JOURNAL OF VIROLOGY, May 1997, p. 4042–4054 Vol. 71, No. 5
0022-538X/97/$04.00+0

Endocytosis and Recycling of Varicella-Zoster Virus Fc Receptor Glycoprotein gE: Internalization Mediated by a YXXL Motif in the Cytoplasmic Tail

JULIE K. OLSON AND CHARLES GROSE*

Department of Microbiology and Immunology Program, University of Iowa College of Medicine, Iowa City, Iowa 52242

Received 17 October 1996/Accepted 16 January 1997

Varicella-zoster virus (VZV) encodes a cell surface Fc receptor, glycoprotein gE. VZV gE has previously been shown to display several features common to nonviral cell surface receptors. Most recently, VZV gE was reported to be tyrosine phosphorylated on a dimeric form (J. K. Olson, G. A. Bishop, and C. Grose, J. Virol. 71:110–119, 1997). Thereafter, attention focused on the ability of VZV gE to undergo receptor-mediated endocytosis. The current transient transfection studies demonstrated by confocal microscopy and internalization assays that VZV gE was endocytosed when expressed in HeLa cells. Endocytosis of gE was shown to be dependent on clathrin-coated vesicle formation within the cells. Subsequent colocalization studies showed that endocytosis of VZV gE closely mimicked endocytosis of the transferrin receptor. The gE cytoplasmic tail and more specifically tyrosine residue 582 were determined by mutagenesis studies to be important for efficient internalization of the protein; this tyrosine residue is part of a conserved YXXL motif. The amount of gE internalized at any given time reached a steady state of 32%. In addition, like the transferrin receptor, internalized gE recycled to the cell surface. The finding of gE endocytosis provided insight into earlier documentation of gE serine/threonine and tyrosine phosphorylation, since these phosphorylation events may serve as sorting signals for internalized receptors. Taken together with the previous discovery that both human and simian immunodeficiency virus envelope proteins can undergo endocytosis, the gE findings suggest that endocytosis of envelope components may be a posttranslational regulatory mechanism among divergent families of enveloped viruses.

Varicella-zoster virus (VZV) is a member of the alphaherpesvirus subfamily and is the prototype of the recently designated *Varicellovirus* genus (29). VZV infection in humans causes two clinically distinct diseases, chicken pox (varicella) and shingles (herpes zoster). The genome of VZV encodes at least six glycoproteins, one of which is gE (previously designated gpI and gp98). Of the six, gE is the most abundant virion envelope glycoprotein (17). In addition, gE is the predominant VZV glycoprotein expressed on the surface of infected cells, as demonstrated by both indirect immunofluorescence and flow cytometric analysis (26, 32). VZV gE has also been expressed on the surface of gE-transfected cells (25, 52). The glycoprotein gE has been designated a typical type I transmembrane glycoprotein, which consists of three regions: a 544-amino-acid hydrophilic extracellular region, a 17-amino-acid hydrophobic transmembrane region, and a 62-amino-acid charged cytoplasmic tail region (8, 17). The amino acid sequence of the extracellular region contains two cysteine-rich regions, three N-linked glycosylation signals, and putative juxtamembrane domain favoring O-linked glycans (25). VZV gE has been shown to be both N-linked and O-linked glycosylated as well as sialylated and sulfated (32, 52). VZV gE has been demonstrated to be modified by serine/threonine casein kinase II phosphorylation (37, 52). Hence, gE displays features commonly observed in several nonviral cell surface receptors (37, 46). Further VZV gE on the surface of infected cells has previously been shown to function as an Fc receptor for nonimmune immunoglobulin G (IgG) (25, 26). Recently, we reported that gE was phosphorylated on a tyrosine residue but only on a dimeric form (37). This observation further increased the likelihood that gE functioned as a surface receptor. Therefore, we have extended the foregoing investigations to include receptor-mediated endocytosis, another prominent function of cell surface receptors which contain both serine and tyrosine consensus phosphorylation motifs in their cytoplasmic tails.

Receptor-mediated endocytosis enables the uptake of macromolecules that are vital for a cell's proper functioning (16, 30). The entry phase of this process is initiated by cell surface receptors collecting in regions of the plasma membrane specialized for internalization, clathrin-coated pits. Other transmembrane proteins are mostly excluded from clathrin-coated pits and are internalized very slowly (2, 39). The clathrin-coated pits are then pinched off into the cell as clathrin-coated vesicles that deliver the receptors to early endosomes. Internalized proteins are sorted into either a lysosomal pathway or a recycling pathway. Those receptors which are recycled first enter the sorting endosome and are then shuttled to the recycling endosome, from which they are directed back to the plasma membrane (14). Recent evidence indicates that the cytoplasmic domains of cell surface receptors are required for endocytosis. Cytoplasmic tail deletions in the low-density lipoprotein receptor (LDLR), transferrin receptor (TR), epidermal growth factor receptor (EGFR), or polymeric Ig receptor (pIgR) diminished internalization (24, 38, 40). Mutations changing a single tyrosine residue in the cytoplasmic tail of several receptors, including LDLR and pIgR, have prevented efficient internalization of the proteins (7, 34).

Due to the homology between VZV gE and nonviral cell surface receptors (37), the current study was designed to examine whether VZV gE undergoes endocytosis in a similar manner to other cell surface receptors. The results described herein not only show remarkable similarities in cell surface

* Corresponding author. Mailing address: University Hospital/2501 JCP, 200 Hawkins Dr., Iowa City, IA 52242. Phone: (319) 356-2288. Fax: (319) 356-4855. E-mail: grose@blue.weeg.uiowa.edu.

internalization patterns between the VZV gE Fc receptor and the human TR but also suggest mechanisms by which gE may mediate cell-to-cell spread of virus (10, 12).

MATERIALS AND METHODS

Cells and plasmids. HeLa cells (ATCC CCL2) were obtained from American Type Culture Collection, Rockville, Md. HeLa cells were grown in Eagle complete medium supplemented with 10% fetal bovine serum (FBS). Recombinant vaccinia virus (T7-vaccinia virus) and the expression plasmid pTM1 have been described elsewhere (33). Construction of plasmid pTM1-gE containing VZV open reading frame 68 has been published previously (52). Two anti-TR monoclonal antibodies (MAbs E2.3 and A27) were obtained from I. Trowbridge (50).

Endocytosis assay with laser scanning confocal microscope. HeLa cells were seeded in 35-mm-diameter tissue culture dishes at a concentration of 6.2×10^5 cells per dish. After overnight incubation at 37°C, the cells were first infected with recombinant T7-vaccinia virus and then transfected with 15% Lipofectin (Gibco BRL) containing 4 μg of pTM1-gE construct. Six hours posttransfection, fresh medium was added, and the cells were incubated overnight at 37°C. The cells were washed two times with cold phosphate-buffered saline (PBS; pH 7.4). The primary MAb, 3B3, was added to each dish (1:2,000 dilution in PBS) and incubated for 30 min at 4°C. MAb 3B3 recognizes an 11-amino-acid epitope in the ectodomain of VZV gE (19). The cells were washed once with PBS and once with Eagle complete medium supplemented with 10% FBS. Then fresh medium was added, and the cells were incubated at 37°C for various intervals. At the given times, the cells were fixed and permeabilized with 2% paraformaldehyde in 0.1 M Na_2HPO_4 with 0.05% Triton X-100. After 1 h of incubation, the cells were washed five times with PBS. The cells were then incubated for 1 h with goat anti-mouse–fluorescein isothiocyanate (FITC) conjugate (Biosource). After one more wash, the cells were viewed with a Bio-Rad MRC 600 or MRC 1024 laser scanning confocal microscope in the University of Iowa Central Microscopy Research Facility (37). Images were saved in X-view (J. Bradley) and Showcase (Silicon Graphics) programs.

Inhibition of endocytosis by sucrose. HeLa cells were transfected as previously described. Prior to the endocytosis assay, the cells were incubated with Eagle complete medium supplemented with 10% FBS containing 0.3 M sucrose for 30 min. The cells were incubated with MAb 3B3 as previously described. The cells were then incubated with medium containing 0.3 M sucrose for different amounts of time during the assay. This method is similar to the sucrose inhibition assay previously described by Ashworth et al. (1). The cells were processed as described above.

Colocalization with clathrin-coated vesicles. HeLa cells were transfected and incubated with MAb 3B3 at 4°C as previously described. The cells were incubated with medium at 37°C for 0, 5, 15, or 30 min, after which they were fixed and permeabilized. The cells were incubated with mouse monoclonal IgM anti-clathrin antibody (10 μg/ml; ICN Biomedicals) for 1 h. The cells were washed and then incubated with goat anti-mouse IgG–Texas red conjugate (1:1,000 dilution) and goat anti-mouse IgM-FITC conjugate (1:1,000 dilution) (Biosource) for 1 h. The cells were prepared and analyzed for laser scanning confocal microscopy as described above.

Colocalization with TR. HeLa cells were transfected as previously described. After 16 h, the cells were first incubated with murine MAb E2.3 or A27 (1:1,000 dilution) and rabbit monospecific polyclonal antiserum which recognizes VZV gE (1:1,000 dilution). The cells were incubated next with secondary antibodies, goat anti-mouse–Texas red conjugate (Molecular Probes), and goat anti-rabbit–FITC conjugate (Biosource). The time interval for each incubation period is given in Results. The cells were processed as described above.

Endocytosis assay of Fc receptor. HeLa cells were transfected as described above. The cells were washed with PBS and incubated with human plasma IgG Fc fragments (1 mg/ml in 0.1% NaN_3; Calbiochem) at 4°C for 30 min. The cells were washed with PBS and then incubated with medium for 15 min at 37°C. After 15 min, the cells were fixed and permeabilized as described above. Next, the cells were incubated with $F(ab')_2$ goat anti-human Ig-FITC conjugate (1:800 dilution) (Biosource) for 1 h. The cells were prepared for analysis by laser scanning confocal microscopy as described above.

Construction of gE tailless mutant and gE-Y582G tyrosine mutant. To construct the tailless gE mutant, plasmid pTM1-gE was digested with *Ssp*I and *Pst*I to remove the intervening nucleotides. The DNA termini were repaired with the Klenow fragments of *Escherichia coli* DNA polymerase and then religated. The final tailless product contained 555 of the 623 codons of the wild-type gE molecule (8). The construct was followed by multiple TAA stop codons in the polylinker region of pTM1 (33, 52).

