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Golgi Complex Localization of the Punta Toro Virus G2 Protein Requires Its Association with the G1 Protein

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The glycoproteins of bunyaviruses accumulate in membranes of the Golgi complex, where virus maturation occurs by budding. In this study we have constructed a series of full length or truncated mutants of the G2 glycoprotein of Punta Toro virus (PTV), a member of the Phlebovirus genus of the Bunyaviridae, and investigated their transport properties. The results indicate that the hydrophobic domain preceding the G2 glycoprotein can function as a translocational signal peptide, and that the hydrophobic domain near the C-terminus serves as a membrane anchor. A G2 glycoprotein construct with an extra hydrophobic sequence derived from the N-terminal NS_M region was stably retained in the ER, and was unable to be transported to the Golgi complex. The full-length G2 glycoprotein, when expressed on its own, was transported out of the ER and expressed on the cell surface, whereas the G1 and G2 proteins when expressed together are retained in the Golgi complex. A truncated anchor-minus form of the G2 glycoprotein was found to be secreted into the culture medium, but was retained in the Golgi complex when coexpressed with the G1 glycoprotein. These results indicate that the G2 membrane glycoprotein is a class I membrane protein which does not contain a signal sufficient for Golgi retention, and suggest that its Golgi localization is a result of association with the G1 glycoprotein.

INTRODUCTION

A central problem in cell biology is to determine how membrane proteins are sorted and targeted to various subcellular compartments. Recent evidence supports the view that selective transport of proteins from the endoplasmic reticulum (ER) to the cell surface in the central vacuolar system could simply occur by bulk flow: according to this model, after folding and assembly of secretory and membrane proteins into their native 3-dimensional structure, they are transported out of the ER to the plasma membrane, and only proteins which have specific retention signals are retained in intracellular compartments (Klausner, 1989; Pelham, 1988; Rose and Doms, 1988; Rothman, 1987; Wieland *et al.*, 1987). Assembly of enveloped viruses of several families, e.g., coronaviruses, bunyaviruses, and flaviviruses, takes place by budding at intracellular membranes, and the corresponding viral glycoproteins accumulate intracellularly rather than being transported to the cell surface (Leary and Blair, 1980; Matsuoka *et al.*, 1988; Murphy *et al.*, 1973; Tooze and Tooze, 1985). Machamer and Rose (1987) found that the first of three hydrophobic membrane-spanning domains of the coronavirus E1 glycoprotein, a Golgi membrane protein, is required for its retention. The NH_2-terminal sequence of a trans Golgi resident membrane protein, β-galactoside α-2,6-sialyltransferase, was found to be involved in Golgi localization (Colley *et al.*, 1989). Portions of the sequences of several ER resident membrane proteins were also recently shown to be required for their retention (Jackson *et al.*, 1990; Kuroki *et al.*, 1989; Nilsson *et al.*, 1989; Poruchynsky *et al.*, 1985; Stirzaker and Both, 1989). However, the precise structure features of the retention signals and the mechanism for the intracellular retention of resident membrane proteins of the ER and Golgi complex are still unknown.

The glycoproteins of bunyaviruses are model Golgi membrane proteins, since the viruses are formed by budding at the smooth membranes of the Golgi complex (Murphy *et al.*, 1973; Smith and Pifat, 1982) and the viral glycoproteins accumulate in the Golgi complex during virus infection (Chen *et al.*, 1991; Gahmberg *et al.*, 1986; Kuismanen *et al.*, 1982, 1984; Madoff and Lenard, 1982) as well as when expressed by recombinant vaccinia viruses (Matsuoka *et al.*, 1988; Pensiero *et al.*, 1988; Pettersson *et al.*, 1988; Wasmoen *et al.*, 1988). Punta Toro virus (PTV) is a member of the phlebovirus genus of the family Bunyaviridae (Bishop *et al.*, 1980). Its structural components include three single-stranded RNA genomic segments, L, M, and S, and three major structural proteins, a nucleoprotein, N, and two major membrane glycoproteins, G1 and G2. These glycoproteins are encoded by the M genomic segment and are translated from a single

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mRNA as a precursor glycoprotein and cleaved cotranslationally into individual proteins (Eshita *et al.*, 1985; Schmaljohn and Patterson, 1990; Suzich and Collett, 1988).

The gene product order, NH_2–NS_M (nonstructural protein)–G1–G2–COOH, of the PTV M RNA segment has been determined by N-terminal amino acid sequence analysis (Ihara *et al.*, 1985). One potential N-linked glycosylation site (Asn–X–Ser/Thr) in the G1 protein and four in the G2 protein were found in the sequence (Ihara *et al.*, 1985). The predicted amino acid sequence of the polyprotein precursor of PTV reveals that distinct hydrophobic regions precede both the G1 and G2 proteins. There are also hydrophobic domains near the carboxy termini of the G1 and G2 proteins, followed by charged amino acids, similar to the "stop transfer" sequences seen in the transmembrane domains of other viral envelope proteins (Garoff *et al.*, 1980; Ihara *et al.*, 1985; Rose *et al.*, 1980). This suggests that each of the two glycoproteins encoded in the polyprotein precursor may have its own signal sequence and membrane anchor sequence, analogous to the internal signal sequence and membrane anchor sequences described for the E1 protein of the alphaviruses, Semliki forest virus, and Sindbis virus (Garoff *et al.*, 1980; Hashimoto *et al.*, 1981; Rice and Strauss, 1981). However, virtually no direct evidence is available about the sequences responsible for targeting of the nascent PTV proteins to the ER membrane or the domains responsible for membrane anchorage. The PTV G1 and G2 glycoproteins, when expressed from a recombinant vaccinia virus, are specifically retained in the Golgi complex (Matsuoka *et al.*, 1988; Chen *et al.*, 1991). The NS_M sequence was ruled out as a retention signal for the glycoproteins, indicating that the structural features of the G1 and/or G2 glycoproteins are responsible for their Golgi retention.

To investigate the molecular basis for ER translocation, membrane anchorage and Golgi retention of the PTV G2 glycoprotein, we constructed a series of full-length and truncated G2 glycoprotein mutants. In the present paper, we have investigated the transport properties of the resulting molecules using vaccinia virus-based expression systems.

