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Biosynthesis of Glycoproteins E and I of Feline Herpesvirus: gE-gI Interaction Is Required for Intracellular Transport

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The biosynthesis of glycoproteins E and I of feline herpesvirus was studied by using the vaccinia virus vTF7-3 expression system. gE and gI were synthesized as N-glycosylated, endoglycosidase H (EndoH)-sensitive precursors with M_rs of 83,000 and 67,000, respectively. When coexpressed, gE and gI formed sodium dodecyl sulfate-sensitive hetero-oligomeric complexes that were readily transported from the endoplasmic reticulum (ER). Concomitantly, the glycoproteins acquired extensive posttranslational modifications, including O glycosylation, leading to an increase in their apparent molecular weights to 95,000 and 80,000 to 100,000 for gE and gI, respectively. In the absence of gE, most gI remained EndoH sensitive. Only a minor population became EndoH resistant, but these molecules were processed aberrantly as indicated by their M_rs (100,000 to 120,000). By immunofluorescence microscopy, gI was detected primarily in the ER but also at the plasma membrane. gE, when expressed by itself, remained EndoH sensitive and was found only in the ER and the nuclear envelope. These results were corroborated by studying the biosynthesis of gE in feline herpesvirus (FHV)-infected cells. In cells infected with wild-type FHV, gE acquired the same co- and posttranslational modifications as during vTF7-3-driven expression. However, an FHV mutant lacking gI failed to produce mature gE. We conclude that gE is retained in the ER, presumably by associating with molecular chaperones, and becomes transport competent only when in a complex with gI.

Feline herpesvirus 1 (FHV), a member of the *Alphaherpesvirinae* subfamily, is the etiologic agent of feline viral rhinotracheitis (for a review, see reference 54). In adult cats, FHV replication is usually restricted to the upper respiratory tract and conjunctiva. The virus establishes neural latency in the trigeminal ganglion (27, 48, 52), from which recrudescent respiratory and ocular infections may arise. In kittens, FHV may cause generalized infections sometimes involving osseous pathology with widespread necrosis in the growth regions of the skeleton (54).

FHV infection can be well controlled by vaccination, and this may explain why, in contrast to other alphaherpesviruses such as herpes simplex virus (HSV), pseudorabies virus (PRV), and varicella-zoster virus (VZV), FHV has long received little attention. Recently, the potential use of FHV in live recombinant vaccines has sparked renewed interest (10, 51, 68). Moreover, FHV provides an attractive system to study herpesvirus-host interactions in the natural host. In contrast to HSV and PRV, FHV is very species specific and replicates only in felid cells (54). Thus, FHV is also an interesting model with which to study factors that determine host and cell tropism.

The FHV genome is about 126 kb in length (29) and appears to be largely colinear with those of other alphaherpesviruses. A number of FHV genes have been identified, including those for glycoproteins gB (44, 60), gC (71), gD, gE, and gI (61, 72). The first three glycoproteins mediate attachment of the virion to the host cell and penetration (for a review, see reference 62). The role of gE and gI is more elusive. For all alphaherpesviruses studied thus far, these proteins are dispensable for viral entry and replication (3, 24, 45, 50, 64). Although gE and gI are often regarded as accessory proteins, they are of crucial importance during natural infection. Mutants lacking gE and/or gI are severely crippled, producing smaller lesions at the primary site of infection and exhibiting reduced neuronal spread (3, 7, 8, 15, 16, 23, 37, 38, 47, 55, 64, 70).

There is ample evidence that gE and gI can form a complex (35, 73, 76), most likely a heterodimer (70). In the case of HSV type 1 and VZV, both gE and the gE-gI complex display Fc receptor activity (4, 5, 18, 36, 42). It has long been thought that gE and gI are primarily involved in immune evasion by protecting against complement-mediated cytolysis and virolysis (19, 25, 65). However, for other alphaherpesviruses, such as PRV (76), binding of immunoglobulin G (IgG) by gE-gI could not be shown. Furthermore, it is becoming increasingly clear that gE and gI play a pivotal role in cell-to-cell spread both in vitro and in vivo (3, 7, 15, 16, 23, 37, 38, 47, 75). Herpesvirus mutants lacking gE and/or gI characteristically display a small-plaque phenotype when grown in tissue culture (3, 15, 45, 50). The mechanism of cell-to-cell spread is still unknown but may entail controlled local fusion of cell membranes (3, 12, 62). In addition to gE and gI, several other proteins are involved, e.g., gB, gK, the gH-gL complex, and the presumptive cytoplasmic protein UL24 (12, 62).

gE and gI of HSV and VZV, when individually expressed in heterologous expression systems, are readily transported from the endoplasmic reticulum (ER) to the plasma membrane (5, 18, 42, 73). In PRV, export of gE and gI from the ER is inefficient unless both proteins are present (70). Here, we present the first biochemical characterization of FHV gE and gI. We provide evidence that both in the heterologous vaccinia virus vTF7-3 expression system and in FHV-infected cells, gE-gI interaction is required to allow exit of gE from the ER.

MATERIALS AND METHODS

Cells, viruses, and antisera. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Inc.), supplemented with 10%

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TABLE 1. Oligonucleotide primers

Primer	Nucleotide sequence (5′ to 3′)[a]	Gene	Position[b]	Polarity
346	TG(C/T)AG(C/T)TT(C/T)ACNTCNCC	gE	790–806	+
348	AC(A/G)AANAC(A/G)TA(C/T)AANCC	gE	992–1008	−
369	ATATTAGGTTTCTTCGTTAATTAC	gI	1158–1181	−
370	ATACTTAATATGTCGTCGATAGCC	gI	−9–15	+
374	CATGCCGGATCCTGTCTACATCCGGAAG	gE	762–781	+
379	AAGTATGAATTCGTACAACCCAGAAGCGTTG	gE	966–985	−

[a] N, an equimolar mixture of all four nucleotides.
[b] Numerical position on the genome of FHV strain B927 counted from the initiation codon of gE or gI.

fetal calf serum and 100 IU of penicillin and 100 μg streptomycin per ml (DMEM–10% FCS). FHV strain B927 (28) was obtained from D. A. Harbour and propagated in Crandell feline kidney (CRFK) cells (American Type Culture Collection) (11). The virus was plaque purified three times before virus stocks were prepared. Recombinant vaccinia virus vTF7-3 expressing the bacteriophage T7 RNA polymerase (26) was obtained from B. Moss. The virus was propagated in Ost7-1 cells (22) and BHK-21 cells (American Type Culture Collection).

A mouse monoclonal antibody directed against bovine protein disulfide isomerase (PDI) was provided by S. Fuller. The polyclonal rabbit serum directed against α-mannosidase II (46) was a gift from K. Moremen. The rabbit antipeptide serum against the mouse hepatitis virus M protein was described previously (56).

Recombinant DNA techniques. Recombinant DNA techniques were performed according to Sambrook et al. (58) and Ausubel et al. (2). Sequence analysis was performed with the T7 sequencing kit (Pharmacia Biotech). PCR was performed as described previously (57) by using the thermostable DNA polymerase of *Thermus aquaticus* (Promega) according to the instructions of the manufacturer.

Cloning and sequence analysis of the FHV strain B927 genes for gE and gI. To clone the gE gene, PCR was performed on total DNA extracted from FHV-infected cells by using degenerate oligonucleotide primers 346 and 348. These primers were designed to correspond to two short amino acid motifs, six residues each, that are conserved in the gE proteins of HSV type 1, PRV, VZV, and equine herpesvirus type 1 (Table 1). A product with the anticipated size of 0.2 kb was obtained. This product, confirmed to be derived from the gE gene by sequence analysis, was then used as a probe in a Southern blot analysis of FHV genomic DNA. A 2-kb *Eco*RI-*Sal*I fragment containing the complete gE gene was identified and cloned into pUC20 (Boehringer Mannheim). For expression studies, a 1.6-kb *Xma*I-*Sal*I fragment was subcloned into pBluescript KS(+) (Stratagene), yielding pBS-gE. While this work was in progress, the complete nucleotide sequence of the unique short region of FHV strain C27 was published (61). This sequence was used to design specific oligonucleotide primers 369 and 370 (Table 1) to PCR amplify the gI gene by using total DNA extracted from FHV-infected cells as a template. The resulting 1.2-kb PCR product was cloned into *Eco*RV-digested pBluescript SK(−) downstream of the T7 promoter, yielding pBS-gI.

The nucleotide sequences of the genes for gE and gI were determined for both orientations and analyzed with the PC-DOS HIBIO DNASIS software (41) from Pharmacia Biochemicals.

Preparation of monospecific rabbit antisera against gE and gI and a cat antiserum against FHV. To prepare an antiserum against gE, residues 765 to 1137 were PCR amplified with oligonucleotide primers 374 and 379 (Table 1). The PCR product was cut with *Bam*HI and *Eco*RI and inserted into pGEX-2T (Pharmacia Biotech). The resulting construct was used to express amino acid residues 255 to 328 of gE as a glutathione *S*-transferase fusion protein in *Escherichia coli* PC2495 (59). The expression product was purified from sodium dodecyl sulfate (SDS)-polyacrylamide gels as described previously (39). To obtain an antiserum directed against the amino terminus of FHV gI, the synthetic peptide MB108 (NH_2-VYRGDHVSLHVDTSSGFC-COOH; residues 20 to 36) was coupled to keyhole limpet hemocyanin as described previously (30); the C-terminal cysteine was added to facilitate the coupling. New Zealand White rabbits were immunized subcutaneously with 100 μg of either glutathione *S*-transferase–gE fusion protein or keyhole limpet hemocyanin-coupled peptide as described previously (9).