To mutate the tyrosine residue at position 582 in the gE cytoplasmic tail, site-directed mutagenesis was performed by recombination PCR (53). Four oligonucleotide primers were prepared in order to generate two linear fragments containing two homologous ends. Two pairs of primers were designed: one pair of mutating primers and one pair of nonmutating primers. The nonmutating primers were prepared on an Applied Biosystems Synthesizer (Applied Biosystems) at the University of Iowa DNA core facility. The mutating primers were prepared by Genosys through the DNA core facility. Sequences of the mutating primers were 5′ AGCATGTATGGCGCCGGCCTTCCAG 3′ and 5′ GGAAG GCCGGCGCCATACATGCTT 3′, with a 23-bp overlap. Sequences of the nonmutating primers were previously described (37). Plasmid pTM1-gE was first linearized with restriction endonuclease *Sac*I or *Spe*I. One mutating and one nonmutating primer were used in pairs to generate linear fragments. Amplification of the DNA fragments from the linearized plasmid template was performed by PCR methods previously described (37, 53). The two linear DNA products were combined and transformed into Max competent *E. coli* DH5α cells (BRL, Life Technologies). Recombination occurred between the homologous fragments to produce a plasmid, called gE-Y582G, containing the desired mutation, and the mutation was verified by sequencing at the University of Iowa DNA core facility.

Quantitative internalization assay with radiolabel analysis. HeLa cells were transfected as described above. Six hours posttransfection, 250 μCi of [^{35}S]methionine-cysteine (specific activity, 7.15 mCi/ml; PRO-MIX; Amersham) per ml was added to each dish, and the cells were incubated overnight at 37°C. The cells were washed, incubated with MAb 3B3 (1:2,000 dilution) for 30 min at 4°C as described above, and then incubated at 37°C for different intervals. At the given times, the cells were treated with 1 mg of trypsin (Sigma) per ml for 30 min at 0°C. After removal of surface proteins, the cells were lysed in radioimmunoassay buffer containing 0.5 mg of soybean trypsin inhibitor (Sigma) per ml on ice for 30 min. This assay closely resembles that previously described by McClain et al. (28). The lysates containing antigen-antibody complexes were incubated with protein A-Sepharose CL-4B beads (Pharmacia) and precipitated as previously described (37). The proteins were eluted from the protein A beads in reducing buffer (125 mM Tris [pH 6.8], 6% glycerol, 10% 2-mercaptoethanol) by incubation at 100°C for 5 min. Immunoprecipitated proteins were analyzed on 10 to 18% gradient polyacrylamide gel containing 0.1% sodium dodecylsulfate (SDS). Gels were analyzed on a Packard Instantimager and exposed to radiographic film.

Endocytosis and recycling of gE analyzed by trypsin treatment. HeLa cells were transfected as previously described. The cells were washed, incubated with MAb 3B3 at 4°C as described above, and then incubated with medium at 37°C for 30 min. After 30 min, the cells were treated with 1 mg of trypsin per ml at 0°C for 30 min. After trypsin treatment, the cells were returned to 37°C with fresh medium containing 0.5 mg of trypsin inhibitor per ml for various amounts of time. At the specified times, the cells were fixed with 2% paraformaldehyde in 0.1 M Na_2HPO_4. Next, the cells were incubated with secondary antibody, goat anti-mouse–FITC conjugate, for 1 h. The cells were processed and viewed by laser scanning confocal microscopy as described above.

RESULTS

Endocytosis of VZV gE. Since VZV gE was previously identified as a phosphorylated Fc receptor, gE was examined for similarities with other nonviral cell surface receptors (26). Further, homology comparisons with other cell surface receptors identified similarities between gE and the LDLR and the Fc gamma receptor II (FcγRII) (37). Because endocytosis is common to these receptors, gE was analyzed to determine if it could be endocytosed from the plasma membrane.

To this end, HeLa cells were transfected with plasmid pTM1-gE, which contains the entire wild-type gE gene. Sixteen hours posttransfection, the cells were incubated with MAb 3B3 at 4°C for 30 min, during which time endocytosis is inhibited. The cells were then returned to 37°C for 0, 5, 10, 15, 30, 45, or 60 min to allow internalization of gE. At the given times, the cells were fixed and permeabilized. Then, secondary antibody was incubated with the cells to identify where the antibody-bound gE proteins localized within the cell. Subsequently, the cells were analyzed by confocal microscopy with laser sectioning in 1-μm increments from the surface of the cell to the plane of attachment (zeta series). The images were analyzed, and the central section from each time point was compared for gE localization. As shown in Fig. 1A, when gE transfected cells were incubated with MAb 3B3 and not returned to 37°C, antibody bound gE was on the surface of the cell and internalization was not evident. When the cells were incubated with MAb 3B3 and then incubated at 37°C for 5 min (Fig. 1B), gE was localized in clumping pattern on the membrane, and some gE was observed within the cell. After 10 and 15 min at 37°C (Fig. 1C and D), more gE was localized within the cytoplasm of the cells in small vesicle-shaped clusters. After 30, 45, and 60 min at 37°C (Fig. 1E to G), gE was detected within the cell as well as on the surface. Further time points (data not shown)

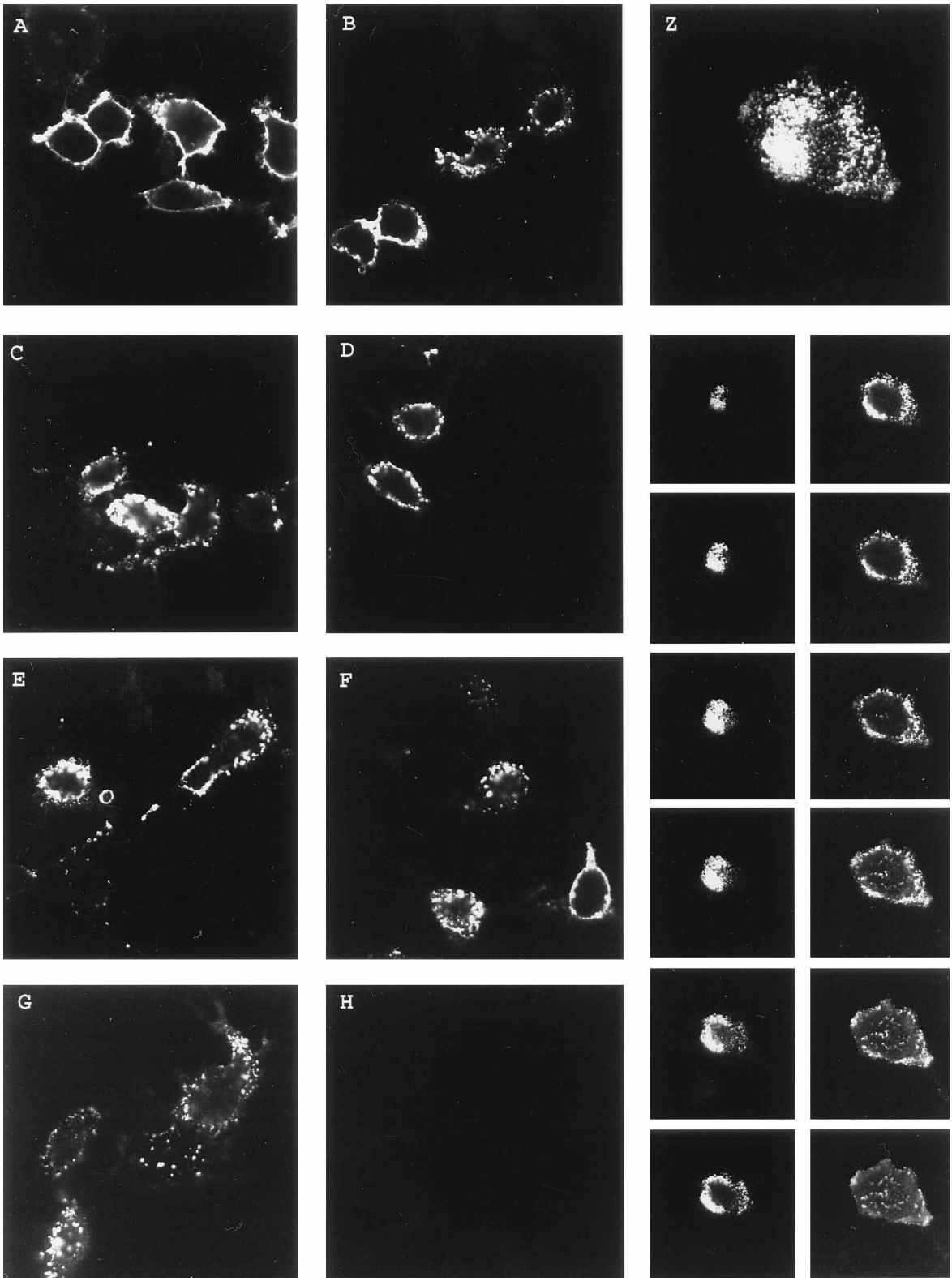

FIG. 1. Endocytosis of VZV gE analyzed by laser scanning confocal microscopy. HeLa cells were transfected with the gE gene. At 16 h posttransfection, the cells were incubated with MAb 3B3 at 4°C for 30 min. The cells were then returned to 37°C with fresh medium for 0 min (A), 5 min (B), 10 min (C), 15 min (D), 30 min (E), 45 min (F), or 60 min (G). The cells were fixed and permeabilized and then incubated with secondary antibody for 1 h. The images shown represent the central section from the cells analyzed by laser scanning confocal microscopy. (H) HeLa cells were mock transfected and stained for a negative control. For each image represented here and in similar figures, we performed a zeta series whereby the cell is laser sectioned in 1-μm increments from cell surface to plane of attachment. (Z) A zeta series of a cell that was incubated for 60 min at 37°C. Twelve smaller individual sections of the composite image are shown below panel Z, with the cell surface section in the upper left and the plane of attachment in the lower right.