MATERIAL AND METHODS

Materials

BHK, CV-1, HeLa, HeLa $T4^+$, TK^-143, and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum (in the presence of BUdR for TK^-143 cells). A recombinant pGEM-G containing the PTV G1 and G2 coding sequence under control of a T7 promoter was described previously (Matsuoka *et al.*, 1988). PTV was obtained from USAMRIID (Frederick, MD) and passaged in Vero cells. Polyclonal and monoclonal antibodies against PTV G1 and G2 glycoproteins were generously supplied by Drs. J. F. Smith and D. Pifat (USAMRIID, Frederick, MD). The monoclonal antibody to immunoglobulin heavy chain binding protein (BiP) (Bole *et al.*, 1986) was kindly supplied by Dr. L. M. Hendershot. Oligonucleotides were synthesized by the UAB core facility and purified by HPLC. GeneAmp DNA amplification kits with AmpliTaq recombinant DNA polymerase were purchased from Perkin–Elmer–Cetus (Norwalk, CT). Lipofectin was purchased from BRL Corp. (Bethesda, MD). Geneclean kits were purchased from Bio 101 Corp. (La Jolla, CA). Plasmids pGEM-3Z and pGEM-4Z were purchased from Promega Corp. (Madison, WI). Restriction endonucleases were purchased from BMB Corp. (Indianapolis, IN).

Construction of G2 recombinants

All DNA manipulation was carried out as described by Maniatis *et al.* (1986). To construct the G2 recombinant designated as pGEM-G2(SH) (Fig. 1), the pGEM-M plasmid (Matsuoka *et al.*, 1988) was linearized by digestion with *Stu*I and partially digested with *Hind* II. A 5.8-kb fragment was recovered by gel-purification, treated with Klenow DNA polymerase, and then religated. The G2 insert of the resulting recombinant was then cut by *Pst*I and *Eco*RI, recovered by gel purification, and finally subcloned into *Pst*I and *Eco*RI-cut pGEM-4Z under control of the T7 promoter. To construct the G2 recombinant designated as pGEM-G2(HH), pGEM-M was partially digested with *Hin*cII, and a 5.6-kb fragment was gel-purified and religated. To construct the G1-truncated G2 recombinant designated as pGEM-G(A−), the plasmid pGEM-G (Matsuoka *et al.*, 1988) was cut at an *Xba*I site located just before the 19 amino acid hydrophobic sequence which is the presumed membrane anchor, in addition to two *Xba*I sites in the G1 sequence and polylinker of the vector. Two DNA fragments were recovered from a low-melting agarose gel, purified with a Geneclean kit, and then religated. The recombinant which lacked the coding region (45 amino acids), for the G2 hydrophobic 19 amino acids and C-terminal sequences was then cut at a *Sal*I site next to an *Xba*I site in the polylinker. The ends were then filled in by the Klenow fragment of DNA polymerase, and ligated with an *Hpa*I linker. The colonies were screened by dot hybridization with [γ^{32}P]ATP terminal labeled *Hpa* I linkers, and the plasmid DNA in positive colonies was further checked by restriction enzyme analyses. The resulting recombinant contained an in-frame TAA sequence, and the

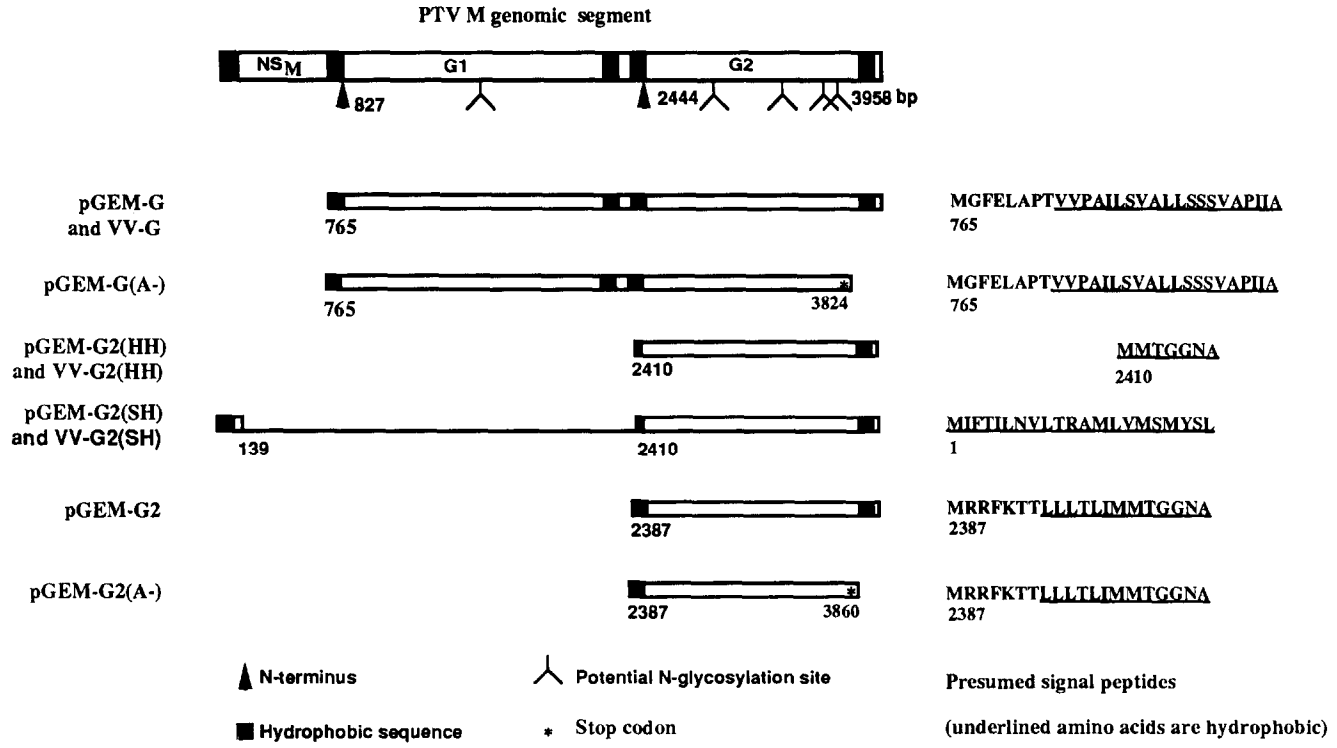

FIG. 1. Molecular organization of the G1 and G2 glycoprotein coding sequences in the PTV M genomic segment and in mutants, and predicted amino acid sequences preceding the G1 or G2 glycoprotein in the mutants. The underlined amino acids are hydrophobic. The N-termini of the G1 and G2 proteins were determined by Ihara *et al.* (1985).

insert DNA was located downstream of the T7 promoter sequence in plasmid pGEM-3.