Polyclonal antisera directed against FHV were obtained by oronasal infection of two 15-week-old cats with 10^6 PFU of FHV B927 in 0.5 ml of phosphate-buffered saline (PBS). At 47 days postinfection (p.i.), virus inoculation was repeated, and the cats were bled at day 75.

Transfection of vTF7-3-infected cells and metabolic labeling. Subconfluent monolayers of Ost7-1 and BHK-21 cells in 35-mm dishes were washed once with DMEM and infected with vaccinia virus vTF7-3 at a multiplicity of infection of 3 in DMEM at 37°C. At 1 h p.i., the cells were washed with DMEM and transfected with plasmid DNA as follows. A transfection mixture consisting of 2 to 5 μg of plasmid DNA, 500 μl of DMEM, and 10 μl of Lipofectin (GIBCO BRL, Life Technologies, Inc.) was added to the monolayers. After 5 min of incubation at room temperature, 500 μl of DMEM was added and incubation was continued at 37°C. At 2 h p.i., the incubation temperature was lowered to 32°C. From 4 to 5 h p.i., the cells were incubated with minimum essential medium with Earle's salts, lacking cysteine and methionine (GIBCO BRL, Life Technologies, Inc.). Then, 100 μCi of ^{35}S in vitro labeling mix (Amersham) was added to the culture medium and the incubation was continued for 1 h. The cells were harvested either immediately or after a 2-h chase with DMEM–10% FCS containing 5 mM (each) L-methionine and L-cysteine.

Metabolic labeling of FHV-infected cells. Subconfluent monolayers of CRFK cells in 35-mm dishes were washed once with DMEM and infected with FHV strain B927 at a multiplicity of infection of 5 at 37°C. At 1 h p.i., the culture supernatant was replaced by DMEM–10% FCS and the incubation was continued. Metabolic labeling was done as described for vTF7-3-infected cells, except that the methionine and cysteine depletion and subsequent labeling procedures were performed 4 h later and at 37°C.

RIPA and SDS-gel electrophoresis. Metabolically labeled cells were washed once with ice-cold PBS and then lysed on ice in 600 μl of lysis buffer (20 mM Tris-Cl [pH 7.5], 1 mM EDTA, 100 mM NaCl, 1% Triton X-100) containing 1 μg of pepstatin A, 40 μg of aprotinin, and 1 μg of leupeptin per ml. Nuclei and cell debris were pelleted for 1 min at 10,000 × *g* at 4°C. Of the supernatant, 200 μl was mixed with 1 ml of detergent mixture (50 mM Tris-Cl [pH 8.0], 62.5 mM EDTA, 0.4% sodium deoxycholate, 1% Nonidet P-40), SDS was added to a final concentration of 0.25% unless indicated otherwise, and incubation was performed for 15 min on ice. Subsequently, the antisera were added (Cat-αFHV, Ra-αgE, and Ra-αM, 3 μl each, and Ra-αgI, 5 μl) and the mixtures were incubated for 16 h at 4°C. Immune complexes were collected with 50 μl of a 10% (wt/vol) suspension of formalin-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem) in the detergent mixture. After 30 min of incubation at 4°C, they were washed three times with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-Cl [pH 7.4], 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40) and finally resuspended in 30 μl of Laemmli sample buffer containing 5% β-mercaptoethanol. The samples were heated for 5 min at 95°C before analysis in SDS-polyacrylamide gels.

Endoglycosidase treatment of immunoprecipitates. Immunoprecipitated FHV proteins were treated with endoglycosidase H (EndoH; Boehringer Mannheim) as described by Machamer et al. (43). Treatment with endoglycosidase F/*N*-glycosidase F (GlycoF; Boehringer Mannheim) was performed as described by de Vries et al. (14).

Indirect immunofluorescence. BHK-21 cells grown on 12-mm-diameter gelatin-coated coverslips were infected with vTF7-3 and transfected as described above. At 6 h p.i., cells were fixed with 3% paraformaldehyde and either permeabilized as described by Opstelten et al. (53) or left untreated for surface immunofluorescence. They were then incubated with cat anti-FHV serum, diluted 1:50 in PBS–10 mM glycine–5% FCS, and stained with fluorescein isothiocyanate-(FITC)-conjugated goat anti-cat IgG (Cappel) that had been diluted 1:100. For double staining, the cells were subsequently incubated with either the PDI-specific monoclonal antibody (diluted 1:40) or the α-mannosidase II-specific rabbit serum (diluted 1:500) and then stained with tetramethyl rhodamine isothiocyanate-(TRITC)-conjugated goat anti-mouse IgG (1:100; Cappel) or TRITC-conjugated goat anti-rabbit IgG (1:100; Cappel). The cells were embedded in FluorSave reagent (Calbiochem) and examined with an Olympus BH2-RFCA microscope.

Construction of the FHVΔgI-LZ recombinant. A 3.9-kb *Eco*RV-*Sal*I fragment of the FHV strain B927 genome containing the genes for gI and gE and the 3′ end of the gD gene (61) was cloned into pUC20. The 0.7-kb *Xho*I-*Bam*HI fragment comprising residues 203 to 923 of the gI gene was excised and replaced by a 4.1-kb *Xho*I-*Bam*HI fragment derived from pTNZ1 (66) carrying the *lacZ* reporter gene downstream of the internal ribosomal entry site of encephalomyocarditis virus, thus yielding transfer vector pFHVΔgI-LZ. CRFK cells were cotransfected as follows. Two hundred fifty nanograms of pFHVΔgI-LZ and 50 ng of FHV DNA, diluted in water to a volume of 100 μl, were mixed with 75 μl of DMEM and 25 μl of Lipofectin (GIBCO BRL, Life Technologies, Inc.). After 15 min of incubation at room temperature, 800 μl of DMEM was added and the transfection mixture was applied to a subconfluent monolayer of cells grown in a 35-mm dish. The cells were incubated at 37°C for 16 h after which the tissue culture supernatant was replaced by 2 ml of DMEM–10% FCS. The culture medium was harvested 5 days after transfection and used for a plaque assay.

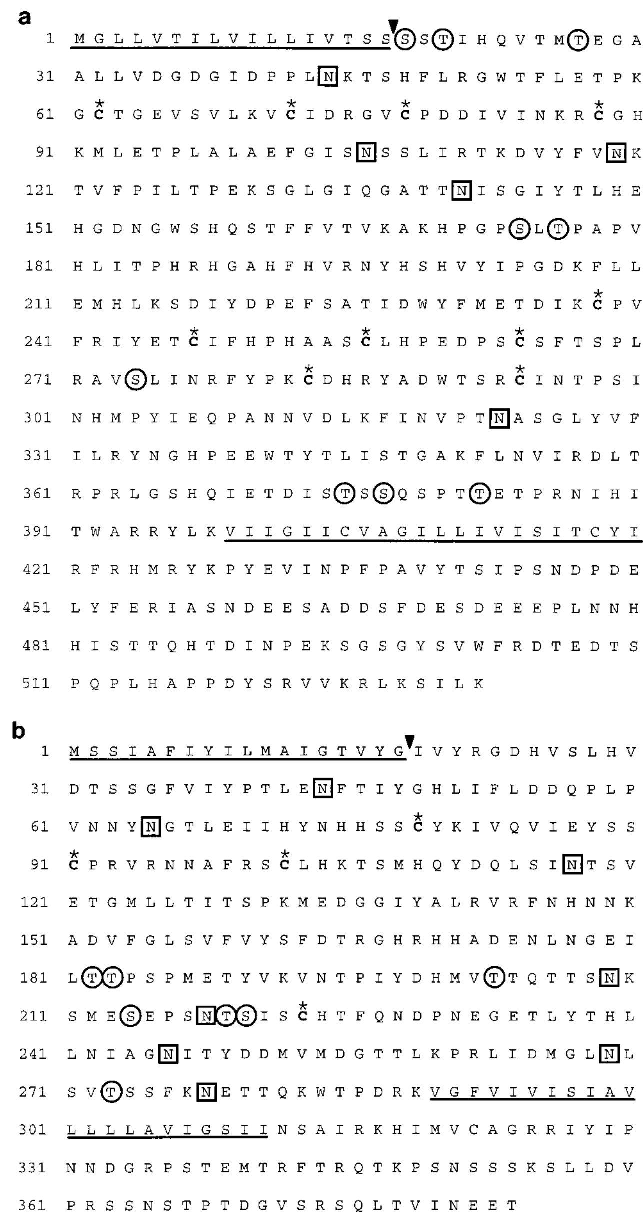

FIG. 1. Amino acid sequences of gE (a) and gI (b) of FHV strain B927. Presumptive N-terminal signal sequences (67) and transmembrane domains (21) are underlined. Arrowheads indicate predicted clevage sites. Potential N-glycosylation sites in the presumptive ectodomains are indicated by boxed asparagine residues. Potential O-glycosylation sites (32) are indicated by encircled serine and threonine residues. Asterisks indicate cysteine residues potentially involved in disulfide formation.

Plaques containing recombinant FHV were identified by in situ staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Boehringer Mannheim), and recombinant virus was purified to homogeneity as described previously (71). Correct insertion of the expression cassette was confirmed by Southern blot analysis using ^{32}P-labeled probes specific for the gI gene or *lacZ.*

Nucleotide sequence accession numbers. The sequences in this study were assigned EMBL accession numbers X98448 and X98449.