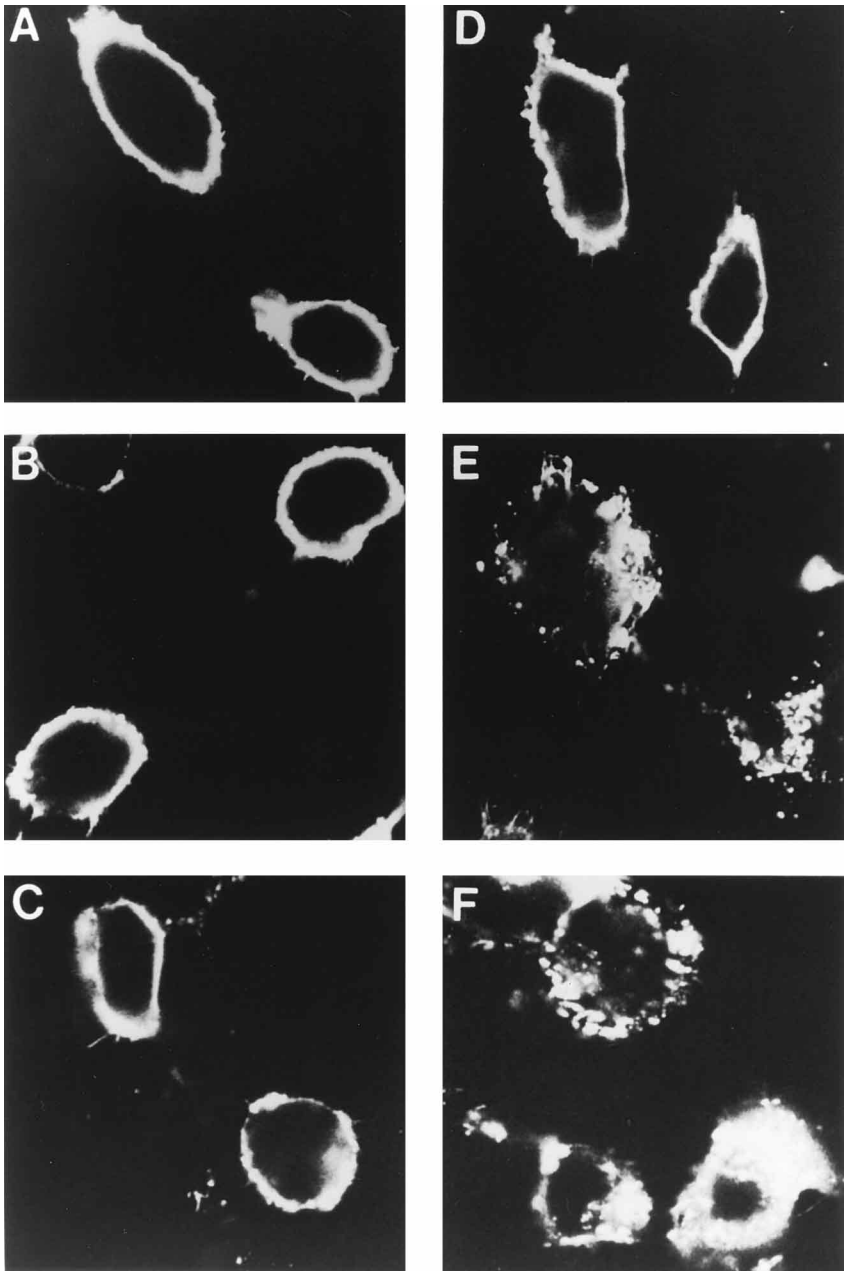

FIG. 2. Sucrose inhibition of VZV gE endocytosis. HeLa cells were transfected with the gE gene. The cells were incubated with sucrose-containing medium for 30 min at 37°C and then incubated with MAb 3B3 at 4°C for 30 min. The cells were returned to 37°C with medium containing 0.3 M sucrose for 0 min (A), 15 min (B), or 30 min (C). Transfected cells which were not incubated with sucrose medium were returned to 37°C for 0 min (D), 15 min (E), or 30 min (F). All the cultures were analyzed by laser scanning confocal microscopy.

displayed similar localization and showed no decrease in the amount of labeled protein. As a control, mock-transfected HeLa cells were not bound by MAb 3B3 during the 30-min incubation (Fig. 1H). Panel Z of Fig. 1 depicts a composite of a complete zeta series as well as each individual section through a gE-transfected cell which was incubated for 60 min at 37°C. This pattern of VZV gE localization demonstrated that gE was endocytosed from the cell membrane in a manner consistent with other cell surface receptors.

Internalization of gE by clathrin-coated vesicles. Endocytosis of receptors is mediated primarily by clathrin-coated vesicles which are derived from the cell surface clathrin-coated pits. The vesicles are responsible for the transfer of receptors from the plasma membrane to the early endosomes. Internalization via clathrin-coated vesicles is disrupted in hypertonic solution (1). Hypertonic medium stabilizes clathrin microcage structures, which reduces the soluble pool of clathrin available for coated pit formation; hence, clathrin-coated vesicles are not formed (30). To determine if gE was internalized by clathrin-coated pits, the cells were incubated in hypertonic medium containing sucrose.

HeLa cells were transfected with the gE gene, and 16 h posttransfection, the medium was replaced with medium containing 0.3 M sucrose for 30 min. The cells were incubated with

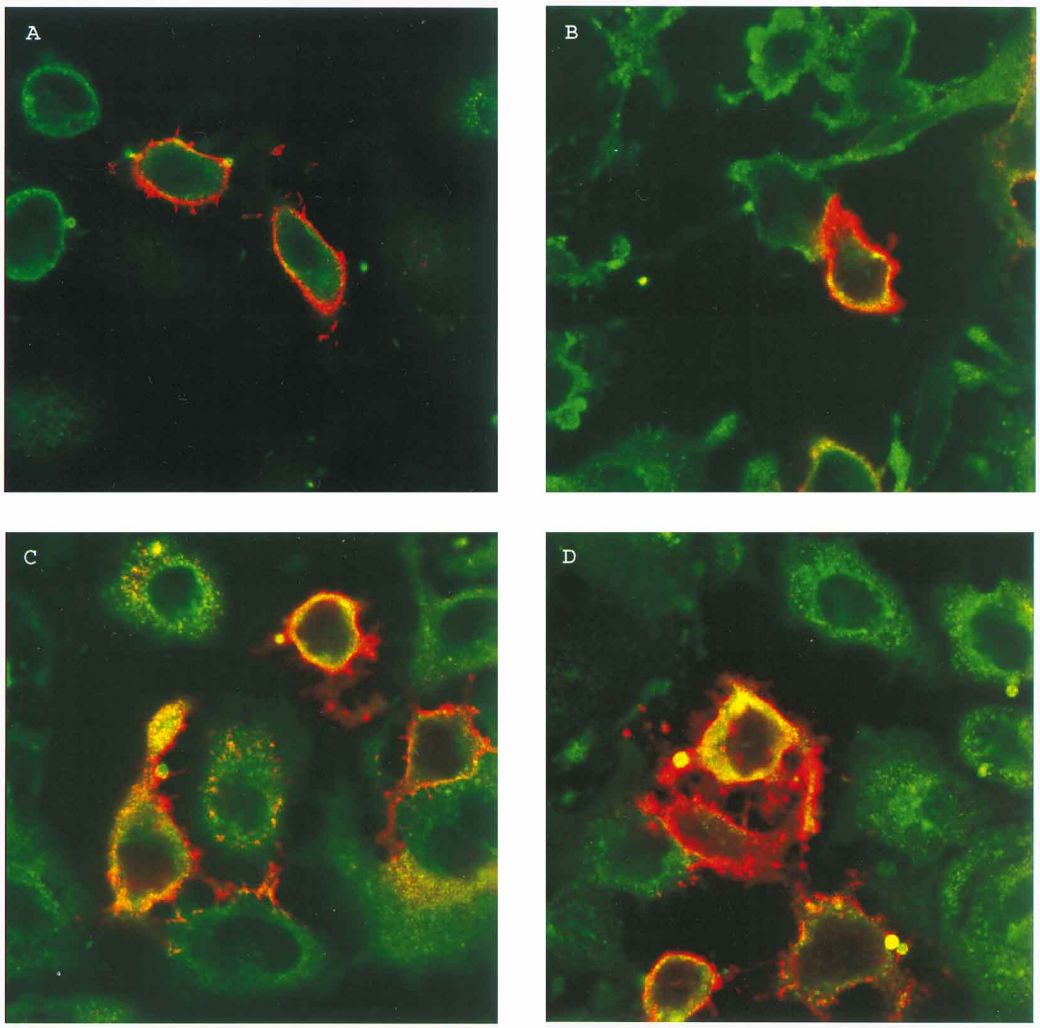

FIG. 3. Colocalization of VZV gE with clathrin-coated vesicles during endocytosis. HeLa cells were transfected with the gE gene. The cells were incubated with MAb 3B3 at 4°C for 30 min. The cells were then returned to 37°C with fresh medium for 0 min (A), 5 min (B), 15 min (C), or 30 min (D). The cells were fixed and permeabilized prior to being incubated with MAb against clathrin-coated vesicles for 1 h. The cells were then incubated with goat anti-mouse IgG–Texas red conjugate and goat anti-mouse IgM–FITC conjugate. The cells were analyzed by laser scanning confocal microscopy with gE (red stain) and clathrin (green stain). Colocalization is designated by the yellow color produced from merging the red and green stains.

MAb 3B3 for 30 min at 4°C. Then, the cells were incubated in hypertonic medium for 15 or 30 min at 37°C. At the given times, the cells were fixed and permeabilized. Secondary antibody was incubated with the cells to identify where the antibody bound proteins were localized. When analyzed by laser scanning confocal microscopy, the gE-transfected cells that were not incubated at 37°C after incubation with MAb 3B3 showed surface expression of gE (Fig. 2A). After 15 min at 37°C in the hypertonic medium (Fig. 2B), gE was localized to the cell surface, in marked contrast to the internalized gE, which was observed in a similar sample not incubated with hypertonic medium (Fig. 2E). After 30 min at 37°C, gE was still not internalized in the cells which were incubated with hypertonic medium (Fig. 2C) but was internalized in the cells which were incubated with regular medium (Fig. 2F). This experiment showed that gE was unable to be internalized when the cells were incubated in hypertonic medium. Therefore, this result demonstrated that internalization of gE was dependent on the clathrin-coated pit formation and clathrin-coated vesicle transport.

