was kept at 4°C until analysis. PCR products were analyzed by agarose gel electrophoresis in Tris-borate-EDTA buffer (TBE). PCR products were visualized after ethidium bromide staining by use of a Universal Hood II Gel Doc XR camera and Quantity One software (Bio-Rad, Hercules, CA).

RT-PCR products of interest were gel purified and cloned using a pCR2.1 TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Products were sequenced at the DNA Research Services Core Facility, University of New Mexico.

Generation of HAdV-3p-9K-HA mutant virus. A schematic of the strategy used to generate HAdV-3-9K-HA is presented in Fig. 2. The highly efficient bacteriophage λ Red recombination system (37) was used to generate a HAdV-3 clone encoding a C-terminally HA epitope-tagged version of the E3-9K ORF. The bacmid pKSB2Ad3wt, harboring the full-length genome of HAdV-3 prototype strain GB, was a kind gift of Silvio Hemmi (44). We generated a shuttle plasmid, pE3-9K, to facilitate modification of the E3-9K ORF within the bacterial shuttle plasmid pKSB2Ad3wt of PCR mix, including 1× GoTaq buffer, 1.25 units of GoTaq polymerase, a 0.2 mM concentration of each deoxyribonucleoside triphosphate, 1.5 mM MgCl2, and 25 pmol of each primer (Promega, Madison, WI). The primers used for RT-PCR are described in Table 2. The reaction mixtures were heated to 95°C for 5 min for the initial denaturation step, followed by 34 cycles of 94°C for 30 s, the annealing temperature for 30 s, and 72°C for 30 s. The annealing temperature used for each primer pair was 2°C lower than the lowest melting temperature for the primer pair. Cycling was followed by a final extension at 72°C for 7 min, at the end of which the reaction mix was kept at 4°C until analysis. PCR products were analyzed by agarose gel electrophoresis in Tris-borate-EDTA buffer (TBE). PCR products were visualized after ethidium bromide staining by use of a Universal Hood II Gel Doc XR camera and Quantity One software (Bio-Rad, Hercules, CA).

RT-PCR products of interest were gel purified and cloned using a pCR2.1 TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Products were sequenced at the DNA Research Services Core Facility, University of New Mexico.

Generation of HAdV-3p-9K-HA mutant virus. A schematic of the strategy used to generate HAdV-3-9K-HA is presented in Fig. 2. The highly efficient bacteriophage λ Red recombination system (37) was used to generate a HAdV-3 clone encoding a C-terminally HA epitope-tagged version of the E3-9K ORF. The bacmid pKSB2Ad3wt, harboring the full-length genome of HAdV-3 prototype strain GB, was a kind gift of Silvio Hemmi (44). We generated a shuttle plasmid, pE3-9K, to facilitate modification of the E3-9K ORF within the bacmid pKSB2Ad3wt. A SalI site was included just upstream of the pKSB2Ad3wt upon recombination.

A C-terminally HA-tagged version of ORF E3-9K was cloned into the pE3-9K shuttle and linearized with HindIII and PvuI. The purified linear fragment was then transformed by electroporation into Escherichia coli strain BW25113, harboring the pKSB2Ad3wt bacmid and the temperature-sensitive replication pKD46, which expresses the λ Red genes under the control of the arabinose-inducible pBAD promoter (9). Electroporation was performed in a 0.2-cm-gap cuvette with a microcylinder (Bio-Rad, Hercules, CA) set to 2,500 V. Electroporated cells were resuspended in SOC plus 0.5% L-arabinose and incubated overnight at 30°C. Induction of the λ Red genes resulted in recombination between the linear pE3-9K-HA fragment and the HAdV-3 bacmid, and recombinant pAd3-9K-HA-FZF bacmids were then purified and electroporated into E. coli strain SW105 (56), which expresses Flp recombinase under arabinose control. Incubation overnight at room temperature in SOC plus 0.5% L-arabinose induces Flp recombinase expression, which collapses the FZF cassette into a single 34-bp FRT site. Transformed cells were plated at 30°C on chloramphenicol (15 μg/ml). Several colonies were picked and screened for sensitivity to zeocin, as proper Flp recombination results in a loss of zeocin resistance (Fig. 2D). Final pAd3-9K-HA clones were screened by restriction digestion and sequenced to confirm the presence of the E3-9K-HA fusion. The recombination described above ultimately resulted in a new SalI site, between the E3-20.5K and E3-9K ORFs, as well as a BamHI site, the 34-bp FRT scar site, and an NheI site between the E3-9K and E3-10.2K ORFs in the E3 region (Fig. 2D). A recombinant pAd3-9Kwt vector containing the unmodified wild-type E3-9K ORF was also constructed to control for the introduction of these additional elements into the HAdV-3 genome.