RESULTS

Cloning of the FHV genes for glycoproteins E and I. The FHV strain B927 genes for gE and gI were cloned and sequenced. FHV gE and gI are 532 and 384 residues in length, respectively (Fig. 1) and have all the characteristics of class I membrane proteins. gE contains 12 cysteine residues, 10 of which are located in the putative ectodomain and thus are possibly involved in disulfide bond formation. There are five and nine predicted sites for N- and O-linked glycosylation (32), respectively. Residues 399 to 420 form a potential transmembrane domain. gI contains eight and seven predicted sites for N- and O-linked glycosylation, respectively, and five cysteine residues, four of which are in the ectodomain. Nucleotide sequence comparison revealed some major differences from the sequence of FHV strain C27 published by Spätz et al. (61). There were four nucleotide substitutions in the gE gene, two of which result in amino acid changes in the ectodomain (G-100→A and E-378→Q; Fig. 1a). More importantly, in the B927 gI sequence there are two frameshifts at nucleotide positions 843 and 909. Consequently, the amino acid sequence predicts a transmembrane region (amino acids 290 to 311) which is lacking in the sequence determined by Spätz et al. (61). Finally, these authors apparently missed a 42-nucleotide *Eco*RI fragment (nucleotides 1051 to 1092) encoding part of the cytoplasmic tail of gI. The FHV strain B927 sequences thus conform to those recently published for FHV strain G2620 (72), except for four amino acid substitutions (gI, S-186→P and Q-260→K; gE, L-102→F and T-346→I).

Expression of the gE and gI genes in the vaccinia virus vTF7-3 system. The biosynthesis of gE and gI was studied by using the vaccinia virus vTF7-3 expression system (26). Plasmids carrying the genes for gE and gI downstream of the T7 promoter were transfected into vTF7-3-infected Ost7-1 cells. The proteins were metabolically labeled during a 1-h pulse and immunoprecipitated with an FHV-specific cat serum. Polypeptides with M_rs of 83,000 and 67,000 were found for gE and gI, respectively (Fig. 2). Products of this size were also obtained when synthetic T7 transcripts encoding gE and gI were translated in rabbit reticulocyte lysates in the presence of dog pancreas microsomes (data not shown). The 83,000-molecular-weight (83K) product was recognized by a rabbit antiserum raised against amino acid residues 255 to 328 of gE (Fig. 2). Upon treatment with EndoH, gE and gI were trimmed to M_rs of 72,000 and 46,000, respectively (Fig. 3a). For gI, this size closely corresponds to the predicted size of the protein backbone (42 kDa). EndoH-treated gE migrated more slowly than anticipated; its predicted size was 58.5 kDa.

Intracellular transport of gE and gI. The intracellular trans-

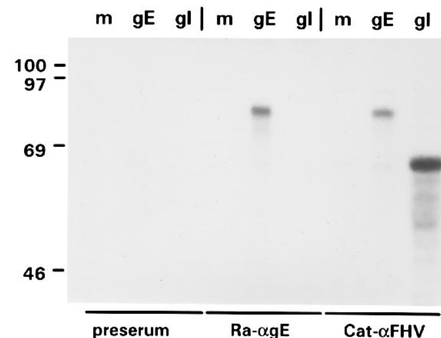

FIG. 2. Radioimmunoprecipitation of vTF7-3-expressed gE and gI. vTF7-3-infected cells were mock transfected (m) or transfected with either pBS-gE (gE) or pBS-gI (gI) at 1 h p.i. and metabolically labeled from 5 to 6 h p.i. Cell lysates were subjected to immunoprecipitation by using either rabbit preserum, a rabbit serum raised against residues 255 to 328 of gE (Ra-αgE), or serum from a cat experimentally infected with FHV (Cat-αFHV). The samples were analyzed in an SDS–7.5% PAGE gel. Molecular sizes are in kilodaltons.

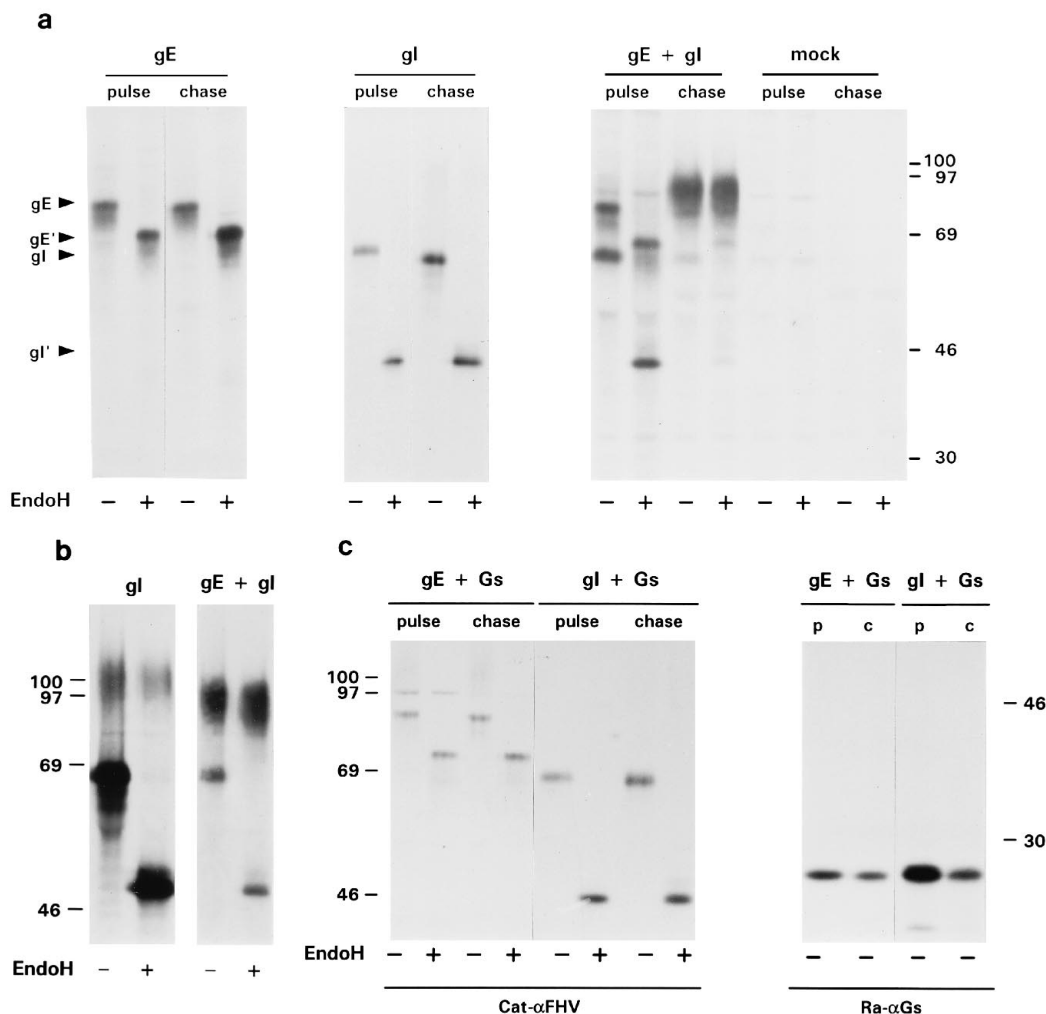

FIG. 3. (a) Intracellular transport of FHV gE and gI in the vTF7-3 expression system. vTF7-3-infected cells were transfected with pBS-gE (gE) or pBS-gI (gI) or cotransfected with both plasmids (gE + gI). Mock-transfected cells were used as a negative control (mock). The cells were metabolically labeled from 5 to 6 h p.i. and either harvested immediately (pulse) or after a 2-h chase (chase). Cell lysates were subjected to RIPA with the Cat-αFHV serum. To monitor ER-to-Golgi transport the immunoprecipitates were treated with EndoH (+) or left untreated (−). Arrowheads on the left indicate the positions of the ER forms of gE and gI and those of their EndoH digestion products, gE′ and gI′. (b) Aberrant processing of gI. gI was expressed in the vTF7-3 system either separately (gI) or together with gE (gE + gI). The proteins were metabolically labeled for 1 h, subjected to a 2-h chase, immunoprecipitated with Cat-αFHV, and either treated with EndoH (+) or left untreated (−). Panels a and b were derived from the same gel. (c) Maturation of gE and gI cannot be induced by other glycoproteins. gE and gI were coexpressed with the Gs protein of equine arteritis virus. The FHV proteins were immunoprecipitated with Cat-αFHV. Intracellular transport was monitored by treatment of immunoprecipitates with EndoH. The samples were analyzed in SDS–7.5% PAGE gels. As a control for Gs expression, lysates were also subjected to RIPA with a rabbit antiserum against Gs (Ra-αGs). These samples were analyzed in SDS–12.5% PAGE gels. Molecular sizes are in kilodaltons.

port of gE and gI was studied biochemically and microscopically. Independently expressed gE remained fully EndoH sensitive during a 2-h chase (Fig. 3a). Immunofluorescence microscopy (IF) to determine its intracellular location revealed a reticular cytoplasmic staining colocalizing with that of PDI, an ER-resident protein (Fig. 4a and b). gE was also present in the nuclear envelope. We did not observe gE-specific staining of the Golgi complex or of the plasma membrane. When expressed separately, most gI remained EndoH sensitive (Fig. 3a). During a 2-h chase, a small fraction of gI was transported from the ER and acquired EndoH resistance concomitant with an increase in molecular weight. These products migrated in SDS-polyacrylamide gel electrophoresis (PAGE) gels as a diffuse smear with apparent molecular weights ranging from 100,000 to 120,000 (Fig. 3b). gI was detected by IF not only in the ER but also at the plasma membrane (Fig. 4c, d, and g). gI was also found by confocal IF in a compartment devoid of PDI, suggestive of the Golgi complex (data not shown). When gE and gI were expressed separately, their ER forms showed a subtle yet reproducible decrease in apparent molecular weight after the 2-h chase (Fig. 3a and c).