To verify that gE internalization was clathrin mediated, gE-transfected HeLa cells were double stained with MAb 3B3 and MAb for clathrin in vesicles. HeLa cells transfected with gE were incubated with MAb 3B3 as described above and incubated at 37°C for 0, 5, 15, or 30 min. After the incubation times, the cells were fixed and permeabilized. The cells were then incubated with mouse IgM MAb for clathrin located in clathrin-coated vesicles. Next, the cells were stained anti-mouse IgG–Texas red conjugate antibody and anti-mouse IgM–FITC conjugate before analysis by laser scanning confocal microscopy. When the gE-transfected cells were not incubated at 37°C and were incubated with clathrin antibody (Fig. 3A), gE was localized on the surface of the cells, as shown by the red staining, and the clathrin was diffused throughout the cells in vesicles, as shown by the green staining. Since not all cells in the field were transfected, only a few cells showed the gE staining (red) whereas clathrin was present in all cells stained (green). After 5 min of incubation at 37°C (Fig. 3B), gE was still localized to the surface with some gE, forming a clumping pattern; however, some yellow staining, which represented co-

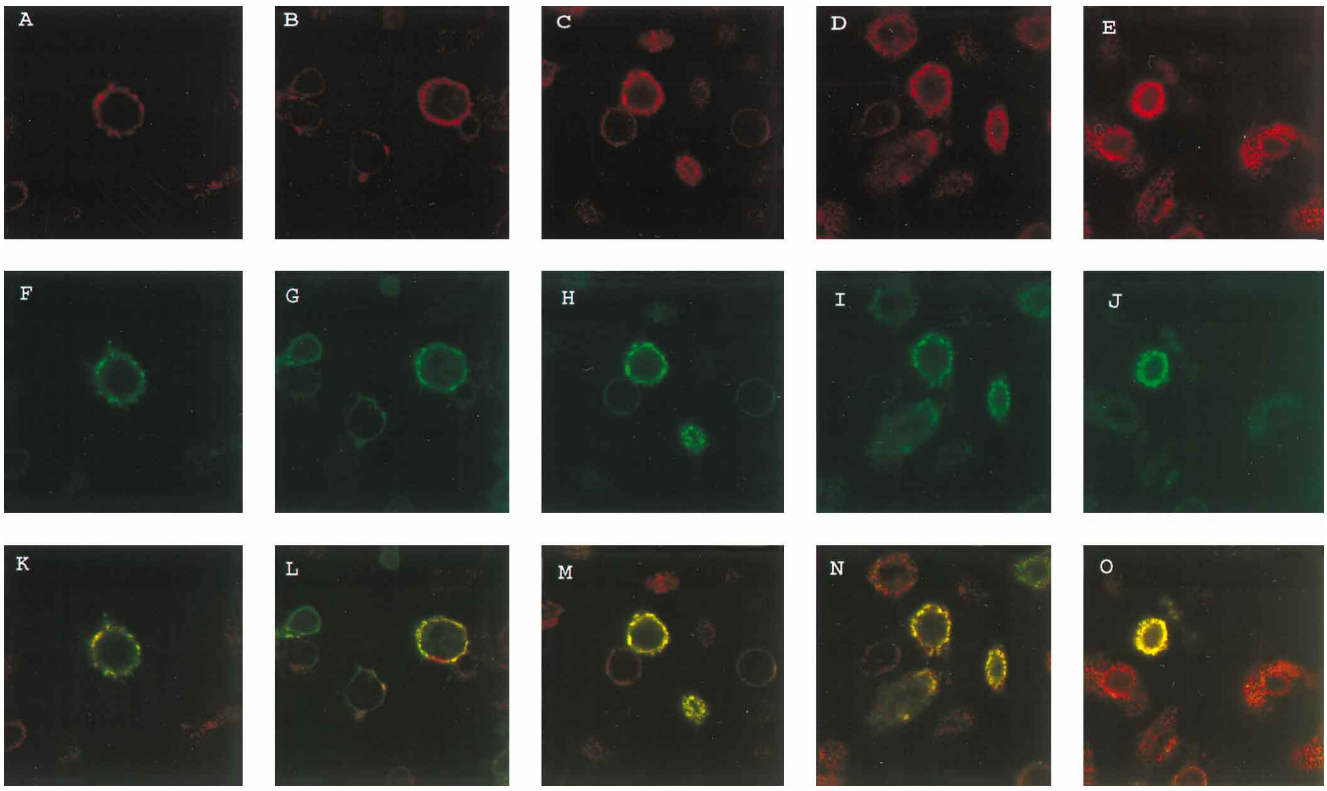

FIG. 4. Colocalization of VZV gE with TR during endocytosis. HeLa cells were transfected with the gE gene. The cells were first incubated at 4°C for 30 min with murine anti-TR MAb E2.3, MAb A27, and polyclonal antiserum against gE. The cells were then returned to 37°C for 0 min (A, F, and K), 15 min (B, G, and L), 30 min (C, H, and M), 45 min (D, I, and N), or 60 min (E, J, and O). The cells were fixed and permeabilized prior to incubating the cells with goat anti-mouse–Texas red conjugate and goat anti-rabbit–FITC conjugate. The cells were analyzed by laser scanning confocal microscopy with TR (red stain) (A to E) and gE (green stain) (F to J); the merged images (K to O) stained yellow.

localization of green and red, was observed. These results indicated that gE was clustering in the clathrin-coated pits forming the clathrin-coated vesicles at the cell membrane. After 15 min of incubation at 37°C (Fig. 3C), gE was internalized, and small vesicle-shaped structures which contained both gE and clathrin were observed. The colocalization of gE and clathrin in vesicles was observed further after 30 min of incubation at 37°C (Fig. 3D). These results demonstrated that gE colocalized with clathrin in coated vesicles during endocytosis.

Colocalization of VZV gE with TR during endocytosis. The TR has been shown to undergo receptor-mediated endocytosis. This process is dependent on clathrin-coated vesicles which mediate the transport of TR to early endosomes after internalization. The receptors are then transported to sorting and recycling endosomes, which facilitate the transport of TR back to the plasma membrane (14, 15). To determine if gE was following the same endocytosis pathway, gE was colocalized with TR during endocytosis by laser scanning confocal microscopy.

For this experiment, transfected cells were incubated with rabbit polyclonal antiserum against gE and mouse MAb against TR (50). The cells were returned to 37°C for 15, 30, or 60 min. At the given times, the cells were fixed and permeabilized. Next, the cells were incubated with goat anti-rabbit–FITC conjugate and goat anti-mouse–Texas red conjugate. When the cells were incubated with primary antibody but not returned to 37°C, gE was localized on the cell surface in the same manner as shown in Fig. 1A (Fig. 4F), and TR was also localized on the cell surface (Fig. 4A). When the two images were merged, the two proteins colocalized to the cell surface, as shown by the yellow color (Fig. 4K). After 15 min at 37°C, gE was internalized in the same manner as in Fig. 1D (Fig. 4G), and TR was also internalized (Fig. 4B). When the two images were merged, the two proteins were localized to the same areas of the cell (Fig. 4L). After 30 min at 37°C, gE was still internalized as was TR (Fig. 4H and C). The two proteins were colocalizing in the cytoplasm, and small vesicles which contained the two proteins were defined within the cell (Fig. 4M). After 45 and 60 min of incubation at 37°C, the two proteins continued to show similar localization patterns (Fig. 4D, E, I, J, N, and O). Therefore, these results showed that gE was internalized and possibly recycled back to the plasma membrane in the same compartments as TR. Since the TR endocytosis pattern has been previously defined (49), gE colocalization with TR suggested a similar pattern of signal-dependent protein sorting.

Endocytosis of a functional VZV Fc receptor. Previous studies have shown that gE functions as an Fc receptor which binds IgG (25). The endocytosis studies described above were conducted with MAb 3B3, which does not discriminate between receptors which have bound Fc and those which have not bound Fc. To determine whether gE was endocytosed when it bound Fc, gE internalization assays were repeated with Fc fragments rather than gE-specific antibody. HeLa cells were transfected with the gE gene. Sixteen hours posttransfection, the cells were incubated with human plasma IgG Fc fragments for 30 min. The cells were then incubated at 37°C for 15 min as previously described. The cells were fixed and permeabilized prior to incubation with $F(ab')_2$ FITC conjugated to goat anti-human Ig. When the cells were analyzed by laser scanning confocal microscopy as previously described, the gE which

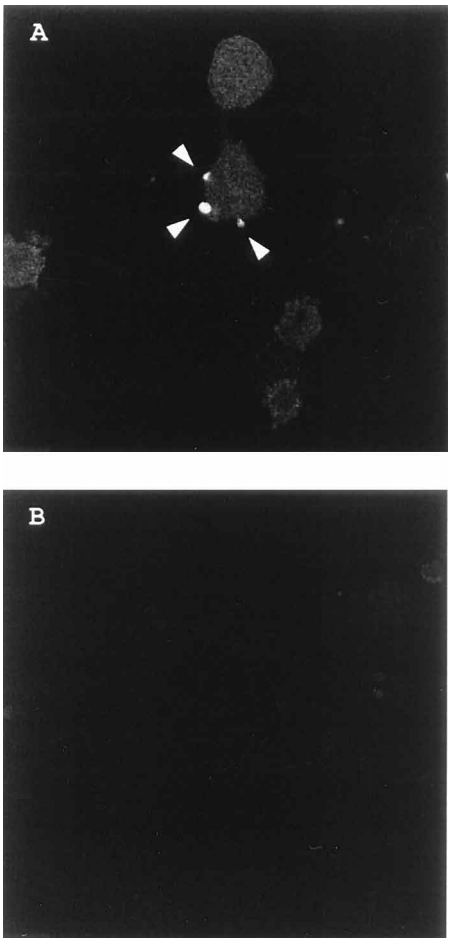

FIG. 5. Endocytosis of VZV gE while functioning as an Fc receptor. HeLa cells were transfected with the gE gene. At 16 h posttransfection, the cells were incubated with human IgG Fc fragments at 4°C for 30 min. The cells were then returned to 37°C for 0 min (A) or 15 min (B). Next, the cells were fixed and permeabilized prior to incubation with $F(ab')_2$-FITC conjugated to goat anti-human Ig for 1 h. The cells were analyzed by laser scanning confocal microscopy. Arrowheads designate the Fc aggregates on the cell surface in panel A.

bound Fc fragments prior to incubation at 37°C displayed distinct patches of staining on the cell surface (Fig. 5A). This pattern of staining was similar to that observed for human Fc receptors (13). However, after 15 min of incubation at 37°C, the gE molecules which bound the Fc fragments were absent from the cell surface, and no staining was observed in the cells (Fig. 5B). These results suggested that all the gE which bound the Fc fragments had been internalized and the internalized Fc fragments had been degraded. In short, gE was able to undergo endocytosis when it functioned as an Fc receptor.