DNAs from pAd3-9K-HA and pAd3wt were digested with MluI and transcribed into A549 cells by use of Effectene reagent (Qiagen, Valencia, CA). Two micrograms of digested bacmid DNA was used per transfection. Transfected cells were incubated for 14 days. For virus recovery, cells were subjected to 3 freeze-thaw cycles at −80°C and 25°C and then clarified by centrifugation. The resultant virus was propagated by passage in A549 cells, and the presence of virus was confirmed by observation of the characteristic adenovirus cytopathic effect. Titrated stocks of HAdV-3-9K-HA and HAdV-3wt were generated as indicated above. The identity of the mutant virus was confirmed by sequencing of the E3 region and restriction enzyme analysis with BamHI and Sall.

Nucleotide sequence accession numbers. Sequences obtained from cloned RT-PCR products were deposited in GenBank under accession numbers GU951413 to GU951425.

RESULTS

Sequence-based predictions of structural motifs for E3-10.9K orthologs. In order to investigate the predicted structural characteristics of various E3-10.9K-encoded proteins, Kyte-Doolittle hydrophobicity plots were analyzed for the orthologs E3-4.8K (encoded by HAdV-7p strain CH4), E3-7.7K (encoded by HAdV-7p strain Gomen), E3-9K (encoded by HAdV-3p strain GB), and E3-10.9K (encoded by HAdV-16p strain Ch.79). All orthologs, except for E3-4.8K, exhibited a region of high hydrophobicity that was predicted to be a transmembrane domain, and E3-7.7K, E3-9K, and E3-10.9K contained aromatic residues within this hydrophobic domain (Fig. 3B). The hydrophobicity plot generated for E3-10.9K is shown as an example in Fig. 3A. The protein topology for E3-10.9K and E3-9K was predicted to be Ccyt (the C terminus of the protein is cytoplasmic). The cytoplasmic tails of the E3-9K and E3-10.9K orthologs contain a region rich in basic residues (labeled as a polybasic region in Fig. 3B) and a putative tyrosine-based sorting signal, YSPM (4). Two potential N-glycosylation sites were identified for E3-4.8K, E3-7.7K, and E3-10.9K, and only one N-glycosylation site was predicted for E3-9K (Fig. 3B). No obvious O-glycosylation sites were predicted for any of the examined orthologs by use of NetOGlyc 3.1 software (data not shown). A palmitoylation site was predicted for the E3-10.9K-encoded proteins.

TABLE 2. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Melting temp (°C)</th>
<th>Nucleotide positionα</th>
<th>Corresponding gene/coding region</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3-10.9K forward</td>
<td>GTTCAATCAATACCC</td>
<td>43.9</td>
<td>29796–29811</td>
<td>E3-9Kβ</td>
</tr>
<tr>
<td>E3-10.9K reverse</td>
<td>CTAATGAGGAGAATAG</td>
<td>42.5</td>
<td>29973–29990</td>
<td>E3-9Kβ</td>
</tr>
<tr>
<td>E3-10.9K reverse Tm60</td>
<td>GGGGACGACTAATGAGAATAG</td>
<td>61.8</td>
<td>29973–29997</td>
<td>E3-9Kβ</td>
</tr>
<tr>
<td>TPL3 forward</td>
<td>CAGTCCGCAATCGCAAG</td>
<td>60.3</td>
<td>9548–9563</td>
<td>Tripartite leader 3β</td>
</tr>
<tr>
<td>Hexon reverse</td>
<td>GTTCTGTTAGCATGCCG</td>
<td>59.1</td>
<td>19253–19369</td>
<td>L3-hexon proteinβ</td>
</tr>
<tr>
<td>RIG/S15 forward</td>
<td>TCTCCGAAGTTCAGCACCTACC</td>
<td>62.6</td>
<td>NA</td>
<td>Small ribosomal subunit RIG/S15β</td>
</tr>
<tr>
<td>RIG/S15 reverse</td>
<td>CGGGCCGCCACTGTCCTAGC</td>
<td>76.9</td>
<td>NA</td>
<td>Small ribosomal subunit RIG/S15β</td>
</tr>
</tbody>
</table>

α Relative to the HAdV-3p strain GB full genome sequence (GenBank accession no. AY159834).