To construct the G2 recombinant designated as pGEM-G2, an upstream primer (P-1,5′-GGGAATTC*ATG*AGAAGATTCAAAACAACT-3′), containing an *Eco*RI site followed by the sequence of nucleotides 2387 to 2419 of the PTV M genomic RNA, and a downstream primer (5′-CACTGCAG*TCA*GTTTTTCTTCTTGATATT-3′), containing the sequence of nucleotides 3941 to 3961 followed by a *Pst*I site, were used to amplify the G2 DNA fragment by the polymerase chain reaction (PCR). The amplified DNA fragment containing an internal ATG, a putative signal peptide, and G2 coding sequence was then cut by *Eco*RI and *Pst*I, purified by a Geneclean kit, and ligated into *Eco*RI and *Pst*I-cut pGEM-3Z. To construct the truncated G2 recombinant designated as pGEM-G2(A-), the upstream primer P-1, and a downstream primer (5′-CAAAGC*TTA*CTTTAGTATGGCCTTCATAGG-3′), containing the sequence of nucleotides 3839 to 3860 of the PTV M RNA followed by a stop Codon TAA, were used to amplify the DNA fragment by PCR. The amplified DNA fragment was cut by *Eco*RI, and then ligated into *Eco*RI and *Sma*I-cut pGEM-3Z. The nucleotide sequence of the PCR-amplified DNA products in pGEM-3Z was analyzed by restriction endonucleases and confirmed by Sanger's dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using a sequenase DNA sequence kit (United States Biochemical Corp., Cleveland, OH). The SP6 promoter and T7 promoter primers (Promega Biotech., Madison, WI) were used for sequencing by the dideoxy method.

PCR amplification

Selected DNA fragments of the PTV M genomic segment were amplified by PCR with the plasmid pGEM-G as template DNA (Saiki *et al.*, 1988), in a DNA thermal cycler (Perkin–Elmer–Cetus, Norwalk, CT) using the following conditions: upstream and downstream primers were 30 p*M* each in final concentration, dNTP were 100 μM each, template plasmid DNA was 100 ng, *Taq* DNA polymerase was 2.5 units, other reaction conditions were used according to the GeneAmp DNA amplification kit instructions in a total volume of 100 μl. The reaction mixtures were overlayed with 75 μl of mineral oil, and the reactions were carried out for 25 cycles. Each cycle included a heat denaturation step at 92° for 1 min, followed by annealing of primers to the DNA template at 50° for 1 min, and DNA chain extension with *Taq* polymerase at 72° for 2 min. After the reaction was completed, the PCR products were purified with a Geneclean kit.

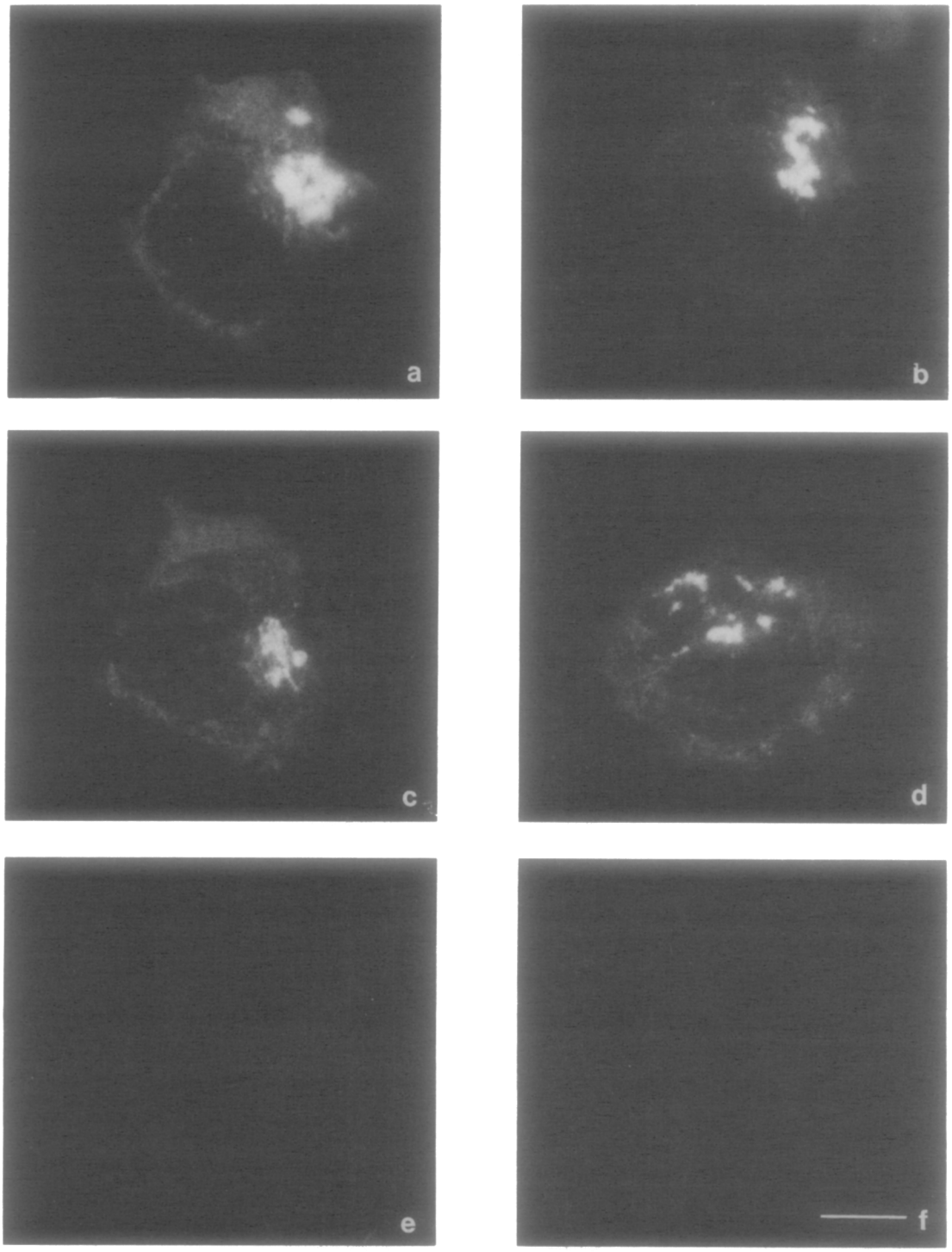

FIG. 2. Localization of the G1 and G2 glycoproteins expressed by pGEM-G in a T7 transient expression system. For expression of the pGEM-G recombinant, HeLa T4$^+$ cells grown on coverslips were infected with VV-T7 for 30 min, transfected with 10 μg of pGEM-G plasmid DNA mixed with lipofectin, and then incubated for 12 hr. For expression of the VV-G recombinant, HeLa T4$^+$ cells were infected with VV-G and incubated for 12 hr. Cells were incubated with cycloheximide (50 μg/ml) at 37° for 4 hr, fixed, and then stained with Mabs against G1 or G2, or with polyclonal antibody to PTV, followed with fluorescein-labeled goat anti-mouse IgG. a. G1 glycoprotein localization in VV-G infected cells. b. G1 glycoprotein localization in pGEM-G transfected cells c. G2 glycoprotein localization in VV-G infected cells. d. G2 glycoprotein localization in pGEM-G transfected cells. e. Cell surface staining for G1 and G2 glycoproteins in VV-G infected cells. f. Cell surface staining for G1 and G2 glycoproteins in pGEM-G transfected cells. (Bar = 3.5 μm.)