Upon coexpression of gE and gI, the EndoH-sensitive species disappeared during a 2-h chase and EndoH-resistant products with apparent molecular weights of 80,000 to 100,000 were found with the FHV-specific cat serum (Fig. 3a). The gE-specific rabbit antiserum precipitated an EndoH-resistant product with an apparent molecular weight of about 95,000, corresponding to the upper part of the 80 to 100K smear (Fig. 5). The mature gI species migrated in SDS-PAGE gels as a smear of 80 to 100K (described below). The 100 to 120K species, seen in cells expressing only gI, were not observed (Fig. 3b). The coexpressed FHV proteins were detected by IF in the Golgi complex (Fig. 4e and f). These data suggested that gE exits from the ER only in the presence of gI and that

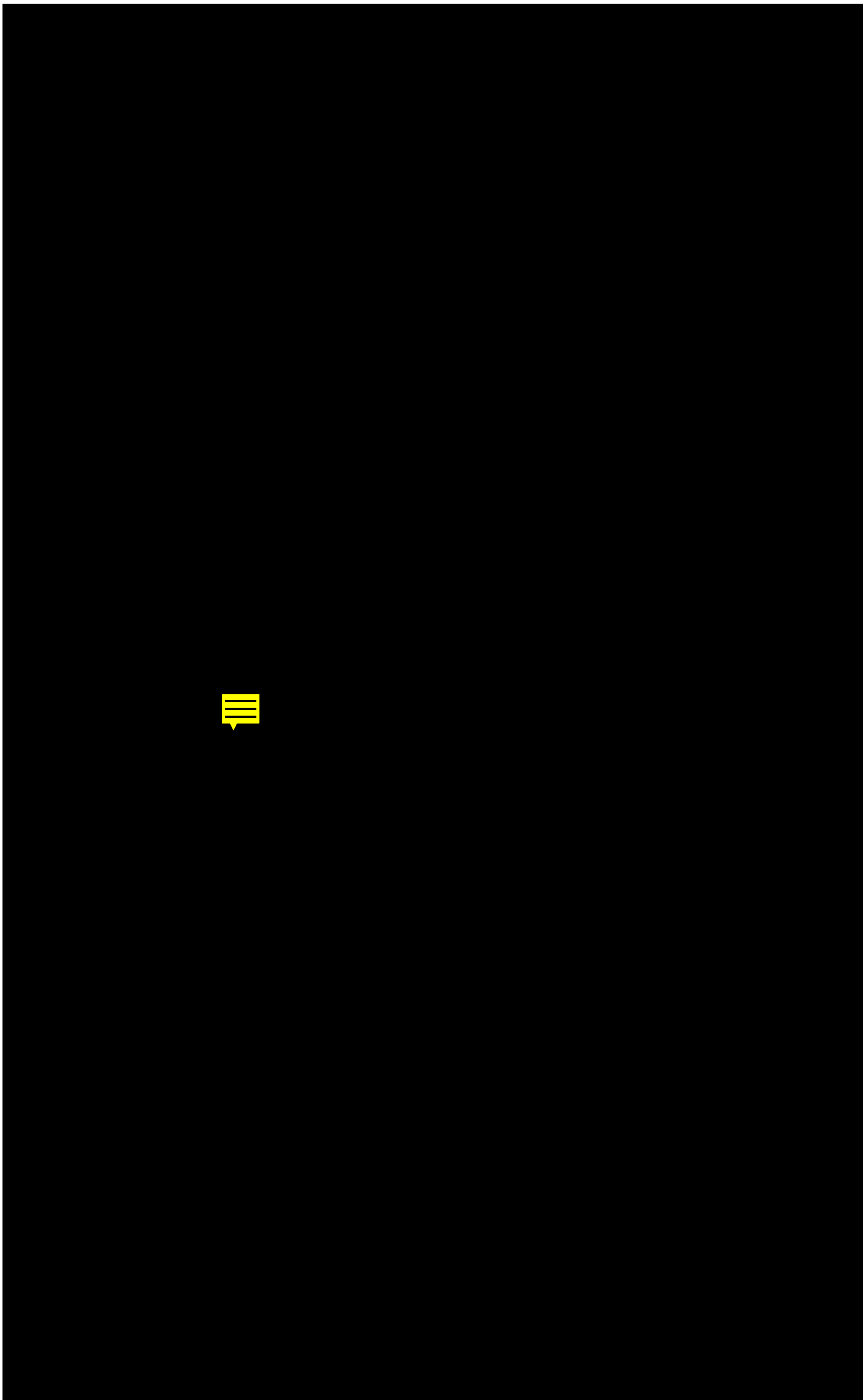

FIG. 4. Localization of FHV gE and gI by IF. BHK-21 cells grown on 12-mm-diameter gelatin-coated coverslips were infected with vTF7-3 and transfected with either pBS-gE (a and b), pBS-gI (c, d, and g), or both (e, f, and h). At 6 h p.i. the cells were fixed with 3% paraformaldehyde and either permeabilized for internal immunofluorescence (a through f) or left untreated for surface immunofluorescence (g and h). Cells were stained for FHV proteins with the Cat-αFHV serum and FITC-conjugated goat anti-cat IgG antibodies (green fluorescence). IF detection of PDI (b and d) and α-mannosidase II (f) was performed with TRITC-labeled conjugates (red fluorescence).

efficient intracellular transport of gI requires the presence of gE.

To determine whether gI specifically induces intracellular transport of gE, pBS-gE was cotransfected with the empty pBluescript vector or with vectors carrying the Gs protein of equine arteritis virus (14) or the hemagglutinin-esterase gene of bovine torovirus (10a). We found that maturation of gE occurred exclusively in the presence of gI. In cells cotransfected with pBluescript or coexpressing bovine torovirus hemagglutinin-esterase (data not shown) or equine arteritis virus Gs (Fig. 3c), only the immature, EndoH-sensitive gE species was seen after a 2-h chase. Similarly, efficient transport of gI occurred only in the presence of coexpressed gE (Fig. 3c).

Identification of mature forms of gI. Detailed analysis of the mature forms of gI and gE was hampered by their extensive posttranslational modifications and by the lack of a proper gI-specific antiserum. An N-terminal antipeptide serum raised against residues 20 to 36 of gI readily precipitated immature gI but not the mature forms. Only after prolonged exposure of gels were mature gI species observed, ranging in size from 80,000 to 100,000 kDa (data not shown).

In an attempt to optimize immunodetection, gI was provided with an epitope tag by exchanging its C-terminal 10 residues for the C-terminal 22 residues of the M protein of mouse hepatitis virus strain A59 (13). In pulse-labeled cells coexpressing gE and the gI-M chimera, a 67K product comigrating with authentic immature gI was detected with a mouse hepatitis virus M-specific antipeptide serum (data not shown). After a 2-h chase, a heterogeneous smear consisting of products with molecular weights of 80,000 to 100,000 was found, apparently representing mature gI-M (Fig. 5a).

Coprecipitation of gI and gE. gE and gI of either HSV, PRV, or VZV form a hetero-oligomeric complex which can be precipitated by using antisera raised against either of the two proteins (35, 73, 76). Our anti-gE serum detected only gE (Fig. 2 and 5). However, RIPA with this serum required the presence of 0.25% SDS, i.e., conditions under which the gE-gI complex is likely to dissociate. To study complex formation, gE and gI-M were coexpressed and RIPA was performed by using the anti-M serum either in the absence or presence of SDS (Fig. 5). In the absence of SDS, coprecipitation of gE was observed and both the mature 95K species and its 83K precursor were detected (Fig. 5b). The precipitation of gE was not caused by nonspecific binding, since gE coexpressed with authentic gI was not detected by the anti-M serum (Fig. 5c). Also, coprecipitation of gI-M and gE was not observed when lysates had been incubated at 95°C in the presence of 1.5% SDS prior to RIPA (Fig. 5b, rightmost lane). These results indicated that coexpressed FHV gE and gI assemble into an SDS-sensitive complex and that complex formation occurs in the ER.

A considerable proportion of gI-M remained EndoH sensitive (Fig. 5). This, however, was not due to the addition of the epitope tag. Complete maturation of gE and gI was observed only when the proteins were expressed in the proper ratio. If either one was expressed in excess, the surplus was retained in the ER. This is illustrated for gI-M in Fig. 5d. Again, these findings are consistent with the formation by gE and gI of a complex with a fixed stoichiometry.