See reference 29.
and the cytoplasmic domain. The same site was predicted to be present in E3-9K (Fig. 3B). No palmitoylation sites were predicted for the backbones of the orthologs E3-7.7K and E3-4.8K. Compared to E3-10.9K and as previously described (28), the E3-9K ortholog contains an in-frame deletion affecting the amino terminus of the predicted protein, and E3-7.7K has a premature stop codon immediately after the hydrophobic domain, resulting in a truncated version of the polypeptide that lacks the cytoplasmic tail. Unique among the E3-10.9K orthologs examined, E3-4.8K does not have a putative transmembrane domain (Fig. 3B). As previously reported (28), this is due to a frameshift mutation and a subsequent premature stop codon.

E3-10.9K orthologs are posttranslationally modified. In order to investigate the use of N-glycosylation sites predicted for E3-10.9K orthologous proteins, we expressed C-terminally EGFP-tagged ORF E3-10.9K-encoded proteins by use of the T-REx tetracycline-regulated expression system, inhibited N-glycosylation with tunicamycin, and examined protein migration patterns by Western blotting (Fig. 4A). As shown in Fig. 4, with the exception of E3-4.8K, all E3-10.9K fusions extracted from untreated cells (panel A) or not digested with glycosidases (panel B) migrated as multiple diffuse bands of higher molecular weights (MW) than those predicted for the corresponding polypeptide backbones (indicated by asterisks in Fig. 4C) in polyacrylamide gels containing SDS, indicating the presence of posttranslational modifications. The electrophoretic mobilities of E3-7.7K-EGFP, E3-9K-EGFP, and E3-10.9K-EGFP, but not that of E3-4.8K-EGFP, were increased by tunicamycin treatment, indicating that these proteins were N glycosylated. In order to further investigate the use of glycosylation sites predicted for E3-10.9K orthologous proteins, EGFP-tagged E3-10.9K orthologous fusion proteins were digested with a panel of glycosidases, and protein migration patterns were examined by SDS-PAGE and Western blotting (Fig. 4C). Some nondiffuse bands appearing smaller than the predicted polypeptide backbone were observed. We postulate that these bands represent products arising from proteolytic cleavage and containing the EGFP epitope. No high-MW bands were observed for the E3-4.8K-EGFP fusion polypeptide, suggesting a lack of any posttranslational modifications for this ortholog. For E3-7.7K-EGFP and E3-10.9K-EGFP fusions, diffuse bands of approximately

![Figure 2](http://jvi.asm.org/)

**FIG. 2.** ORF E3-9K shuttle plasmid and recombination strategy. (A) Map of the pE3-9K shuttle plasmid and its unique restriction sites. Sequences cloned into the SalI and BamHI sites replace ORF E3-9K of the HAdV-3p genomic bacmid upon recombination. (B) Transformation of the linearized shuttle plasmid containing the E3-9K-HA fusion into λ Red recombination E. coli strain BW25113, containing the HAdV-3p genomic bacmid, results in recombination across homologous regions. (C) Recombinant bacmid PA3-9K-HA-FZF is selected by growth under chloramphenicol and zeocin selection. (D) Transformation of this recombinant into E. coli strain SW105 causes collapse across the FRT sites and deletion of the zeocin resistance cassette to generate pAd3-9K-HA.
60 kDa were readily detectable. For E3-9K-EGFP, these diffuse bands were approximately 50 kDa (Fig. 4B). Removal of N-linked glycans with PNGase resulted in increased electrophoretic mobility for E3-7.7K-EGFP, E3-9K-EGFP, and E3-10.9K-EGFP, confirming the tunicamycin data and indicating that these fusion proteins contained N-linked glycans (Fig. 4C). E3-9K has only one potential glycosylation site, at residue 7 (Fig. 3B), and these results indicate that the site is used.