Endocytosis of gE mediated by a tyrosine motif. Receptor endocytosis has been shown to be dependent on specific cytoplasmic tail sequences (30). Deletional analyses of the cytoplasmic tail from several receptors, including FcγIIR, LDLR, pIgR, EGFR, TR, and insulin receptor, have shown less efficient internalization of the tailless receptor (24, 28, 31, 35, 38, 40). To determine if gE internalization was likewise dependent on the cytoplasmic tail, a tailless gE mutant was constructed and analyzed for efficient internalization. HeLa cells were transfected with the tailless gE gene, which lacked the entire cytoplasmic domain and seven transmembrane domain codons. The cells were incubated with MAb 3B3 and processed for confocal microscopy as previously described for wild-type gE. When the tailless gE was incubated with MAb 3B3 without being returned to 37°C (Fig. 6A), the protein was localized on the surface of the cell; therefore, the deletion did not prevent protein transport to the cell membrane. When the cells were returned to 37°C for 15 min (Fig. 6B), tailless gE remained on the surface of the cell, in contrast to wild-type gE (Fig. 6E). After 30 min (Fig. 6C), tailless gE was still not efficiently internalized, in contrast to the rapid internalization observed with wild-type gE (Fig. 6F). Further time points showed similar results (data not shown). These micrographs confirmed that gE required the cytoplasmic tail sequence for efficient internalization.

Specific amino acids within the cytoplasmic tails of receptors are commonly associated with endocytosis. The requirement for a cytoplasmic tyrosine motif in maintaining high-efficiency internalization has been determined for a number of receptors internalized by endocytosis, including the LDLR and TR (6, 30). The gE cytoplasmic tail contains six tyrosine residues, but one tyrosine is located within a previously defined internalization motif; i.e., the sequence YXXV(L) has been shown to facilitate internalization of the mannose 6-phosphate receptor and the FcγRII (4, 13, 31). Since gE contains a YAGL sequence in its cytoplasmic tail, the tyrosine residue in this motif was replaced by a glycine residue, and the internalization efficiency of this substitution was evaluated. HeLa cells were transfected with a mutant encoding the gE-Y582G gene. When the gE-Y582G mutant was observed immediately after incubation with MAb 3B3, the protein was located at the cell surface (Fig. 7A). When the transfected cells were incubated with MAb 3B3 and returned to 37°C for 15 or 30 min (Fig. 7B and C), the protein remained on the surface and was not internalized like the wild-type gE (Fig. 7E and F). Further time points showed similar results (data not shown). Therefore, these results suggested that the tyrosine in the YAGL motif in the cytoplasmic tail was required for efficient internalization of gE.

Endocytosis and recycling of VZV gE. By confocal microscopy, gE was found to be internalized and to be continually present throughout the assay in a similar distribution. By colocalization with the TR, gE was shown to enter the sorting and recycling compartments where the TR was localized. These results suggested that gE may be continually recycling to the cell surface after internalization. To determine if gE was following such a pathway, gE was first analyzed by a quantitative internalization protein assay. HeLa cells were transfected with either wild-type gE or tailless mutant gE and radiolabeled with [^{35}S]methionine-cysteine. Sixteen hours posttransfection, the cells were incubated with MAb 3B3 at 4°C for 30 min. The cells were then returned to 37°C at increasing time intervals. At each time point, the cells were treated with trypsin for 30 min at 0°C and then lysed in lysis buffer containing trypsin inhibitor. Trypsin degrades receptors on the surface of cells without affecting internalized receptors (28). The lysates were precipitated with protein A beads. After the precipitates were separated by SDS-polyacrylamide gel electrophoresis, the protein bands on the gel were analyzed by an Instantimager for densitometric determination of internalized protein at each time point. The percent internalized protein was calculated based on the total amount of protein precipitated from the cells which were incubated with MAb 3B3 and not treated with trypsin at the beginning of the assay. The percent internalized protein represented the amount of protein which was bound by MAb 3B3 at the surface that was subsequently internalized during the various timed incubations at 37°C. The amount of protein on the surface that was bound to MAb 3B3 prior to

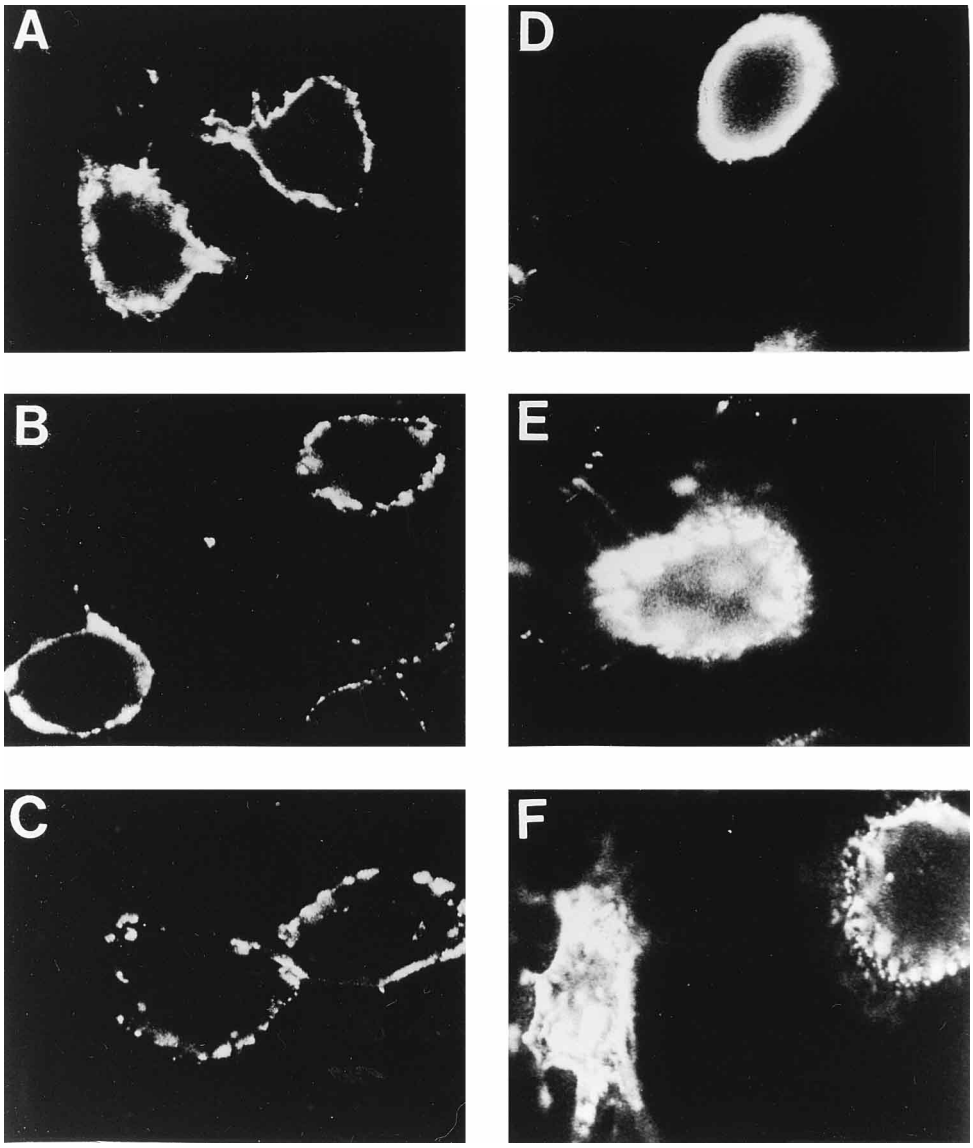

FIG. 6. Diminished endocytosis of a tailless VZV gE mutant. Deletion of the cytoplasmic domain of the gE receptor gene was carried out as described in Materials and Methods. HeLa cells were transfected with the tailless gE gene. The cells were incubated with MAb 3B3 at 4°C for 30 min. Then, the cells were incubated at 37°C for 0 min (A), 15 min (B), or 30 min (C). Simultaneously, HeLa cells were transfected with the wild-type gE gene and similarly processed at 0 min (D), 15 min (E), or 30 min (F). Cultures were fixed and permeabilized prior to incubation with secondary antibody, after which they were analyzed by laser scanning confocal microscopy.

incubation at 37°C represented 100%; the amount of protein which remained after trypsin treatment of these same cells represented 0%. Previous reports demonstrate that all surface gE is removed by trypsin treatment (37). The internalization of gE is shown in a graphical representation in Fig. 8. As the cells were incubated at increasing intervals at 37°C, the amount of gE internalized increased linearly until 15 min (Fig. 8). Thereafter, the amount of gE internalized reached a steady state of 32% at any given time. This curve was indicative of a protein which was internalized and continually recycled. The equilibrium of internalized protein was observed after the initial burst of internalization when the protein was bound to antibody on the surface. Cells which were not trypsin treated after the endocytosis time points were used as controls to ensure steady-state levels of antibody-bound protein throughout the assay. In contrast to full-length gE, the tailless gE was unable to be internalized under the same experiment conditions (Fig. 8).