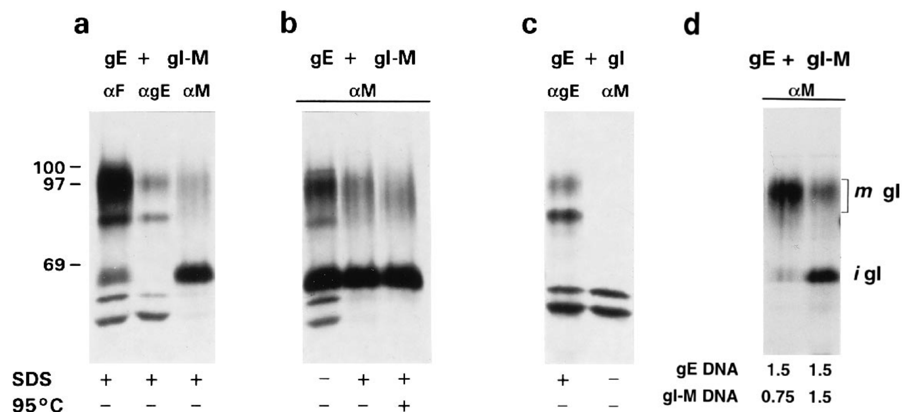

FIG. 5. Coexpression of gE and gI-M. (a) gI-M induces maturation of gE. vTF7-3-infected cells were cotransfected with pBS-gE and pBS-gI-M and metabolically labeled for 1 h; this was followed by a 2-h chase. Lysates were subjected to RIPA with the Cat-αFHV serum (αF), the Ra-αgE serum (αgE), and a rabbit antipeptide serum raised against the C-terminal 18 residues of mouse hepatitis virus M (αM). RIPA was performed in the presence of 0.25% SDS. (b) Coprecipitation of gE and gI-M. Lysates were prepared as described above. RIPA was performed with the Ra-αM serum (αM) under one of three sets of conditions: in the absence of SDS (left lane), in the presence of 0.25% SDS (middle lane), or after heating at 95°C for 5 min in the presence of 1.5% SDS (final SDS concentration, 0.25%). (c) Coprecipitation of gE and gI-M is not caused by nonspecific binding of gE to the Ra-αM serum. To test for nonspecific binding of gE to the Ra-αM serum, lysates of cells coexpressing gE and authentic gI were subjected to RIPA with the Ra-αM serum in the absence of SDS (αM). To test for gE expression, RIPA was also performed with Ra-αgE (αgE). The samples were analyzed in SDS–7.5% PAGE gels. The 55 and 59K polypeptides represent vaccinia virus products nonspecifically binding to the immunosorbent. (d) Overexpressed gI-M is retained in the ER. vTF7-3-infected cells transfected with 1.5 μg of pBS-gE were cotransfected with either 1.5 or 0.75 μg of pBS-gI-M thus varying the relative expression levels of gE and gI-M. Cells were metabolically labeled for 1 h and then subjected to a 2-h chase. Cell lysates were subjected to RIPA with the Ra-αM serum (αM) in the presence of SDS. The immature ER gI species (*i* gI) and the mature gI-M species (*m* gI) are indicated. Molecular sizes are in kilodaltons.

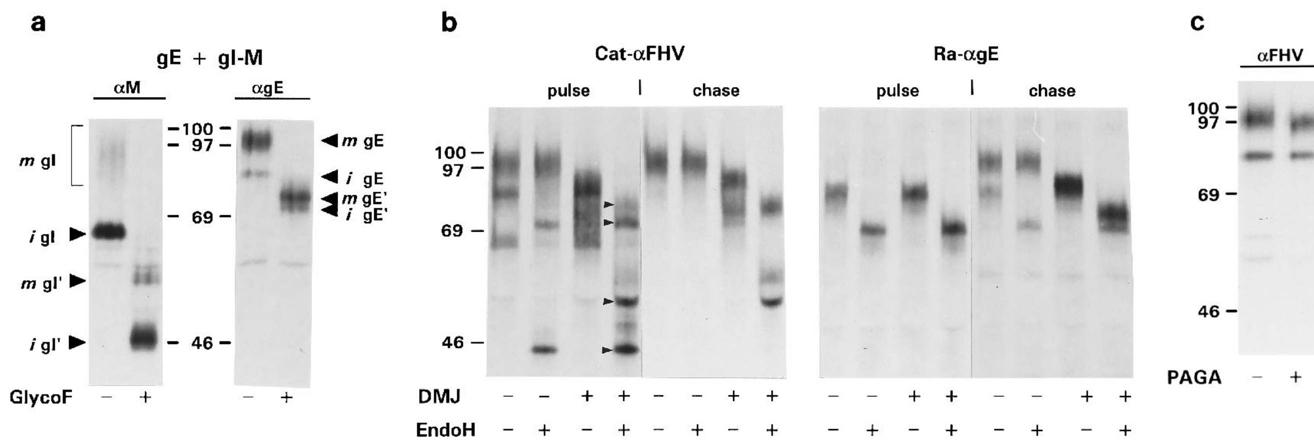

FIG. 6. Posttranslational modifications of FHV gE and gI. (a) Removal of N-linked glycans by GlycoF. Lysates of vTF7-3-infected cells coexpressing gE and gI-M were labeled for 1 h and then subjected to a 2-h chase. gI and gE were immunoprecipitated with the Ra-αM (αM) and Ra-αgE (αgE) sera, respectively, in the presence of 0.25% SDS. The immunoprecipitates were treated with GlycoF (+) or left untreated (−). The arrowheads and the bracket indicate the mature forms of gI and gE (*m* gI and *m* gE), their immature precursors (*i* gI and *i* gE), and the fully deglycosylated precursors (*i* gI′ and *i* gE′). The mature forms of gI and gE from which the N-glycans have been removed by GlycoF are also indicated (*m* gI′ and *m* gE′). (b) Metabolic labeling in the presence of DMJ. vTF7-3-infected cells coexpressing gE and gI were labeled for 1 h in the absence (−) or presence (+) of 1 mM DMJ and harvested either immediately (pulse) or after a 2-h chase (chase). The lysates were subjected to RIPA using Cat-αFHV or Ra-αgE. The immunoprecipitates were treated with EndoH to remove N-linked glycans (+) or left untreated (−). Arrowheads indicate the 78K and 72K species corresponding to mature and immature forms of gE, respectively, and the 52K and 46K species corresponding to mature and immature forms of gI, respectively. (c) Metabolic labeling in the presence of PAGA. vTF7-3-infected cells coexpressing gE and gI-M were labeled for 1 h and then subjected to a 2-h chase in the absence (−) or presence (+) of 2 mM PAGA. gE and gI were immunoprecipitated with the Cat-αFHV serum. The samples were analyzed in SDS–7.5% PAGE gels. Molecular sizes are in kilodaltons.

Posttranslational modification of gE and gI. Maturation of gE and gI was accompanied by a considerable increase in apparent molecular weight. To test whether this was caused exclusively by modification of the N-linked glycans, immunoprecipitates were treated with GlycoF. In addition to the unglycosylated core proteins, gE and gI species with molecular weights of 78,000 and 52,000 respectively, were found (Fig. 6a). The latter products presumably correspond to mature forms of gE and gI, suggesting that both proteins undergo additional modifications.

To confirm these results, the vTF7-3-expressed FHV proteins were metabolically labeled in the presence of deoxymannojirimycine (DMJ). This sugar analog inhibits the processing of N-linked oligosaccharides but does not affect protein transport (6). Thus, the N-linked sugar chains remain EndoH sensitive and can be removed to reveal additional posttranslational modifications. EndoH treatment of coexpressed gE and gI, pulse labeled in the presence of DMJ, yielded four products with M_rs of 78,000, 72,000, 52,000, and 46,000, as detected by the cat anti-FHV serum (Fig. 6b). The 78 and 72K species were recognized by the rabbit anti-gE serum (Fig. 6b). The 72 and 46K species correspond to the unglycosylated core proteins of gE and gI, respectively. After a 2-h chase, mainly the 78 and 52K products were detected, again indicating that these correspond to mature forms of gE and gI. The most likely interpretation is that gE and gI also acquire O-glycans. Indeed, when the proteins were labeled in the presence of the O-glycosylation inhibitor phenyl-α-*N*-acetylgalactosaminide (PAGA) (40), the apparent molecular weights of mature gE and gI were reduced by approximately 6,000 (Fig. 6c).

Biosynthesis of gE in FHV-infected cells. To corroborate our observations, we studied the biosynthesis of gE in FHV-infected cells. In CRFK cells infected with FHV strain B927, gE was also made as an EndoH-sensitive 83K precursor which was converted into a 95K EndoH-resistant form during a 2-h chase (Fig. 7a). Upon metabolic labeling in the presence of DMJ and after EndoH treatment, a 78K gE species was found, suggesting that gE acquired posttranslational modifications similar to those in the vTF7-3 system (Fig. 7a). Thus, the biosynthesis of gE was essentially identical in vTF7-3- and FHV-infected cells, although maturation occurred slightly faster in the latter.

We then asked whether intracellular transport of gE in FHV-infected cells also depended on the presence of gI. To this end, an FHV recombinant, FHVΔgI-LZ, was constructed in which the *Xho*I-*Bam*HI fragment of gI (nucleotide residues 203 to 923) had been replaced by an expression cassette consisting of the internal ribosomal entry site of encephalomyocarditis virus and the *lacZ* reporter gene (Fig. 7c). Recombinant viruses, identified by X-Gal staining, displayed a small-plaque phenotype (data not shown). By using the gI-specific antipeptide serum, gI was detected in pulse-labeled cells infected with the parental strain B927 but not in cells infected with FHVΔgI-LZ. After a 2-h chase, mature gE was detected in B927-infected cells. In cells infected with FHVΔgI-LZ, however, only immature gE was found (Fig. 7a and b). As for vTF7-3-expressed gE, a subtle decrease in apparent molecular weight occurred during the chase.

DISCUSSION

We have studied the biosynthesis of gE and gI of FHV both by vTF7-3-driven expression of their cloned genes and in FHV-infected cells. FHV gE and gI are made as N-glycosylated precursors and, when coexpressed, form a hetero-oligomeric complex. Complex formation occurs in a pre-Golgi compartment, most likely the ER. The gE-gI complex is SDS sensitive, indicating that the glycoproteins are held together through noncovalent interactions. Upon release of gE-gI from the ER, the glycoproteins undergo extensive posttranslational modifications, including the processing of N-linked oligosaccharides and the addition of O-linked oligosaccharides. O-linked glycosylation of gE and gI was also reported for VZV (73).