For E3-9K-EGFP, digestion with PNGase resulted in a loss of all diffuse bands. No major shifts in electrophoretic mobility were detected when E3-9K-EGFP was sequentially digested with neuraminidase and O-glycosidase to remove sialic acid residues and O-linked glycans, respectively, suggesting that the 17-amino-acid deletion present in the N-terminal domain of this protein defines a region subject to O glycosylation. In contrast, sequential digestions of E3-7.7K-EGFP and E3-10.9K-EGFP with neuraminidase and O-glycosidase resulted in slightly increased electrophoretic mobility of the bands, indicating the presence of O-linked glycans in these proteins. Loss of one of the high-MW species observed in PNGase-treated E3-7.7K-EGFP was accomplished by sequential digestion with neuraminidase, O-glycosidase, and PNGase. For E3-10.9K-EGFP, a slight shift in electrophoretic mobility was observed upon digestion to remove O-linked glycans. Sequential digestion with neuraminidase, O-glycosidase, and PNGase resulted in diffuse bands of approximately 40 kDa. These data suggest that E3-7.7K, E3-9K, and E3-10.9K proteins are N glycosylated and that E3-7.7K and E3-10.9K...
proteins are also O glycosylated. Since complete digestion of proteins was confirmed by simultaneous digestion with an equal amount of fetuin as a control (data not shown), the presence of high-MW bands after sequential digestion with neuraminidase, O-glycosidase, and PNGase indicates the occurrence of other types of posttranslational modification, which may include palmitoylation of the predicted sites in the border region between the transmembrane domain and the cytoplasmic domain of the backbones of E3-9K and E3-10.9K (Fig. 3B).

E3-10.9K orthologs with a hydrophobic domain and a C terminus localize to the plasma membrane. Structural predictions suggested that E3-7.7K, E3-9K, and E3-10.9K are integral membrane proteins. Due to the lack of a suitable antibody, we examined the subcellular localization of C-terminally EGFP-tagged orthologs ectopically expressed in HeLa T-REx cells. Clear localization to the plasma membrane was observed for E3-10.9K-EGFP and E3-9K-EGFP, which colocalized with wheat germ agglutinin-Alexa Fluor 647 conjugate (Fig. 5). In contrast, E3-7.7K-EGFP displayed only faint plasma membrane localization in some cells but showed distinct localization to an intracellular juxtanuclear compartment that could not be identified. The E3-7.7K-EGFP fusion protein did not colocalize with the endoplasmic reticulum (ER) marker calnexin, the trans-Golgi network marker Golgin 97, or the general Golgi apparatus marker 58K-Golgi protein (Fig. 5). Because the juxtanuclear localization and aggregate-like appearance of E3-10.9K-EGFP, E3-9K-EGFP, and E3-7.7K-EGFP were suggestive of cytoplasmic aggresomes, which are structures for degradation or virus assembly that can be induced by protein aggregation (57), we stained HeLa T-REx cells expressing E3-10.9K orthologs with an anti-vimentin an-

\[ \text{FIG. 5. Subcellular localization of E3-10.9K orthologous proteins ectopically expressed as C-terminal EGFP fusions. HeLa T-REx cells were transfected with plasmids expressing EGFP-tagged E3-10.9K orthologs under the control of a tetracycline-regulated promoter. Expression was induced with 1 \mu g/ml of tetracycline for 24 h. Cells were fixed, and localization to either the plasma membrane (WGA-647), endoplasmic reticulum (calnexin), or Golgi apparatus (Golgin 97 and 58K-Golgi protein) was determined by immunofluorescence microscopy with WGA-647 or with anti-calnexin, anti-Golgin 97, or anti-58K-Golgi antibodies. Staining with anti-vimentin was carried out to investigate the presence of cytoskeleton rearrangements. Cy5-conjugated donkey anti-mouse IgG was used as a secondary antibody in all experiments. Pseudocolored merged images show EGFP (green), DAPI (blue), subcellular structures (red), and overlapping EGFP and subcellular structures (yellow). Schematics of the corresponding EGFP-tagged E3-10.9K protein orthologs indicating the N-terminal domain (N), transmembrane domain (TM), C-terminal domain (C), and EGFP tag (EGFP) are shown.} \]
However, neither obvious cytoskeleton rearrangements nor the formation of a typical vimentin cage around the aggregates was observed (Fig. 5). The E3-4.8K-EGFP fusion displayed a diffuse cellular localization similar to that observed for the EGFP control protein.