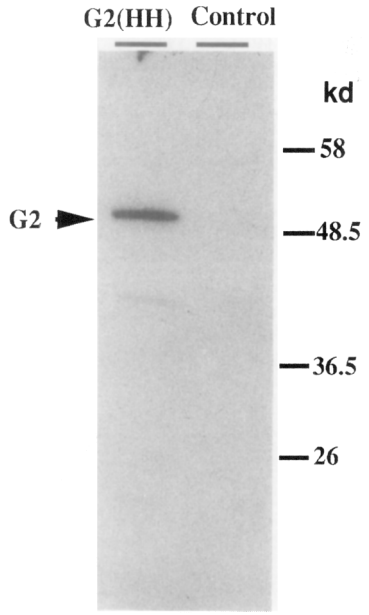

FIG. 3. Immunoprecipitation analysis of the cell-free translation products synthesized from the recombinant pGEM-G2(HH) *in vitro*. The recombinant pGEM-G2(HH) plasmid DNA was linearized and RNA was synthesized using SP6 polymerase. The SP6-G2 transcripts or control samples without RNA transcripts were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. The protein products were immunoprecipitated with polyclonal antibody to PTV and analyzed by SDS–PAGE. The molecular weight markers were pyruvate kinase, 58 kDa; fumarase, 48.5 kDa; lactic dehydrogenase, 36.5 kDa; and triosephosphate isomerase, 26.6 kDa.

Construction and expression of recombinant vaccinia viruses

To construct the vaccinia recombinant viruses designated as VV-G2(SH) and VV-G2(HH) (Fig. 1), the G2 glycoprotein coding sequence was excised from pGEM-G2 (SH) by *Sma*I digestion or from pGEM-G2(HH) by *Sma*I–*Hin*cII digestion, ends filled in with the Klenow DNA polymerase, inserted into the *Sma*I site of pSC11, and then checked for orientation (Chakrabarti *et al.*, 1985). For isolation of vaccinia recombinants, CV-1 cells were infected with vaccinia virus (strain IHD-J) at an m.o.i. of 0.05. At 2 hr p.i., cells were transfected with a calcium phosphate precipitate of 10 μg of a pSC11 recombinant plasmid and 15 μg of salmon sperm DNA/ml in HEPES-buffered saline (Graham and Van der Eb, 1973). To select recombinants, TK$^-$143 cells were infected with 50–100 PFU of virus in the presence of BUdR (25 μg/ml). At 48 p.i. the monolayers were overlayered with 1% low-melting agarose containing 300 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase (X-Gal), and blue plaques were picked and purified by several rounds of plaque purification. Stocks of recombinant viruses were prepared in CV-1 cells, and titrated in TK$^-$143 cells. For vaccinia recombinant virus expression, HeLa T4$^+$ cells, which are relatively resistant to cytopathic effects of vaccinia virus (Matsuoka *et al.*, 1988), were infected with recombinant vaccinia virus with an m.o.i. of 10 for 30 min and incubated for 16 hr before radiolabeling and immunoprecipitation.

T7 polymerase transient expression

A recombinant vaccinia virus containing the T7 RNA polymerase gene (VV-T7) was obtained from Dr. B. Moss (Fuerst *et al.*, 1986). HeLa T4$^+$ cells were infected with VV-T7 at an m.o.i. of 20 for 30 min and washed with PBS three times, and then 10 μg of recombinant plasmid DNA mixed with 50 μl of water and 50 μl of lipofectin was added into the serum-free DMEM medium of the infected cells and incubation continued for 12 hr before radiolabeling and immunoprecipitation.

In vitro transcription and translation

Recombinant plasmid DNA under control of the SP6 promoter (4.0 μg) was linearized by *Kpn*I digestion, and was then incubated at 37° for 2 hr in a reaction mixture containing 100 m*M* dithiothreitol, 2.5 m*M* ATP, 2.5 m*M* GTP, 2.5 m*M* UTP, 2.5 m*M* CTP, 1 unit RNasin (Promega Biotech., Madison, WI), and 10 units SP6 polymerase (Promega Biotech., Madison, WI) in a final volume of 50 μl. The reaction was terminated by the addition of 1 unit of RQ 1 DNase (Promega Biotech., Madison, WI) at 37° for 10 min. The mixture was extracted with phenol/chloroform, before being precipitated with ethanol. RNA transcripts (1 μg) were incubated at 30° for 90 min with 17.5 μl of rabbit reticulocyte lysate (Promega Biotech., Madison, WI), 0.5 m*M* of each amino acid except for methionine, and 25 μCi [^{35}S]methionine/cysteine in final volume of 25 μl. The translation product was immunoprecipitated and analyzed by SDS–PAGE.

Labeling of cells and precipitation of viral proteins

Transfected or infected cells were washed once with phosphate-buffered saline (PBS) at indicated time points postinfection or transfection and incubated in methionine-free medium for 3 hr. Cells were then labeled with [^{35}S]methionine/cysteine (100 μCi/ml) in methionine-free medium for 15 min and chased in Eagle's medium containing 10 m*M* methionine for indicated periods. For surface immunoprecipitation, these cells were incubated with polyclonal antibody against PTV diluted in PBS for 60 min in 4°, washed three times in ice-cold PBS, and lysed with 0.3 ml of cell lysis buffer (50 m*M* Tris–HCl (pH 7.5), 0.15 *M* NaCl, 1% Triton X-100, 0.1% SDS, 20 m*M* EDTA). Nuclei were removed by centrifugation at 13,000 *g* for