The biosynthesis of vTF7-3-expressed gE was essentially identical to that in FHV-infected cells. The immature and mature gE species produced in the vTF7-3 system were also found in FHV-infected cells. Consistently, pulse-chase exper-

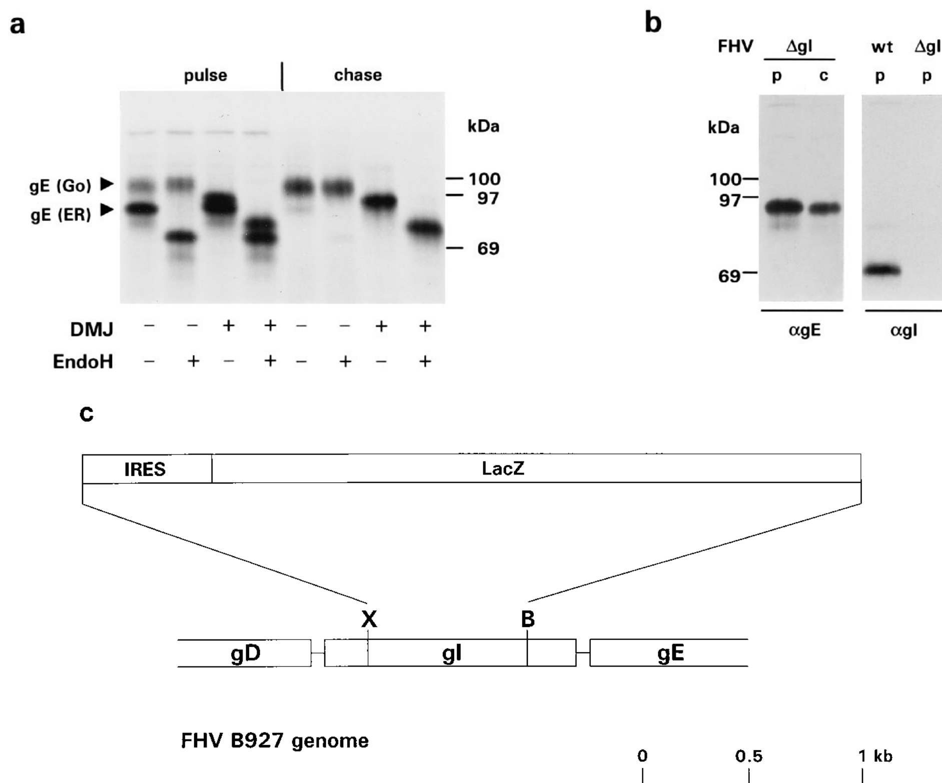

FIG. 7. The biosynthesis of gE in FHV-infected cells. (a) Cells infected with FHV at a multiplicity of infection of 5 were metabolically labeled from 9 to 10 h p.i. in the absence (−) or presence (+) of DMJ. The cells were harvested either immediately (pulse) or after a 2-h chase (chase). gE was immunoprecipitated with Ra-αgE. Immunoprecipitates were treated with EndoH (+) or left untreated (−). (b) Biosynthesis of gE in FHVΔgI-LZ-infected cells. (Left panel) Cells infected with FHVΔgI-LZ were metabolically labeled and harvested either immediately (p) or after a 2-h chase (c). gE was immunoprecipitated with Ra-αgE (αgE). (Right panel) As a control, RIPA was performed on lysates of cells infected with the parental strain, FHV strain B927 (wt), or its gI-defective derivative FHVΔgI-LZ (ΔgI) by using the gI-specific rabbit antipeptide serum (αgI). The samples were analyzed in SDS–7.5% PAGE gels. Molecular sizes are in kilodaltons. (c) Construction of FHVΔgI-LZ. The lower panel shows the genomic region of FHV containing the genes for gD, gI, and gE. The genes are shown as boxes. The gI gene was knocked out via homologous recombination between the FHV B927 genome and transfer vector pFHVΔgI-LZ. Thus, the internal 0.7-kb *Xho*I (X)-*Bam*HI (B) fragment of gI was replaced by an expression cassette consisting of the encephalomyocarditis virus internal ribosomal entry site (IRES) and the *lacZ* reporter gene.

iments in which the glycoproteins were labeled in the presence of the mannosidase II inhibitor DMJ suggested that vTF7-3- and FHV-expressed gEs acquire the same posttranslational modifications. The maturation of gE was slightly faster in FHV-infected cells, probably as a consequence of the difference in incubation temperatures during FHV and vTF7-3 infection, i.e., 37 and 32°C, respectively. Unfortunately, we were not able to monitor the fate of gI in FHV-infected cells because of the lack of a proper antiserum. An antipeptide serum raised against the N terminus of this protein efficiently precipitated the ER species but failed to detect mature gI. The analysis of PRV gI was hampered by similar problems (70). Possibly, the antibody binding sites were shielded by the processing or addition of oligosaccharides.

Our observations concerning processing and complex formation thus confirm and extend those previously made for gE and gI of PRV, HSV, and VZV. Separate expression of FHV gE and gI revealed some interesting differences, however. gE and gI of HSV and VZV, when expressed separately, are readily transported from the ER to the plasma membrane (5, 18, 42, 73). In contrast, gI of FHV is transported inefficiently in the absence of gE: only a minor amount escapes from the ER, and these gI molecules are processed aberrantly, as indicated by their increased M_rs (100,000 to 120,000). gE, when expressed by itself, remained fully EndoH sensitive. Mature gE species were not detected by RIPA, not even after prolonged fluorography. By IF, gE was detected only in the nuclear envelope and in the ER and appeared to be absent both from the Golgi apparatus and at the cell surface. The ER retention of FHV gE was not an artifact of the vTF7-3 expression system: FHVΔgI-LZ, an FHV mutant lacking gI, failed to produce mature gE. Both in vTF7-3- and in FHVΔgI-LZ-infected cells, the ER species of gE showed a subtle decrease in size after a 2-h chase. Removal of the N-glycans with EndoH revealed that this was not caused by modification of the protein moieties but by processing of oligosaccharides. Presumably, the shift in apparent molecular weight results from the trimming of mannose residues. This phenomenon is associated with prolonged ER retention and has been proposed to prelude protein degradation (63). Our combined data strongly suggest that intracellular transport of gE requires a specific interaction with gI, i.e., gE becomes transport competent only when in the gE-gI complex. Our results are reminiscent of those found for gE and gI of PRV (70).

It remains an open question why FHV gE is only transported as part of a gE-gI complex, while for other alphaherpesviruses maturation of gE can occur independently of gI (5, 18, 42, 70, 73). It is possible that during natural infection, complex formation occurs very efficiently and that, even in the case of HSV and VZV, only small amounts of the individual glycoproteins leave the ER. These would merely be dead-end products. Alternatively, for some viruses gE and gI may have functions

different from those of their hetero-oligomer. In this respect, it is of interest that gE and gE-gI of HSV both can act as Fc receptors (5) but differ in their binding preference for complexed versus noncomplexed IgG (18). Also, it has been suggested that PRV mutants lacking gE are less neurovirulent than those lacking gI (37, 38).

ER retention of incomplete oligomeric complexes has been described for many glycoproteins, including various viral membrane proteins (for a review, see reference 17). The molecular chaperones calnexin and calreticulin play a pivotal role in this process (31, 33, 49). These chaperones are structurally related and are thought to interact with their ligands via similar mechanisms. The binding of calnexin has been studied in the most detail and apparently involves recognition of both carbohydrate and protein determinants (1, 69, 74). We assume that gE and gI are retained in the ER by calnexin and calreticulin. Indeed, individually expressed gE can be coprecipitated with calnexin (45a). Further experiments are in progress to identify the chaperone(s) involved in ER retention of gE and gI.

The results obtained for FHVΔgI-LZ indicate that other FHV glycoproteins were not able to compensate for the loss of gI and induce gE maturation. Assistance in the transport of incompletely assembled monomers by proteins other than their authentic partners would be unusual but not unprecedented. The alphaherpesvirus glycoproteins gH and gL also form a hetero-oligomeric complex, and the latter is required for gH transport (20, 34). Remarkably, however, in the case of VZV, transport of gH could also be induced by either gE or gI (20).

Although there is ample evidence for a role in cell-to-cell transmission (3, 15, 75), the function of the gE-gI complex remains to be elucidated. Is gE-gI involved in receptor binding as recently proposed (15) and if so, what is its ligand? It would be interesting to determine whether FHV gE-gI can substitute for the homologous complex of other alphaherpesviruses and whether it would function in a host cell-specific manner. Our data provide a framework for future experiments to answer such questions.

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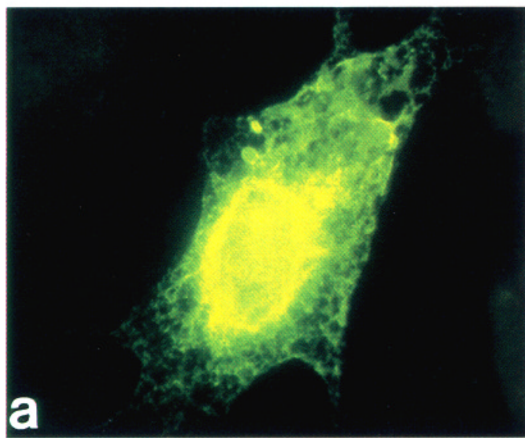
REFERENCES

1. **Arunachalam, B., and P. Cresswell.** 1995. Molecular requirements for the interaction of class II major histocompatibility complex molecules and invariant chain with calnexin. J. Biol. Chem. **270:**2784–2790.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York.
3. **Balan, P., N. Davis-Poynter, S. Bell, H. Atkinson, H. Browne, and T. Minson.** 1994. An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. J. Gen. Virol. **75:**1245–1258.
4. **Baucke, R. B., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein. J. Virol. **32:**779–789.
5. **Bell, S., M. Cranage, L. Borysiewicz, and T. Minson.** 1990. Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J. Virol. **64:**2181–2186.
6. **Burke, B., K. Matlin, E. Bause, G. Legler, N. Peyrieras, and H. Ploegh.** 1984. Inhibition of N-linked oligosaccharide trimming does not interfere with surface expression of certain integral membrane proteins. EMBO J. **3:**551–556.
7. **Card, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist.** 1992. Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. J. Virol. **66:**3032–3041.
8. **Card, J. P., M. E. Whealy, A. K. Robbins, R. Y. Moore, and L. W. Enquist.** 1991. Two alphaherpesvirus strains are transported differentially in the rodent visual system. Neuron **6:**957–969.
9. **Chirnside, E. D., A. A. de Vries, J. A. Mumford, and P. J. Rottier.** 1995. Equine arteritis virus-neutralizing antibody in the horse is induced by a determinant on the large envelope glycoprotein G_L. J. Gen. Virol. **76:**1989–1998.
10. **Cole, G. E., S. Stacy-Phipps, and J. H. Nunberg.** 1990. Recombinant feline herpesviruses expressing feline leukemia virus envelope and *gag* proteins. J. Virol. **64:**4930–4938.