ORF E3-10.9K transcripts are expressed from the adenovirus major late promoter. Total RNAs were isolated from A549 cells infected with HAdV-B1-infected A549 cells. (A) A549 cells were infected at an MOI of 5 PFU/cell with HAdV-3p, HAdV-7p, HAdV-16p, or HAdV-7 genome type 7 h or 7d2 or mock infected (lane m) and then were harvested for total RNA extraction and analysis by RT-PCR at 8 and 24 h postinfection. Primer pairs were as follows: E3-10.9K forward and E3-10.9K reverse (E3-10.9K internal), TPL3 forward and E3-10.9K reverse Tm60 (TPL/E3-10.9K), TPL3 forward and hexon reverse (TPL/hexon), and RIG/s15 forward and RIG/s15 reverse (RIG). RIG was a positive control for RT, and TPL/hexon was a positive control for viral late gene expression. MM, molecular size marker. (B) Products were cloned and sequenced. Predicted splice acceptor sites (AG) are shown in large italics.
E3-20.5K, continuing through ORF E3-10.9K (Fig. 6B), and that the 300-bp products included the TPL followed by four nucleotides upstream of ORF E3-10.9K (Fig. 6B). This was true for all viruses examined. Sequence analysis of the 62-bp product detected in HAdV-3p-infected cells showed that it resulted from amplification of a transcript containing the TPL sequence and a truncated form of E3-9K, with no detectable start codon within this ORF. Our sequence data also showed that at late times of infection, transcripts from the MLP appeared to be spliced to AG acceptor sites just upstream of the E3-20.5K and E3-10.9K start codons.

HA-tagged E3-9K is detectable in the membrane fraction at late times p.i. To investigate ORF E3-10.9K expression at the protein level, and due to the lack of adequate antibodies, A549 cells were infected with an HAdV-3p mutant virus encoding the 9-kDa ortholog, E3-9K, as a C-terminal HA fusion (HAdV-3p-9K-HA). Expression of E3-9K-HA was investigated in cell lysates collected at 8 and 48 h p.i. by immunoprecipitation and Western blotting (Fig. 7A). A band of approximately 10 kDa and several high-MW diffuse bands were detected at 48 h p.i. but not at 8 h p.i. In order to confirm the late expression of E3-9K-HA during mutant HAdV-3p infection, araC was used to inhibit DNA replication and to arrest HAdV-3-9K-HA-infected cells at early stages of infection. The araC-treated infected cells showed no detectable HA-tagged E3-9K at 48 h p.i., indicating that expression of the gene requires viral DNA replication (Fig. 7A). Although we were unable to detect the E3-9K-HA protein by immunoprecipitation and Western blotting at 8 h p.i., RT-PCR of HAdV-3-9K-HA-infected cells showed the presence of the corresponding transcripts at early time points (Fig. 7B). No amplification was obtained for no-RT controls (data not shown). Sequence analysis of the most abundant PCR product (816 bp) detected at 24 h p.i. confirmed that it corresponded to the same late transcript detected in HAdV-3p-infected cells (Fig. 6). Cell fractionation experiments allowed the detection of the E3-9K-HA fusion protein by Western blotting of membrane but not cytosolic fractions (Fig. 7C).

DISCUSSION

The E3 region is present only in the genomes of the mastadenoviruses and siadenoviruses and represents a locus for genus-specific genes (11). The diversified genetic content of the E3 regions of HAdVs belonging to species A to F has been considered a strong indicator of the existence of species-specific genes that have evolved to carry out functions required for unique virus-host interactions (5). Of particular interest is the cluster of highly divergent genes encoding membrane proteins located between ORFs E3-19K and RId (Fig. 7A). A band of approximately 10 kDa and several high-MW diffuse bands were detected at 48 h p.i. but not at 8 h p.i. In order to confirm the late expression of E3-9K-HA during mutant HAdV-3p infection, araC was used to inhibit DNA replication and to arrest HAdV-3-9K-HA-infected cells at early stages of infection. The araC-treated infected cells showed no detectable HA-tagged E3-9K at 48 h p.i., indicating that expression of the gene requires viral DNA replication (Fig. 7A). Although we were unable to detect the E3-9K-HA protein by immunoprecipitation and Western blotting at 8 h p.i., RT-PCR of HAdV-3-9K-HA-infected cells showed the presence of the corresponding transcripts at early time points (Fig. 7B). No amplification was obtained for no-RT controls (data not shown). Sequence analysis of the most abundant PCR product (816 bp) detected at 24 h p.i. confirmed that it corresponded to the same late transcript detected in HAdV-3p-infected cells (Fig. 6). Cell fractionation experiments allowed the detection of the E3-9K-HA fusion protein by Western blotting of membrane but not cytosolic fractions (Fig. 7C). Detection of GADPH in the cytosolic fraction, but not the membrane fraction, confirmed the correct cell fractionation.
in the genomes of HAdV-B2s, and this feature has been correlated with the distinct pathobiology of infection by some serotypes classified within this group of HAdVs (12).