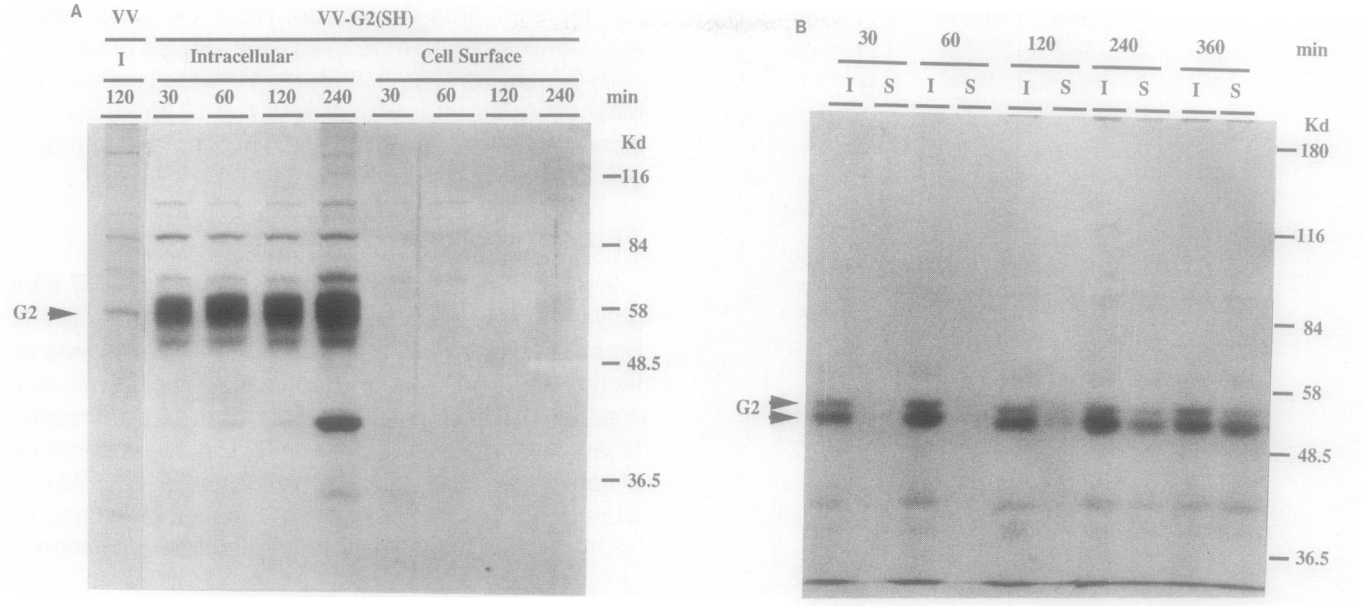

FIG. 4. Comparison of the transport of the G2 glycoprotein expressed by VV-G2(SH) or pGEM-G2. For expression of the VV-G2(SH) recombinant, HeLa T4$^+$ cells were infected with VV-G2(SH) or wild-type vaccinia virus (VV) and incubated for 16 hr. For pGEM-G2 recombinant expression, HeLa T4$^+$ cells infected with recombinant VV-T7 were transfected with 10 μg of pGEM-G plasmid DNA in lipofectin and incubated for 16 hr. HeLa T4$^+$ cells were then labeled with [^{35}S]methionine for 15 min at 37°, and chased in the presence of excess methionine for indicated times. The G2 protein was immunoprecipitated from the cell lysate or cell surface, and then analyzed by SDS–PAGE. A. Intracellular (I) and cell surface immunoprecipitation of the G2 protein expressed by VV-G2(SH). B. Intracellular (I) and cell surface (S) immunoprecipitation of the G2 protein expressed by pGEM-G2.

5 min at 4°. For immunoprecipitation of intracellular proteins, cells were lysed, and monoclonal or polyclonal antibodies were then added to supernatants of the cell lysates and incubated at 37° for 90 min or at 4° overnight. All samples were incubated with Protein A–Sepharose CL-4B at 37° for 90 min or at 4° overnight (Pharmacia Inc. Piscataway, NJ). The precipitates were pelleted, washed three times with cold lysis buffer, resuspended in Laemmli sample buffer with or without β-mercaptoethanol and analyzed by SDS–PAGE (Laemmli, 1970).

Endo H digestion

Immunoprecipitated samples were resuspended in 200 μl of 0.1 *M* sodium acetate, pH 5.5, and then divided into two equal aliquots. Samples were incubated with 8 mU of endo H/ml or without endo H for 16 hr at 37°, and then centrifuged at 15,000*g* for 5 min (Kuismanen, 1984). The precipitates were resuspended in Laemmli sample buffer, boiled for 5 min, and then analyzed by SDS–PAGE.

Indirect immunofluorescence

HeLa T4$^+$ cells grown on glass coverslips were infected with vaccinia virus at an m.o.i. of 10, or VV-T7 at an m.o.i. of 20 following transfection with recombinant plasmid DNA. At indicated times post-infection, cells were washed with PBS and fixed with ethanol containing 5% acetic acid for 10 min at −20° for intracellular immunofluorescence or with 1% formaldehyde for 5 min at room temperature for surface immunofluorescence. Cells were then washed with PBS and reacted with monoclonal or polyclonal antibodies at 37° for 30 min followed by a fluorescein-conjugated goat anti-mouse IgG incubation. After a final washing, coverslips were mounted and observed by a Nikon Optiphot microscope equipped with a modified B2 tube.

RESULTS

Localization of PTV membrane glycoproteins expressed using a T7 transient expression system

We used a vaccinia T7 transient expression system to compare PTV glycoprotein localization with that observed with a recombinant vaccinia virus in several cell lines. This transient expression system is based on infection of the cells with a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase and subsequent transfection with the plasmid DNA containing the gene to be expressed under control of the T7 promoter. Transcription of the gene is mediated by the T7 polymerase produced in the cytoplasm by the recombinant vaccinia virus (Fuerst *et al.*, 1986). We