To further determine whether gE was being recycled to the surface of the cells, gE was analyzed by the confocal microscopy internalization assay after treatment with trypsin. HeLa cells were transfected with the gE gene as previously described. Sixteen hours posttransfection, all of the cells were incubated with MAb 3B3 at 4°C and returned to 37°C for 30 min to allow internalization. Some of the cells were then incubated with trypsin at 0°C for 30 min before being returned to 37°C for 15 or 30 min with medium containing trypsin inhibitor. When the cells are returned to 37°C, internalized proteins are then able to return to the cell surface (51). At the given time points, the cells were fixed, stained with goat anti-mouse–FITC conjugate, and analyzed by laser scanning confocal microscopy. Since the cells were not permeabilized, the secondary antibody was able

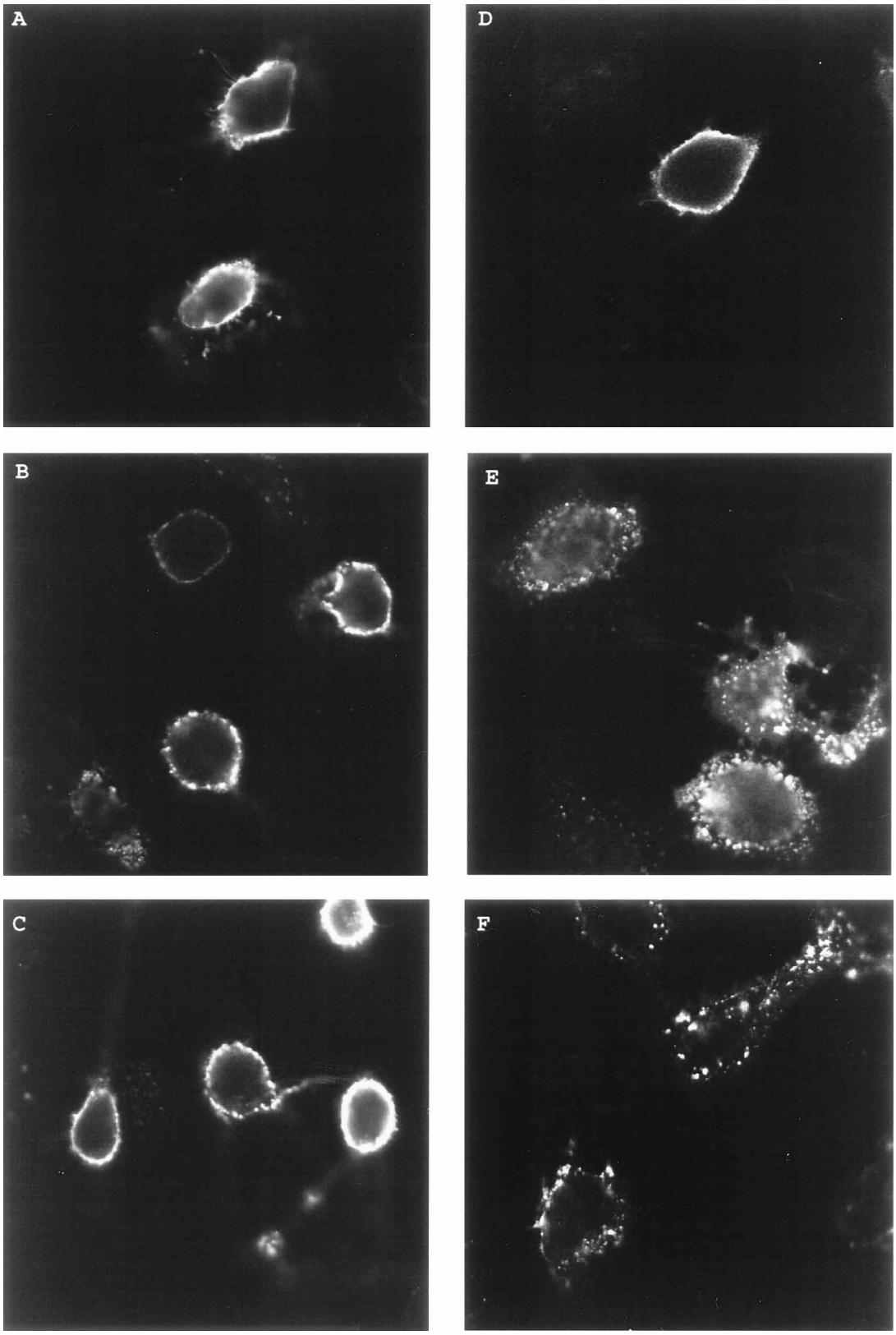

FIG. 7. Diminished endocytosis of a VZV gE tyrosine mutant. A mutation in the tyrosine codon at position 582 in the cytoplasmic tail produced a glycine codon as described in Materials and Methods. HeLa cells were transfected with the gE-Y582G gene (A to C) or wild-type gE gene (D to F). The cells were incubated with MAb 3B3 at 4°C for 30 min. The cells were then incubated at 37°C for 0 min (A and D), 15 min (B and E), or 30 min (C and F). Cultures were fixed and permeabilized. Next, the cells were incubated with secondary antibody and analyzed by laser scanning confocal microscopy.

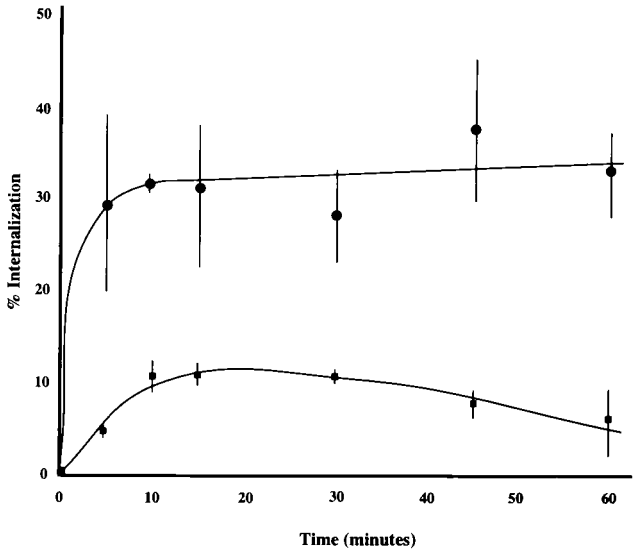

FIG. 8. Endocytosis of VZV gE analyzed by a quantitative radiolabel assay. HeLa cells were transfected with the wild-type gE gene (circles) or with the tailless gE gene (squares) and radiolabeled with [^{35}S]methionine-cysteine. Sixteen hours posttransfection, the cells were incubated with MAb 3B3 at 4°C for 30 min. The cells were then incubated with medium at 37°C for 0, 5, 10, 15, 30, 45, or 60 min. After the various incubation periods, the cells were treated with trypsin at 0°C for 30 min. Then the cells were lysed and precipitated. The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, and the gel was analyzed on an Instantimager. The percent internalized protein was derived from a formula comparing the total protein labeled on the cell surface at 0 min (not treated with trypsin) with the amount of protein internalized at the given time points (treated with trypsin).

to bind only surface antibody-bound protein. In the cells which were not trypsin treated after the 30 min of incubation at 37°C, some gE was still detectable on the surface during endocytosis (Fig. 9A). Examination of the cells which were treated with trypsin but not returned to 37°C after treatment demonstrated that all gE (shown in Fig. 9A) was removed from the surface of the cells by trypsin treatment (Fig. 9B). However, when the cells were returned to 37°C for 15 or 30 min after trypsin treatment (Fig. 9C and D), gE was observed again on the surface of the cells. That gE which was initially on the surface and bound by MAb 3B3 was internalized during the first incubation at 37°C. Next, the trypsin was unable to affect the internalized protein; therefore, by incubating the cells at 37°C again, the internalized labeled gE was recycled back to the surface of the cells, where the antibody bound to gE was detected by laser scanning confocal microscopy. This assay was also conducted with the tailless gE mutant and the gE-Y582G mutant. These mutants were unable to be recycled back to the surface after trypsin treatment and incubation at 37°C (data not shown). These results are summarized in Fig. 10 (see Discussion).

DISCUSSION

Recently published studies have demonstrated that the envelope protein of human immunodeficiency virus type 1 (HIV-1) undergoes endocytosis from the cell membrane (41). The endocytosis of the HIV-1 envelope protein was dependent on a tyrosine-containing motif in the cytoplasmic tail region of gp41. Likewise, a similar conserved tyrosine-containing motif in the envelope protein of simian immunodeficiency virus (SIV) was identified as important for endocytosis of that protein (22, 43). Endocytosis of the HIV-1 envelope protein was inhibited during HIV-1 infection by the HIV-1 Gag precursor protein Pr55^{gag} (11). The current studies have focused on the endocytosis of a herpesvirus glycoprotein, VZV gE. As with HIV and SIV, VZV gE was internalized via a tyrosine motif in its cytoplasmic tail. Thus, endocytosis may be a common regulatory mechanism among diverse enveloped viruses, including other alphaherpesviruses. For example, the pseudorabies virus gE homolog contains a similar conserved tyrosine motif in its cytoplasmic domain (GenBank accession number M14336). Whether the herpesviruses also specify a negative regulatory protein to limit endocytosis is not known.

The findings with VZV gE parallel those of recent endocytosis studies conducted on several cell surface receptors. Endocytosis is the process by which integral membrane proteins are selectively internalized from the plasma membrane in a signal-dependent manner. Receptor endocytosis is an important mechanism for cellular uptake of macromolecules which are bound to cell surface receptors. Endocytosis is also important for the cellular regulation of protein composition of the plasma membrane. During endocytosis, membrane proteins which are internalized are segregated from the resident plasma membrane proteins and are concentrated in clathrin-coated pits at the plasma membrane (45). The coated pits are then pinched off from the plasma membrane into the cytoplasm, forming transport vesicles that deliver the proteins to the endosomal compartment. The clustering of receptors into the coated pits is dependent on internalization signals, which are structural motifs identified as short linear arrays of amino acids that differ in specific sequence but share a common three-dimensional conformation. The signal is most frequently a tetrapeptide sequence with a pattern of aromatic-X-X-large hydrophobic residue, where the aromatic residue is commonly a tyrosine residue and the X represents any amino acid (48). The tetrapeptide internalization sequences identified thus far favor a conformational tight turn, a reverse-turn conformation (β turn) or a terminal turn of α helix (5). The gE tetrapeptide conforms to the internalization motifs previously identified in several receptors, including the TR, LDLR, pIgR, and FcγRII (4, 48). The tyrosine residue appears to be an important determinant for internalization, since substituting a tyrosine for a native cysteine residue in the influenza virus hemagglutinin protein endodomain converted a nonendocytosed protein into an efficiently internalized protein (23).

The VZV studies of gE endocytosis were aided considerably by laser scanning confocal microscopy. Figure 10 depicts our experimental protocol designed to demonstrate endocytosis and recycling of VZV gE. The current studies also clarified that VZV gE can function as an Fc receptor when it bound its ligand; however, ligand binding was not required for internalization. Most likely, the Fc fragments were separated from gE after internalization and then degraded in the lysosomal pathway. This process would be similar to that of the LDLR, which binds its ligand prior to internalization; once internalized, the ligand dissociates from the receptor, with the receptor entering the recycling pathway and the ligand entering the lysosomal pathway (16).