Our sequence-based predictions show that the E3-10.9K ORF encodes a family of orthologous polypeptides with fairly conserved N termini and variable C termini that includes integral membrane proteins but also some naturally occurring mutants without a hydrophobic domain and/or cytoplasmic tail. The circulation of strains of HAdV-3 and HAdV-7 carrying truncating or null mutations in this gene has been documented (27, 28; Kajon, unpublished data), but other than their apparent higher prevalence as causative agents of respiratory disease, no other distinct phenotypic characteristic has been identified for these viruses. Three of the four orthologs examined in this paper, E3-10.9K, E3-9K, and E3-7.7K, have a hydrophilic domain but differ in the sequences of their amino- and carboxyl-terminal domains. The three of them share basic structural characteristics with E3-11.6K/ADP encoded by HAdV-C, and also with a class of diverse virus-encoded membrane-active proteins known as viroporins (7, 14). Like viroporins and ADP, the HAdV-B1 E3-10.9K, E3-9K, and E3-7.7K orthologs exhibit a highly hydrophobic region which is rich in aromatic residues. In addition, and also like the viroporins and ADP, E3-10.9K and E3-9K exhibit a stretch of basic amino acids in their carboxyl-terminal domains that may confer membrane-destabilizing activity to these proteins (7). Studies are under way to characterize the potential membrane-permeabilizing/desaturating activity of these two ORF E3-10.9K-encoded orthologous proteins and their role in facilitating progeny exit, since it is known that not all viral proteins with viroporin-like characteristics induce membrane permeability directly (40).

The predicted presence of a palmitoylation site in the extreme C-terminal portion of the transmembrane domain and the presence of a putative tyrosine-based YXXΦ transport motif in the cytoplasmic tails of E3-10.9K and E3-9K are also structural features that these subspecies B1 orthologous E3 proteins share with ADP, and the roles of these sites in protein trafficking and function warrant further studies.

Like other E3 proteins encoded between ORFs E3-19K and E3-10.4K/RIDα, the products of HAdV-B1 E3-10.9K are expressed late during infection. In addition to HAdV-C E3-11.6K/ADP, the E3-30K protein of species E HAdV-4 and the E3-49K protein of species D HAdV-19a have also been shown to be expressed as late products (31, 48, 59). Interestingly, HAdV-B E3-20.5K, which is encoded immediately upstream of E3-10.9K, is also expressed at both early and late times p.i. (18). It is possible that these two subspecies B1 E3 proteins cooperate to exert their function late in infection. Studies are in progress to examine the occurrence of protein-protein interactions in infected cells.

The examination of C-terminal EGFP fusions by SDS-PAGE revealed the presence of bands of a variety of sizes for all orthologs examined, with the exception of E3-4.8K. The banding pattern observed was similar to that described for ADP (41) and provided evidence of posttranslational modification of the proteins. The occurrence of N glycosylation of the E3-7.7K, E3-9K, and E3-10.9K orthologs was confirmed by tunicamycin inhibition. Our experiments using digestion of EGFP fusion proteins with a variety of glycosidases confirmed that ectopically expressed E3-7.7K-EGFP, E3-9K-EGFP, and E3-10.9K-EGFP contained N-linked glycans and showed that the E3-7.7K and E3-10.9K fusion polypeptides also contained O-linked glycans. The primary difference between the backbones of the E3-9K and E3-10.9K orthologs is an N-terminal in-frame deletion of 17 amino acids, indicating that this domain of the polypeptide comprises an O-glycosylation site.