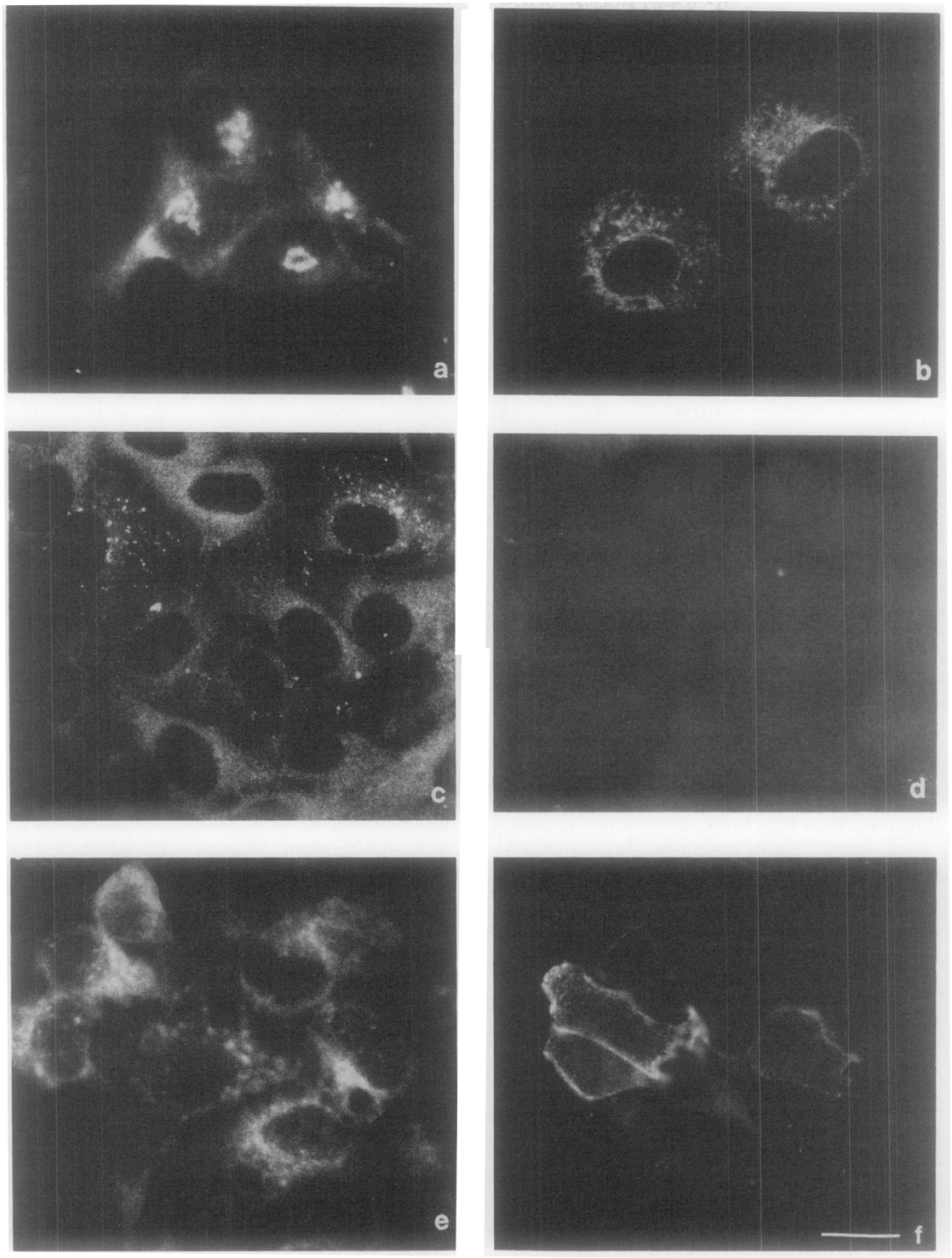

FIG. 5. Localization of the G2 glycoproteins expressed by VV-G2(SH) and pGEM-G2. For vaccinia recombinant expression, HeLa $T4^+$ cells grown on coverslips were infected with VV-G2(SH) or VV-G and incubated for 16 hr, after which cells were incubated with cycloheximide (50 μg/ml) at 37° for 4 hr prior to fixation. For expression of pGEM-G2, HeLa $T4^+$ cells infected with VV-T7 were transfected with 5 μg of pGEM-G plasmid DNA mixed with lipofectin and incubated for 16 hr. Cells were then fixed, and stained with Mabs against G1 or G2, followed with fluorescein-labeled goat anti-mouse IgG. a. G2 glycoproteins in VV-G-infected cells after cycloheximide treatment. b. Anti-BiP staining in HeLa $T4^+$ cells. c. G2 glycoprotein in VV-G2(SH)-infected cells after cycloheximide treatment. d. Cell surface staining for G2 glycoproteins on VV-G2(SH)-infected cells. e. G2 glycoprotein in pGEM-G2-transfected cells. f. G2 glycoprotein on the cell surface of pGEM-G2-transfected cells. (Bar = 4.5 μm.)

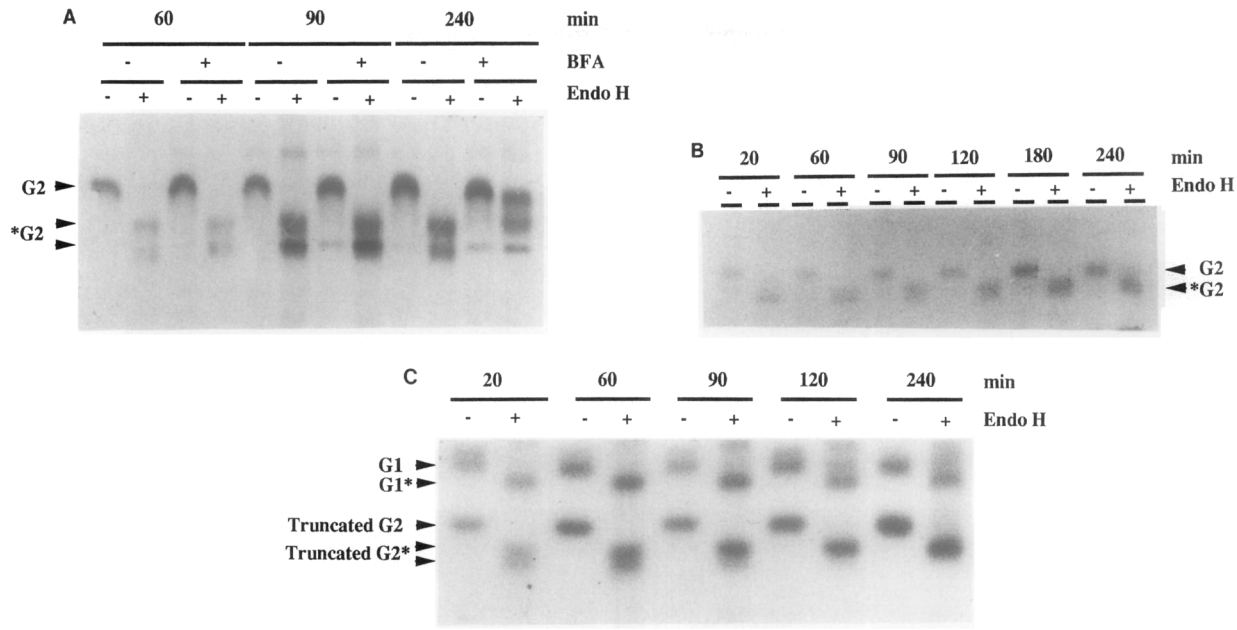

FIG. 6. Processing of glycoproteins expressed from recombinants. HeLa T4$^+$ cells were infected or transfected as described for Fig. 4. At 12 hr, cells were pulse-labeled with [^{35}S]methionine for 15 min at 37°, and then chased in the presence of excess methionine for the indicated times. The cells were lysed, and the PTV glycoproteins were immunoprecipitated with polyclonal antibodies to PTV. The precipitated samples were divided into two parts; one was treated with endo H (+), another not treated with endo H (−) and both were incubated at 37° for 16 hr. The samples were then analyzed by SDS–PAGE under reducing conditions. A. G2 protein from VV-G2(SH)-infected cells which were chased in the presence or absence of BFA (10 μg/ml). B. G2 protein expressed by pGEM-G2. C. G1 and truncated G2 proteins expressed by pGEM-G(A−). *G1 and *G2 indicate the endo H-sensitive forms of the proteins.