The VZV gE phosphorylation events have been extensively characterized (17, 52, 53). Most recently, VZV gE was shown to be tyrosine phosphorylated when it forms a homodimeric complex (37). Tyrosine phosphorylation may be important for differential sorting and trafficking of the monomers and dimers after internalization. Earlier studies have firmly established that VZV gE contains a consensus sequence for casein kinase II phosphorylation in its cytoplasmic tail, and this sequence is phosphorylated by casein kinase II (17, 37, 52). The consensus sequence for casein kinase II phosphorylation is defined as a

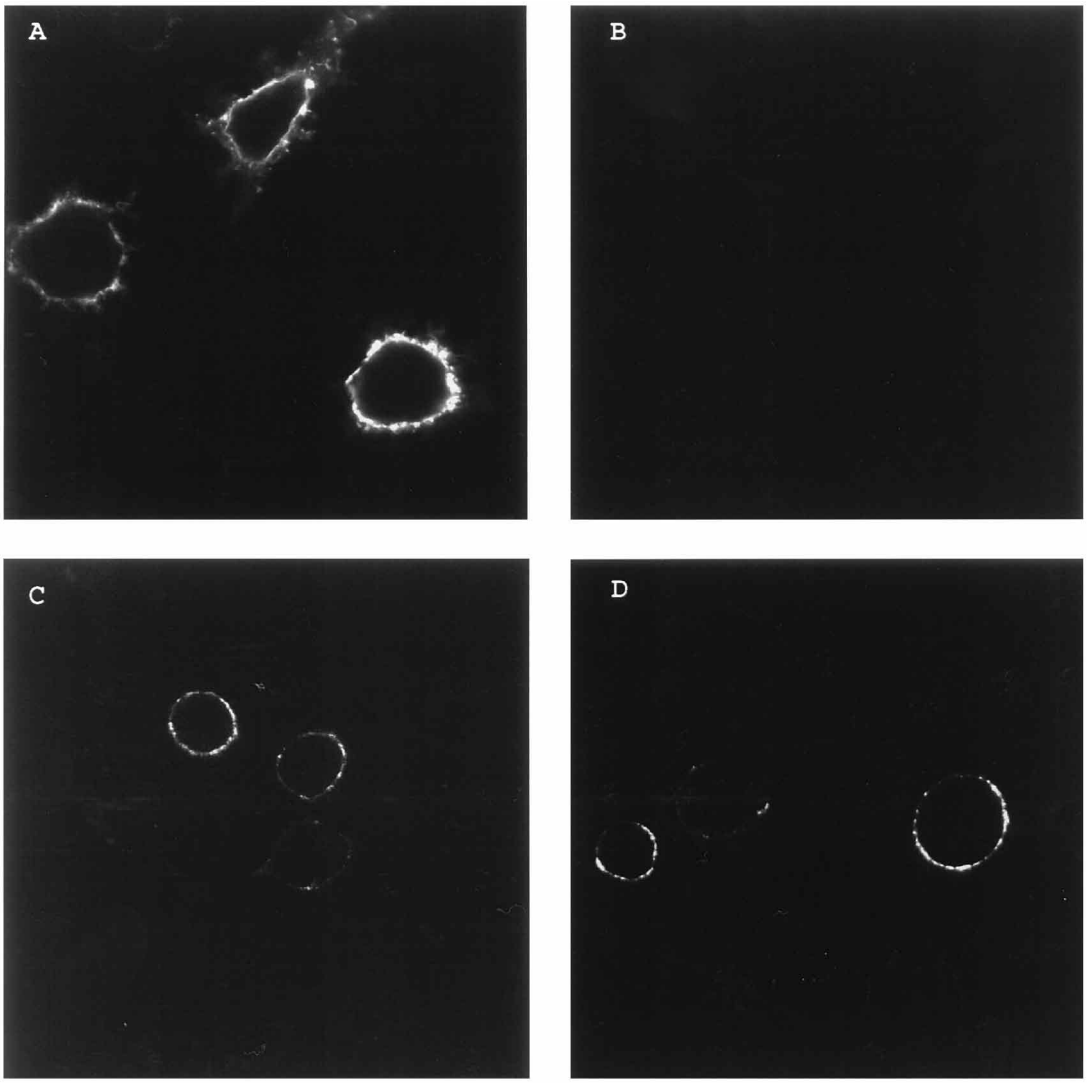

FIG. 9. Endocytosis and recycling of VZV gE. Replicate cultures of HeLa cells were transfected with the gE gene. The cells were incubated with MAb 3B3 at 4°C for 30 min, incubated at 37°C for 30 min to allow internalization, and then incubated with trypsin at 0°C for 30 min to remove surface proteins. The cells were returned to 37°C with fresh medium containing trypsin inhibitor for 0 min (B), 15 min (C), or 30 min (D). At the given time points, the cells were fixed and stained with secondary antibody to identify the gE which had been internalized and recycled to the cell surface. Transfected cells that were not trypsin treated or returned to 37°C represented the positive control (A). The cells were analyzed by laser scanning confocal microscopy.

serine or threonine followed by acidic amino acid residues; the sequence may form an α helix (20). The same acidic amino acid stretches have also been implicated in endocytosis and trafficking of internalized proteins (42). Other studies have detected a casein kinase II-like activity associated with coated vesicles (44). Most recently, studies with the mannose 6-phosphate receptor demonstrated that casein kinase II phosphorylation of the receptor was required for its binding to clathrin-associated complex, AP-1, at the *trans*-Golgi network (TGN) (27). The AP-1 and AP-2 complexes bind to receptors prior to clathrin pit formation at the TGN and plasma membrane, respectively (36). Current studies have also suggested that a novel class of clathrin-coated vesicles enable exit from endosomes during recycling of the TR (47). Therefore, the casein kinase II consensus sequence and its phosphorylation/dephosphorylation may play a role in endocytosis and recycling of receptors which have been internalized (21, 49). In this regard, the acidic amino acid region in VZV gE cytoplasmic tail has recently been shown to function as a sorting signal to the TGN (54).

VZV, like other alphaherpesviruses, is highly neurotropic. Studies with pseudorabies virus (PRV) and herpes simplex virus have implicated gE in neuronal cell-to-cell spread (10, 12). In the PRV rat model, gE is not required for entry into the primary neuron, but gE is required for transneuronal passage of PRV, either from retinal afferents or into postsynaptic retinorecipient neurons (3). Recently, synaptic vesicles have been shown to be clathrin-coated vesicles which continually recycle in a pattern similar to the recycling which occurs at the surface of all cells (9). Since VZV gE can enter the recycling pathway in cells via clathrin-coated vesicles, gE may also be incorporated in the synaptic recycling pathway in neurons. Likewise, endocytosis may be one pathway by which gE is incorporated into cytoplasmic vacuoles containing virions, prior to their egress onto the cell surface (18). Very importantly, VZV gE endocytosis and recycling explain the abundant expression of

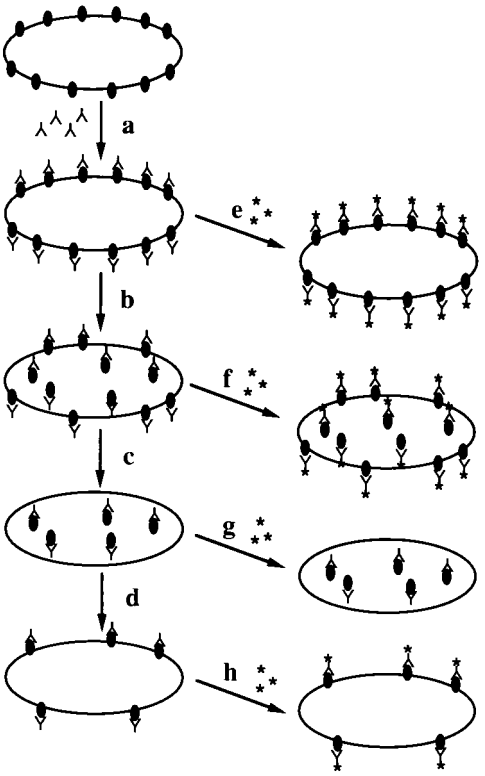

FIG. 10. Model for endocytosis and recycling of VZV gE. Replicate cultures of HeLa cells (large open ovals) were transfected with the gene encoding VZV gE, and gE proteins (small solid ovals) were expressed on the cell surface. The cells were incubated at 4°C for 30 min with MAb 3B3 (a). Some cultures were immediately fixed and permeabilized and then incubated with secondary antibody-FITC conjugate (asterisks) prior to analysis by laser scanning confocal microscopy (e). The remaining cultures were incubated at 37°C for increasing intervals to allow internalization of the surface gE (b). When these cultures were fixed and permeabilized and incubated with FITC conjugate, the confocal microscopy showed that some surface gE had been internalized (f). Further experiments to demonstrate recycling of internalized gE were performed by treating the cells with trypsin to remove all surface gE remaining after incubation at 37°C (c). Some of these cells were fixed and incubated with FITC conjugate (g). Since the cells were not permeabilized, no secondary antibody could enter the cell to label internalized protein. As expected, no surface gE was present when the cells were analyzed by microscopy (g). Posttrypsinization, the remaining cultures were incubated at 37°C for various intervals (d). Recycling back to the cell surface was demonstrated by observing gE on the surface after these cells were fixed and incubated with secondary antibody-FITC conjugate (h).

the protein observed on the surface of both infected and transfected cells (17, 18, 25, 26).

ACKNOWLEDGMENTS

This research was supported by USPHS grant AI22795.

We thank I. S. Trowbridge (Salk Institute) and John Kemp (University of Iowa) for supplying reagents used in this report.