10a. **Cornelissen, L. A. H. M., and R. J. de Groot.** Unpublished data.

11. **Crandell, R. A., C. G. Fabricant, and W. A. Nelson-Rees.** 1973. Development, characterization, and viral susceptibility of a feline (*Felis catus*) renal cell line (CRFK). In Vitro (Rockville) **9:**176–185.
12. **Davis-Poynter, N., S. Bell, T. Minson, and H. Browne.** 1994. Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J. Virol. **68:**7586–7590.
13. **Den Boon, J. A., E. J. Snijder, J. K. Locker, M. C. Horzinek, and P. J. Rottier.** 1991. Another triple-spanning envelope protein among intracellularly budding RNA viruses: the torovirus E protein. Virology **182:**655–663.
14. **de Vries, A. A. F., M. J. B. Raamsman, H. A. van Dijk, M. C. Horzinek, and P. J. M. Rottier.** 1995. The small envelope glycoprotein (G_S) of equine arteritis virus folds into three distinct monomers and a disulfide-linked dimer. J. Virol. **69:**3441–3448.
15. **Dingwell, K. S., C. R. Brunetti, R. L. Hendricks, Q. Tang, M. Tang, A. J. Rainbow, and D. C. Johnson.** 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J. Virol. **68:**834–845.
16. **Dingwell, K. S., L. C. Doering, and D. C. Johnson.** 1995. Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. J. Virol. **69:**7087–7098.
17. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
18. **Dubin, G., I. Frank, and H. M. Friedman.** 1990. Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes. J. Virol. **64:**2725–2731.
19. **Dubin, G., E. Socolof, I. Frank, and H. M. Friedman.** 1991. Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J. Virol. **65:**7046–7050.
20. **Duus, K. M., C. Hatfield, and C. Grose.** 1995. Cell surface expression and fusion by the varicella-zoster virus gH:gL glycoprotein complex: analysis by laser scanning confocal microscopy. Virology **210:**429–440.
21. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. **179:**125–142.
22. **Elroy-Stein, O., and B. Moss.** 1990. Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells. Proc. Natl. Acad. Sci. USA **87:**6743–6747.
23. **Enquist, L. W., J. Dubin, M. E. Whealy, and J. P. Card.** 1994. Complementation analysis of pseudorabies virus gE and gI mutants in retinal ganglion cell neurotropism. J. Virol. **68:**5275–5279.
24. **Flowers, C. C., and D. J. O'Callaghan.** 1992. The equine herpesvirus type 1 (EHV-1) homolog of herpes simplex virus type 1 US9 and the nature of a major deletion within the unique short segment of the EHV-1 KyA strain genome. Virology **190:**307–315.
25. **Frank, I., and H. M. Friedman.** 1989. A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. J. Virol. **63:**4479–4488.
26. **Fuerst, T. R., E. G. Niles, S. F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
27. **Gaskell, R. M., P. E. Dennis, L. E. Goddard, F. M. Cocker, and J. M. Wills.** 1985. Isolation of felid herpesvirus I from trigeminal ganglia of latently infected cats. J. Gen. Virol. **66:**391–394.
28. **Gaskell, R. M., and R. C. Povey.** 1979. The dose response of cats to experimental infection with feline viral rhinotracheitis virus. J. Comp. Pathol. **89:**179–191.
29. **Grail, A., D. A. Harbour, and W. Chia.** 1991. Restriction endonuclease mapping of the genome of feline herpesvirus type 1. Arch. Virol. **116:**209–220.
30. **Gullick, W. J., J. Downward, and M. D. Waterfield.** 1985. Antibodies to the autophosphorylation sites of the epidermal growth factor receptor protein-tyrosine kinase as probes of structure and function. EMBO J. **4:**2869–2877.
31. **Hammond, C., I. Braakman, and A. Helenius.** 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc. Natl. Acad. Sci. USA **91:**913–917.
32. **Hansen, J. E., O. Lund, J. Engelbrecht, H. Bohr, J. O. Nielsen, and J.-E. S.**