used a modification of the system by cloning the genes of interest into pGEM-3, pGEM-3Z, or pGEM-4Z plasmids under the control of a T7 promoter, but lacking a transcriptional termination sequence (Pattnaik and Wertz, 1990). The pGEM-G plasmid, which contains the PTV G1 and G2 coding sequence under control of the T7 promoter, was initially used to investigate the expression and localization of the glycoproteins (Fig. 1). It was found that the G1 and G2 glycoproteins were expressed, cleaved, and glycosylated in the transfected cells as detected by immunoprecipitation and SDS–PAGE (not shown), indicating that expression and processing of the G1 and G2 proteins occurred efficiently using the T7 expression system. HeLa T4$^+$ cells retained relatively normal morphology for over 24 hr post-transfection while expressing high levels of the G1 and G2 glycoproteins. The localization of the G1 and G2 proteins was found to be in a perinuclear Golgi pattern, as observed previously in vaccinia recombinant VV-G-infected cells (Fig. 2). Furthermore, a similar pattern of Golgi localization of the proteins was also detected in the Vero, HeLa, or BHK cell lines when the proteins were expressed by the T7 transient system at early time points post-transfection. Thus, the Golgi targeting of PTV glycoproteins occurs using various cell types and expression systems, and either the T7 transient expression system or the vaccinia recombinant system can be used to study the targeting of glycoprotein constructs.

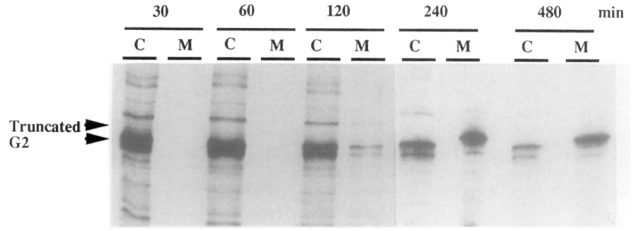

FIG. 7. Secretion of a truncated G2 glycoprotein expressed by pGEM-G2(A−). HeLa T4$^+$ cells infected with VV-T7 were transfected with 10 μg of pGEM-G2 (A−) plasmid DNA in lipofectin, incubated for 12 hr, and then labeled with [^{35}S]methionine for 30 min at 37°. The cells were then chased in the presence of excess methionine for indicated times. The truncated G2 protein was immunoprecipitated from the medium (M) and cell lysate (C), and immunoprecipitates were analyzed by SDS-PAGE under reducing conditions (in a mini-protean II gel apparatus, BioRad Corp. Richmond, CA).

Signal peptide requirement for the G2 glycoprotein

To determine whether the hydrophobic domain preceding the G2 protein is required as a translocational signal peptide, the recombinant pGEM-G2(HH), which

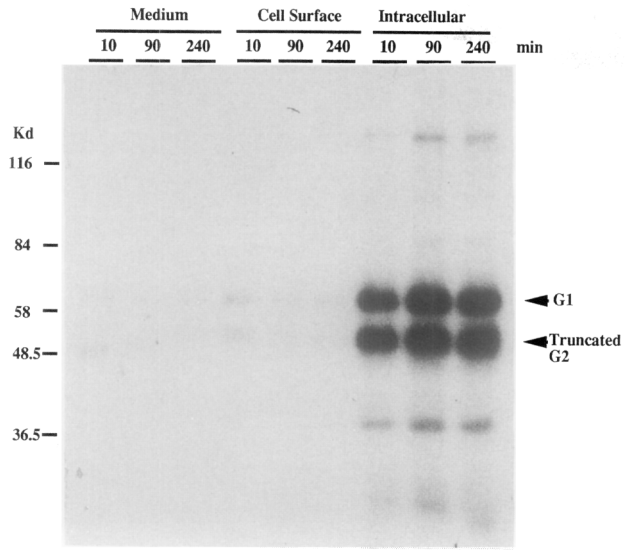

FIG. 8. Intracellular accumulation of the glycoproteins expressed by pGEM-G(A−). HeLa T4$^+$ cells were infected with the VV-T7 and then transfected with 10 μg of the recombinant plasmid DNA. At 12 hr postinfection, cells were pulse-labeled with [^{35}S]methionine for 15 min at 37°, and then chased in the presence of excess methionine for indicated times. The G1 and truncated G2 protein were immunoprecipitated from the culture medium, cell surface, or cell lysate. All samples were analyzed by SDS–PAGE under reducing condition.

lacks the coding sequence for six hydrophobic amino acids in the hydrophobic sequence preceding the G2 protein, was constructed (Fig. 1). To identify the products produced *in vitro,* pGEM-G2(HH) plasmid DNA was analyzed by *in vitro* transcription and translation followed by immune precipitation. A translation product of molecular weight around 52 kDa was found in the pGEM-G2(HH) plasmid translation samples (Fig. 3). To characterize the expressed protein product *in vivo,* a recombinant vaccinia virus designated as VV-G2(HH) was constructed (Fig. 1). HeLa T4$^+$ and CV-1 cells were infected with the VV-G2(HH) recombinant and newly synthesized proteins were immunoprecipitated with antibody against PTV. However, no detectable G2 specific protein was found in the infected cell lysates (data not shown). The lack of a detectable protein product may reflect lack of translocation into the ER. In contrast, when the hydrophobic domain preceding G2 was preserved, the G2 protein was detected *in vivo* (see below), indicating an essential role of this hydrophobic sequence for protein expression.

Retention in the ER of a modified G2 glycoprotein

In order to express the individual G2 protein *in vivo,* a recombinant pGEM-G2(SH), which contains an extra sequence from the NS_M region including a possible signal peptide sequence, was initially constructed using convenient restriction sites, and a corresponding recombinant vaccinia virus VV-G2(SH) was obtained (Fig. 1). To identify the expressed proteins, HeLa T4$^+$ cells were infected with the recombinant VV-G2(SH), and radiolabeled proteins were immunoprecipitated with antibody against PTV. A distinct polypeptide band, migrating slightly slower than native G2, was observed in the cell lysates of VV-G2 (SH)-infected cells. Transport of the G2 protein was determined by surface and intracellular immunoprecipitation and immunofluorescence staining. In VV-G2(SH)-infected cells, the G2 protein was exclusively detected intracellularly, not on the cell surface, and it appeared to accumulate without degradation when analyzed by immunoprecipitation and SDS–PAGE (Fig. 4A). Consistent with this, the G2 protein was also only detected in the cytoplasm by indirect immunofluorescence staining (Figs. 5c and 5d). A perinuclear Golgi pattern as seen in VV-G-infected cells was not observed in the infected cells even after a 4-hr cycloheximide treatment, and the distribution pattern of the protein was similar to that of BiP, a resident ER protein (Figs. 5a–5c), indicating that the protein product is accumulating in the ER. Similar results were obtained using a recombinant pGEM-G2(SH) and the VV-T7 transient expression system (not shown).