Identifying the subcellular location of a protein is an important step toward the elucidation of protein function. Although the limitations of using large tags cannot be ignored (45), the inducible expression of N-terminal EGFP fusions in HeLa T-Rex cells allowed us to make important observations. Ectopically expressed EGFP-tagged E3-10.9K and E3-9K orthologs featuring the hydrophobic and C-terminal domains localized primarily to the plasma membrane, while the E3-7.7K fusion protein lacking the cytoplasmic tail localized primarily to a juxtanuclear compartment that could not be identified by our efforts, as it did not stain positive for Golgin 97, 58K-Golgi protein, or calnexin and was not contained within a vimentin cage for cytoplasmic aggresomes (57). The E3-4.8K fusion protein had a diffuse cellular distribution that was not distinguishable from that observed for the EGFP control and was not detectable as a glycosylated product. Taken together, these data indicate that the presence of a hydrophobic domain and a cytoplasmic tail containing a putative tyrosine-based sorting signal (4) is necessary for correct intracellular trafficking, posttranslational processing, and subcellular localization.

The localization observed for the EGFP fusion proteins requires further verification by staining with an antibody against the native protein or a smaller epitope tag to confirm that the EGFP moiety did not disrupt protein trafficking and localization (45) and that the observed juxtanuclear aggregates were not artifacts resulting from overexpression. Our data, however, show a strong correlation between the subcellular distribution of the expressed fusion proteins and the features of their primary sequence, such as the presence of a hydrophobic domain and a cytoplasmic tail with putative palmitoylation and sorting signals. In addition, the results of our initial characterization of naturally occurring mutants of the E3-10.9K ORF also show that, as observed for ADP (52), genetic variability resulting in the lack of a hydrophobic domain impacts the ability of the encoded products to undergo posttranslational modifications, as well as their subcellular localization.

With the exception of HAdV-D E3-49K (59), localization to the plasma membrane has not been reported for any other E3 protein encoded between ORFs E3-19K and RIDα. E3-encoded members of the RID (receptor internalization and degradation) complex of proteins (E3-10.4K/RIDα and E3-14.5K/RIDβ) have been shown to localize to the plasma membrane when expressed during viral infection or coexpressed simultaneously in transfected cells (21, 22, 32, 47, 51). When expressed in the absence of any other viral protein, RIDα localizes to the Golgi apparatus and RIDβ localizes to the ER and Golgi apparatus (33, 47, 51). Although some degree of localization to the plasma membrane has also been described for E3-6.7K (2), this protein localizes primarily to the ER (58). E3-6.7K coimmunoprecipitates with RIDβ (2) and is necessary for RID complex degradation and internalization of TRAIL receptor 2 (32), suggesting that localization to the plasma membrane may
be an important feature contributing to its function. ORF E3-10.9K protein isoforms share little to no sequence similarity with RIDα, RIDβ, or E3-6.7K, and because they are expressed primarily during the late phase of infection, it is very likely that despite their similar subcellular localization, they carry out a completely different function.

ORF E3-10.9K is located immediately upstream of RIDα, in an analogous region of the E3 cassette to that of HADV-C E3-11.6K/ADP, and references to this ORF in the literature assumed that it represented the HADV-B1 homolog of ADP (41). ADP is expressed from the MLP during late stages of infection and plays a role in the efficient release of progeny virions as well as in the induction of host cell death at the end of infection (48–50). ADP is N and O glycosylated and palmitoylated and localizes to the nuclear envelope (16, 41, 52). Localization to the nuclear envelope is necessary for the activity of ADP (50). ADP interacts with the cellular protein MAD2B, although the functional significance of this interaction is unknown (61). Like ADP, E3-10.9K orthologous proteins are glycosylated and are expressed from the MLP at late times p.i. However, none of the E3-10.9K orthologs investigated in this study were detected at the nuclear envelope.

When expressed as EGFP fusions, E3-9K and E3-10.9K showed a distinct plasma membrane localization, while the truncated ortholog E3-7.7K showed a juxtanuclear localization to a compartment that could not be identified. Taking into consideration that localization to the plasma membrane has not been reported for ADP, our data suggest that despite their similar structural features and temporal expression, ORF E3-10.9K-encoded proteins are not the HADV-B1 counterparts of HADV-C ADP, although their role in facilitating viral progeny release at late stages of infection by a different mechanism cannot be ruled out and should be addressed specifically by future work.

E3 is the only region of the HADV genome that encodes integral membrane proteins, and for those E3-encoded proteins with an identified function, localization to specific subcellular membranes is required for functionality (41, 52). Our data strongly suggest that the naturally occurring genetic variability documented for ORF E3-10.9K and particularly affect- data strongly suggest that the naturally occurring genetic variability documented for ORF E3-10.9K and particularly affect-

**REFERENCES**


We thank the anonymous reviewers for their insightful suggestions for improvement of this paper.