Hansen. 1995. Prediction of O-glycosylation of mammalian proteins: specific patterns of UDP-GalNAc:polypeptide N-acetylgalactosaminyl-transferase. Biochem. J. **308:**801–813.
33. **Hebert, D. N., B. Foellmer, and A. Helenius.** 1995. Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. Cell **81:**425–433.
34. **Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. **66:**2240–2250.
35. **Johnson, D. C., and V. Feenstra.** 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. **61:**2208–2216.
36. **Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stow.** 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62:**1347–1354.
37. **Kritas, S. K., M. B. Pensaert, and T. C. Mettenleiter.** 1994. Invasion and spread of single glycoprotein deleted mutants of Aujeszky's disease virus (ADV) in the trigeminal nervous pathway of pigs after intranasal inoculation. Vet. Microbiol. **40:**323–334.
38. **Kritas, S. K., M. B. Pensaert, and T. C. Mettenleiter.** 1994. Role of envelope glycoproteins gI, gp63 and gIII in the invasion and spread of Aujeszky's disease virus in the olfactory nervous pathway of the pig. J. Gen. Virol. **75:**2319–2327.
39. **Krone, W. J. A., C. Debouk, L. G. Epstein, P. Heutink, R. Meloen, and J. Goudsmit.** 1988. Natural antibodies to HIV-tat epitopes and expression of HIV-1 gene in vivo. J. Med. Virol. **26:**261–270.
40. **Kuan, S.-F., J. C. Byrd, C. Basbaum, and Y. S. Kim.** 1989. Inhibition of mucin glycosylation by Aryl-N-acetyl-alpha-galactosaminides in human colon cancer cells. J. Biol. Chem. **264:**19271–19277.
41. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227:**1435–1441.
42. **Litwin, V., W. Jackson, and C. Grose.** 1992. Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. J. Virol. **66:**3643–3651.
43. **Machamer, C. E., S. A. Mentone, J. K. Rose, and M. G. Farquhar.** 1990. The E1 glycoprotein of an avian coronavirus is targeted to the cis Golgi complex. Proc. Natl. Acad. Sci. USA **87:**6944–6948.
44. **Maeda, K., T. Horimoto, J. Norimine, Y. Kawaguchi, K. Tomonaga, M. Niikura, C. Kai, E. Takahashi, and T. Mikami.** 1992. Identification and nucleotide sequence of a gene in feline herpesvirus type 1 homologous to the herpes simplex virus gene encoding the glycoprotein B. Arch. Virol. **127:**387–397.
45. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, and T. Ben-Porat.** 1987. Role of pseudorabies virus glycoprotein gI in virus release from infected cells. J. Virol. **61:**2764–2769.
45a. **Mijnes, J. D. F., and R. J. de Groot.** Unpublished observations.
46. **Moremen, K. W., O. Touster, and P. W. Robbins.** 1991. Novel purification of the catalytic domain of Golgi alpha-mannosidase. II. J. Biol. Chem. **266:**16876–16885.
47. **Mulder, W. A., L. Jacobs, J. Priem, G. L. Kok, F. Wagenaar, T. G. Kimman, and J. M. Pol.** 1994. Glycoprotein gE-negative pseudorabies virus has a reduced capability to infect second- and third-order neurons of the olfactory and trigeminal routes in the porcine central nervous system. J. Gen. Virol. **75:**3095–3106.
48. **Nasisse, M. P., B. J. Davis, J. S. Guy, M. G. Davidson, and W. Sussman.** 1992. Isolation of feline herpesvirus 1 from the trigeminal ganglia of acutely and chronically infected cats. J. Vet. Intern. Med. **6:**102–103.
49. **Nauseef, W. M., S. J. McCormick, and R. A. Clark.** 1995. Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. J. Biol. Chem. **270:**4741–4747.
50. **Neidhardt, H., C. H. Schröder, and H. C. Kaerner.** 1987. Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. J. Virol. **61:**600–603.
51. **Nunberg, J. H., D. K. Wright, G. E. Cole, E. A. Petrovskis, L. E. Post, T. Compton, and J. H. Gilbert.** 1989. Identification of the thymidine kinase gene of feline herpesvirus: use of degenerate oligonucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs. J. Virol. **63:**3240–3249.
52. **Ohmura, Y., E. Ono, T. Matsuura, H. Kida, and Y. Shimizu.** 1993. Detection of feline herpesvirus 1 transcripts in trigeminal ganglia of latently infected cats. Arch. Virol. **129:**341–347.
53. **Opstelten, D.-J. E., P. de Groote, M. C. Horzinek, H. Vennema, and P. J. M. Rottier.** 1993. Disulfide bonds in folding and transport of mouse hepatitis coronavirus glycoproteins. J. Virol. **67:**7394–7401.
54. **Povey, R. C.** 1979. A review of feline viral rhinotracheitis (feline herpesvirus 1 infection). Comp. Immunol. Microbiol. Infect. Dis. **2:**373–387.
55. **Rajcani, J., U. Herget, and H. C. Kaerner.** 1990. Spread of herpes simplex virus (HSV) strains SC16, ANG, ANGpath and its glyC minus and GlyE minus mutants in DBA-2 mice. Acta Virol. **34:**305–320.
56. **Rottier, P., J. Armstrong, and D. I. Meyer.** 1985. Signal recognition particle-dependent insertion of coronavirus E1, an intracellular membrane glycoprotein. J. Biol. Chem. **260:**4648–4652.
57. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
59. **Smith, D. B., M. R. Rubira, R. J. Simpson, K. M. Davern, W. U. Tiu, P. G. Board, and G. F. Mitchell.** 1988. Expression of an enzymatically active parasite molecule in *E. coli*: *Schistosoma japonicum* glutathione-S-transferase. Mol. Biochem. Parasitol. **98:**503–516.
60. **Spätz, S. J., and R. K. Maes.** 1993. Immunological characterization of the feline herpesvirus-1 glycoprotein B and analysis of its deduced amino acid sequence. Virology **197:**125–136.
61. **Spätz, S. J., P. A. Rota, and R. K. Maes.** 1994. Identification of the feline herpesvirus type 1 (FHV-1) genes encoding glycoproteins G, D, I and E: expression of FHV-1 glycoprotein D in vaccinia and raccoon poxviruses. J. Gen. Virol. **75:**1235–1244.
62. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Inc., Boca Raton, Fla.
63. **Su, K., T. Stoller, J. Rocco, J. Zemsky, and R. Green.** 1993. Pre-Golgi degradation of yeast prepro-alpha-factor expressed in a mammalian cell. Influence of cell type-specific oligosaccharide processing on intracellular fate. J. Biol. Chem. **268:**14301–14309.
64. **van Engelenburg, F. A. C., M. J. Kaashoek, F. A. M. Rijsewijk, L. van den Burg, A. Moerman, A. L. J. Gielkens, and J. T. van Oirschot.** 1994. A glycoprotein E deletion mutant of bovine herpesvirus 1 is avirulent in calves. J. Gen. Virol. **75:**2311–2318.
65. **Van Vliet, K. E., L. A. De Graaf-Miltenburg, J. Verhoef, and J. A. Van Strijp.** 1992. Direct evidence for antibody bipolar bridging on herpes simplex virus-infected cells. Immunology **77:**109–115.
66. **Vennema, H., R. Rijnbrand, L. Heijnen, M. C. Horzinek, and W. J. Spaan.** 1991. Enhancement of the vaccinia virus/phage T7 RNA polymerase expression system using encephalomyocarditis virus 5′-untranslated region sequences. Gene **108:**201–209.
67. **Von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
68. **Wardley, R. C., P. J. Berlinski, D. R. Thomsen, A. L. Meyer, and L. E. Post.** 1992. The use of feline herpesvirus and baculovirus as vaccine vectors for the gag and env genes of feline leukaemia virus. J. Gen. Virol. **73:**1811–1818.
69. **Ware, F. E., A. Vassilakos, P. A. Peterson, M. R. Jackson, M. A. Lehrman, and D. B. Williams.** 1995. The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. J. Biol. Chem. **270:**4697–4704.
70. **Whealy, M. E., J. P. Card, A. K. Robbins, J. R. Dubin, H.-J. Rziha, and L. W. Enquist.** 1993. Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J. Virol. **67:**3786–3797.
71. **Willemse, M. J., W. S. Chalmers, A. M. Cronenberg, R. Pfundt, I. G. Strijdveen, and P. J. Sondermeijer.** 1994. The gene downstream of the gC homologue in feline herpes virus type 1 is involved in the expression of virulence. J. Gen. Virol. **75:**3107–3116.
72. **Willemse, M. J., I. G. Strijdveen, S. H. van Schooneveld, M. C. van den Berg, and P. J. Sondermeijer.** 1995. Transcriptional analysis of the short segment of the feline herpesvirus type 1 genome and insertional mutagenesis of a unique reading frame. Virology **208:**704–711.
73. **Yao, Z., W. Jackson, B. Forghani, and C. Grose.** 1993. Varicella-zoster virus glycoprotein gpI/gpIV receptor: expression, complex formation, and antigenicity within the vaccinia virus-T7 RNA polymerase transfection system. J. Virol. **67:**305–314.
74. **Zhang, Q., M. Tector, and R. D. Salter.** 1995. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. J. Biol. Chem. **270:**3944–3948.
75. **Zsak, L., F. Zuckermann, N. Sugg, and T. Ben-Porat.** 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J. Virol. **66:**2316–2325.
76. **Zuckermann, F. A., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat.** 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62:**4622–4626.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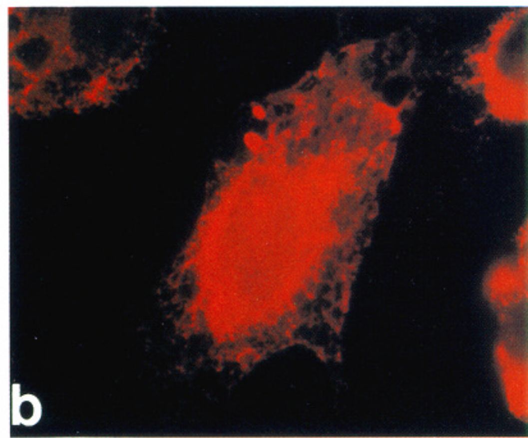
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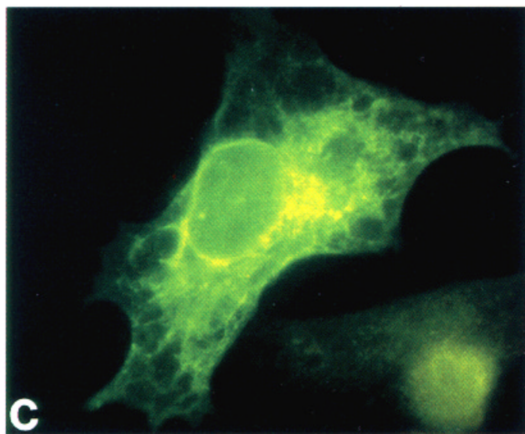
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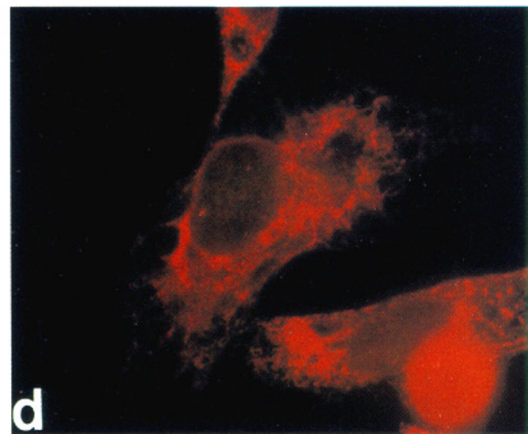
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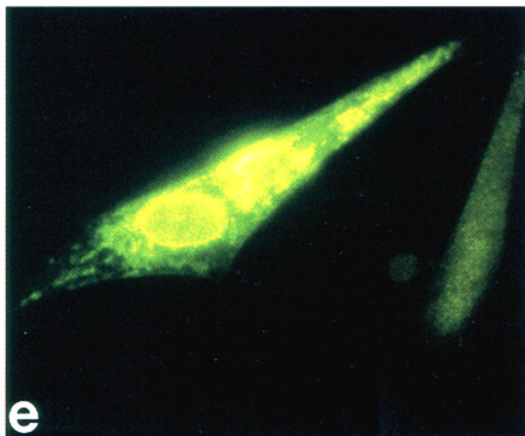
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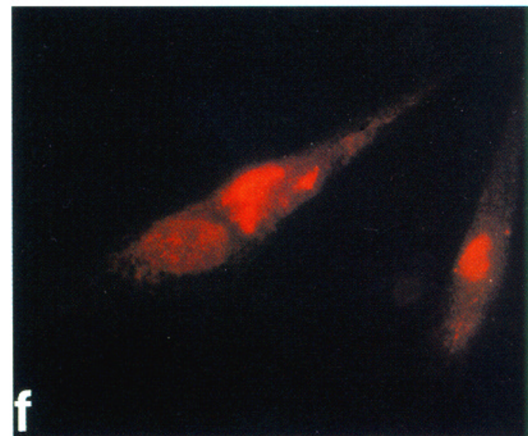
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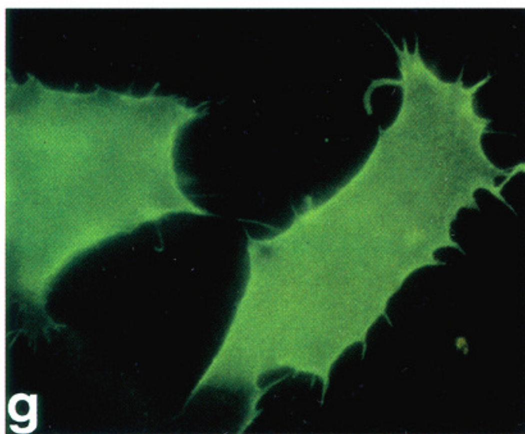
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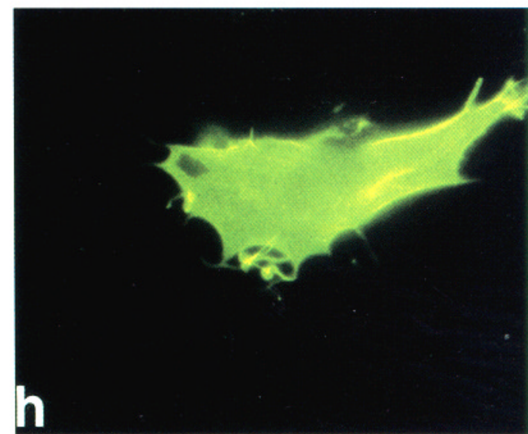
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