REFERENCES

1. **Ashworth, R., R. Yu, E. J. Nelson, S. Dermer, M. C. Gershengorn, and P. M. Hinkle.** 1995. Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling. Proc. Natl. Acad. Sci. USA **92:**512–516.
2. **Bretscher, M. S., J. N. Thompson, and B. M. F. Pearse.** 1980. Coated pits act as molecular filters. Proc. Natl. Acad. Sci. USA **77:**4156–4159.
3. **Card, J. P., and L. W. Enquist.** 1995. Neurovirulence of pseudorabies virus. Crit. Rev. Neurobiol. **9:**137–162.
4. **Collawn, J. F., L. A. Kuhn, L. S. Liu, J. A. Tainer, and I. S. Trowbridge.** 1991. Transplanted LDL and mannose 6-phosphate receptor internalization signals promote high-efficiency endocytosis of the transferrin receptor. EMBO J. **10:**3247–3253.
5. **Collawn, J. F., M. Stangel, L. A. Kuhn, V. Esekogwu, S. Jing, I. S. Trowbridge, and J. A. Trainer.** 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. Cell **63:**1061–1072.
6. **Davis, C. G., I. R. van Driel, D. W. Russel, M. S. Brown, and J. L. Goldstein.** 1987. The low density lipoprotein receptor. J. Biol. Chem. **262:**4075–4082.
7. **Davis, C. G., M. A. Lehrman, D. W. Russell, R. G. W. Anderson, M. S. Brown, and J. L. Goldstein.** 1986. The J.D. mutation in familial hypercholesterolemia: amino acid substitutions in the cytoplasmic domain impedes internalization of LDL receptors. Cell **45:**15–24.
8. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67:**1759–1816.
9. **De Camilli, P.** 1995. The eighth Datta lecture. Molecular mechanisms in synaptic vesicle recycling. FEBS Lett. **369:**3–12.
10. **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.
11. **Eagen, M. A., L. M. Carruth, J. F. Rowell, X. Yu, and R. F. Siliciano.** 1996. Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the $Pr55^{gag}$ precursor protein. J. Virol. **70:**6547–6556.
12. **Enquist, L. W.** 1994. Infection of the mammalian nervous system by pseudorabies virus (PRV). Semin. Virol. **5:**221–231.
13. **Ghazizadeh, S., and H. B. Fleit.** 1994. Tyrosine phosphorylation provides an obligatory early signal for FcγRII-mediated endocytosis in the monocytic cell line THP-1. J. Immunol. **152:**30–41.
14. **Ghosh, R. N., D. L. Gelman, and F. R. Maxfield.** 1994. Quantification of low density lipoprotein and transferrin endocytic sorting in HEp2 cells using confocal microscopy. J. Cell Sci. **107:**2177–2189.
15. **Ghosh, R. N., and F. R. Maxfield.** 1995. Evidence for nonvectorial retrograde transferrin trafficking in the early endosomes of HEp2 cells. J. Cell Biol. **128:**549–561.
16. **Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider.** 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. **1:**1–39.
17. **Grose, C.** 1990. Glycoproteins encoded by varicella-zoster virus: biosynthesis, phosphorylation, and intracellular trafficking. Annu. Rev. Microbiol. **44:**59–80.
18. **Harson, R., and C. Grose.** 1995. Egress of varicella-zoster virus from the melanoma cell: a tropism for the melanocyte. J. Virol. **69:**4994–5010.
19. **Hatfield, C., K. M. Duus, D. H. Jones, and C. Grose.** 1997. Epitope mapping and tagging by recombination PCR mutagenesis. BioTechniques **22:**332–337.
20. **Hathaway, G. M., and J. A. Traugh.** 1982. Casein kinases—multipotential protein kinases. Curr. Top. Cell. Regul. **21:**101–127.
21. **Kishimoto, A., M. S. Brown, C. A. Slaughter, and J. C. Goldstein.** 1987. Phosphorylation of serine 833 in cytoplasmic domain of low density lipoprotein receptor by a high molecular weight enzyme resembling casein kinase II. J. Biol. Chem. **262:**1344–1351.
22. **LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, M. Marsh, and J. A. Hoxie.** 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J. Virol. **69:**5217–5227.
23. **Lazarovits, J., and M. Roth.** 1988. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. Cell **53:**743–752.
24. **Lehrman, M. A., J. L. Goldstein, M. S. Brown, D. W. Russell, and W. J. Schneider.** 1985. Internalization-defective LDL receptors produced by genes with nonsense and frameshift mutations that truncate the cytoplasmic domain. Cell **41:**735–743.
25. **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.
26. **Litwin, V., M. Sandor, and C. Grose.** 1990. Cell surface expression of varicella-zoster virus glycoproteins and Fc receptor. Virology **178:**263–272.
27. **Mauxion, F., R. LeBorgne, H. Munier-Lehmann, and B. Hoflack.** 1996. A casein kinase II phosphorylation site in the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor determines the high affinity interactions of the AP-1 Golgi assembly proteins with membranes. J. Biol. Chem. **271:**2171–2178.
28. **McClain, D. A., H. Maegwa, J. Lee, J. Dull, and A. Ullrich.** 1987. A mutant insulin receptor with defective tyrosine kinase displays no biological activity and does not undergo endocytosis. J. Biol. Chem. **262:**14663–14667.
29. **McGeoch, D. J., S. Cook, A. Dolan, F. E. Jamieson, and E. A. R. Telford.** 1995. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J. Mol. Biol. **247:**443–458.
30. **McGraw, T. E., and F. R. Maxfield.** 1991. Internalization and sorting of macromolecules: endocytosis, p. 11–41. *In* R. L. Juliano (ed.), Targeted drug delivery. Springer-Verlag, Berlin, Germany.
31. **Miettinen, H. M., J. K. Rose, and I. Mellman.** 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic

domain heterogeneity. Cell **58:**317–327.
32. **Montalvo, E. A., R. T. Parmley, and C. Grose.** 1985. Structural analysis of the varicella-zoster virus gp98-gp62 complex: posttranslational addition of N-linked and O-linked oligosaccharide moieties. J. Virol. **53:**761–770.
33. **Moss, B., O. Elroy-Stein, T. Mijukani, W. A. Alexander, and T. R. Fuerst.** 1990. New mammalian expression vector. Nature (London) **348:**91–92.
34. **Mostov, K. E., J. Casanova, and P. Brisfeld.** 1988. Trancytosis and sorting of polymeric immunoglobulin receptor. J. Cell Biol. **107:**439.
35. **Mostov, K. E., A. de Bruyn Kops, and D. L. Deitcher.** 1986. Deletion of the cytoplasmic domain of the polymeric immunoglobulin receptor prevents basolateral localization and endocytosis. Cell **47:**359–364.
36. **Ohno, H., J. Stewart, M.-C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J. S. Bonifacino.** 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science **269:**1872–1875.
37. **Olson, J. K., G. A. Bishop, and C. Grose.** 1997. Varicella-zoster virus Fc receptor gE glycoprotein: serine/threonine and tyrosine phosphorylation of monomeric and dimeric forms. J. Virol. **71:**110–119.
38. **Prywes, R., E. Livneh, A. Ullrich, and J. Schlessinger.** 1986. Mutations in the cytoplasmic domain of the polymeric immunoglobulin receptor prevents basolateral localization and endocytosis. Cell **47:**359–364.
39. **Roth, M. G., C. Doyle, J. Sambrook, and M.-J. Gething.** 1986. Heterologous transmembrane and cytoplasmic domains direct functional chimeric influenza virus hemagglutinins into the endocytic pathway. J. Cell Biol. **102:**1271–1283.
40. **Rothenberger, S., B. J. Iacopetta, and L. C. Kuhn.** 1987. Endocytosis of the transferrin receptor requires the cytoplasmic domain but not its phosphorylation site. Cell **49:**423–431.
41. **Rowell, J. F., P. E. Stanhope, and R. F. Siliciano.** 1995. Endocytosis of endogenously synthesized HIV-1 envelope protein. J. Immunol. **155:**473–488.
42. **Sandoval, I. V., and O. Bakke.** 1994. Targeting of membrane proteins to endosomes and lysosomes. Trends Cell Biol. **4:**292–297.
43. **Sauter, M. M., A. Pelchen-Mathews, R. Bron, M. Marsh, C. C. LaBranche, P. J. Vance, T. Romano, B. S. Haggarty, T. K. Hart, W. M. F. Lee, and J. A. Hoxie.** 1996. An internalization signal in the simian immunodeficiency virus transmembrane protein cytoplasmic domain modulates expression of envelope glycoproteins on the cell surface. J. Cell Biol. **132:**795–811.
44. **Schmid, S. L.** 1992. The mechanisms of receptor-mediated endocytosis: more questions than answers. Bioessays **14:**589–596.
45. **Schwartz, A. L.** 1995. Receptor cell biology: receptor-mediated endocytosis. Pediatr. Res. **38:**835–843.
46. **Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Bechman, R. Jerzey, S. K. Dower, D. Cosman, and R. G. Goodwin.** 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science **248:**1019–1023.
47. **Stoorvogel, W., V. Oorschot, and H. J. Geuze.** 1996. A novel class of clathrin-coated vesicles budding from the endosomes. J. Cell Biol. **132:**21–33.
48. **Trowbridge, I. S., J. F. Collawn, and C. R. Hopkins.** 1993. Signal-dependent trafficking in the endocytic pathway. Annu. Rev. Cell Biol. **9:**129–161.
49. **Trowbridge, I. S.** 1991. Endocytosis and signals for internalization. Curr. Opin. Cell Biol. **3:**634–641.
50. **White, S., R. Taetle, P. A. Seligman, M. Rutherford, and I. S. Trowbridge.** 1990. Combinations of anti-transferrin receptor monoclonal antibodies inhibit human tumor cell growth in vitro and in vivo: evidence for synergistic antiproliferative effects. Cancer Res. **50:**6295–6301.
51. **Wileman, Y., C. Harding, and P. Stahl.** 1985. Receptor-mediated endocytosis. Biochem. J. **232:**1–14.
52. **Yao, Z., W. Jackson, and C. Grose.** 1993. Identification of the phosphorylation sequence in the cytoplasmic tail of the varicella-zoster virus Fc receptor glycoprotein gpI. J. Virol. **67:**4464–4473.
53. **Yao, Z., D. H. Jones, and C. Grose.** 1992. Site-directed mutagenesis of herpesvirus glycoprotein phosphorylation sites by recombination polymerase chain reaction. PCR Methods Appl. **1:**205–207.
54. **Zhu, Z., Y. Hao, M. Gershon, R. T. Ambron, and A. A. Gershon.** 1996. Targeting of glycoprotein I (gE) of varicella-zoster virus to the trans-Golgi network by an AYRV sequence and an acidic amino acid-rich patch in the cytosolic domain of the molecule. J. Virol. **70:**6563–6575.