Endo H treatment, which cleaves high-mannose N-linked oligosaccharides, was used to monitor whether the proteins are transported out of the ER and reach the medial Golgi complex, where processing reactions convert the high mannose forms to the endo H-resistant complex form of oligosaccharides (Kornfeld and Kornfeld, 1985). The G2 proteins expressed by VV-G2(SH) remained sensitive to endo H treatment after a 4-hr chase. In the cells treated with Brefeldin A (BFA), which causes the Golgi enzymes to redistribute to the ER (Lippincott-Schwartz *et al.,* 1989), a small fraction of the protein became endo H resistant (Fig. 6A), indicating that glycosylation sites of the protein are accessible to glycosyltransferases under this condition. Thus, the G2 protein with an extra NS_M sequence may have a structural defect which blocks its transport out of the ER, possibly due to the presence of extra amino acids.

Transport to cell surface of the full-length G2 protein

To investigate the possibility that the transport defect of the G2 protein with an extra N-terminal sequence is due to a structural defect, another construct which only contains the thirteen hydrophobic amino acids preceding the G2 protein and the complete G2 protein sequence was constructed using PCR (Fig. 1). To detect protein expression, HeLa T4$^+$ cells infected with VV-T7 were transfected with this construct desig-

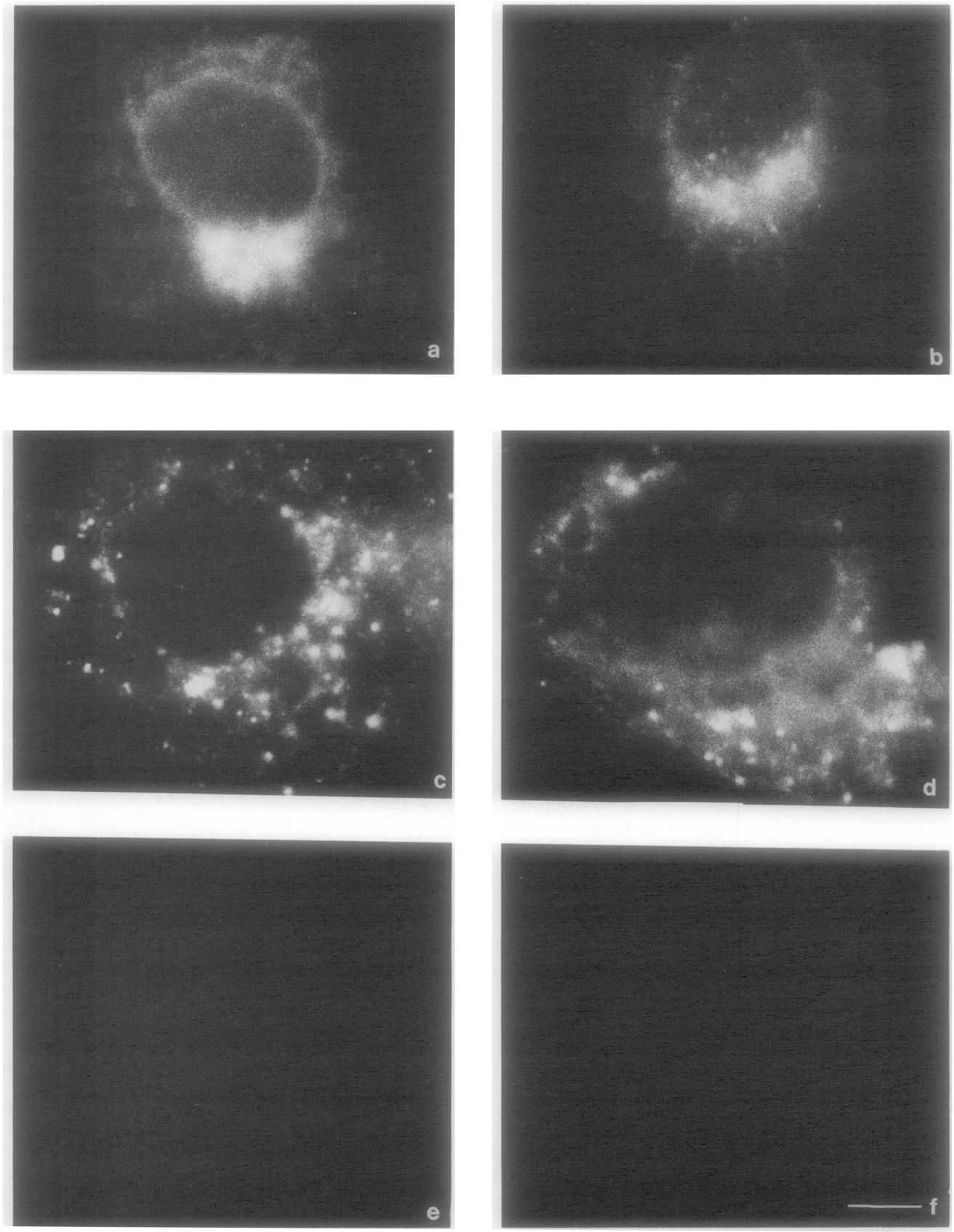

FIG. 9. Localization of the glycoproteins expressed by pGEM-G(A−). HeLa T4$^+$ cells grown on coverslips were infected with VV-T7 for 30 min, and then transfected with the plasmid DNA. After incubating for 12 hr, the cells were incubated with cycloheximide (50 μg/ml) at 37° for 4 hr prior

nated pGEM-G2. A polypeptide band around 55 kD, indistinguishable from native G2 in PTV-infected cells, was found in the cell lysates of pGEM-G2 transfected cells (Fig. 4B). To determine the transport properties of the G2 protein, surface and intracellular immunoprecipitation and immunofluorescence staining were used. In contrast to VV-G2(SH)-infected cells, a fraction of the G2 protein was detected on the surface of pGEM-G2-transfected cells by immunoprecipitation and SDS–PAGE after a 120-min chase, and the fraction of the G2 proteins expressed on the cell surface gradually increased as the chase continued (Fig. 4B). The proportion of the surface proteins expressed by pGEM-G2 was quantitated by densitometric scanning of the autoradiograms, and the results indicate that the $T_{1/2}$ for transport of the molecules to the cell surface was about 6 hr (Fig. 4B). Furthermore, the surface G2 protein was detected by staining nonpermeabilized cells with MAbs to the G2 protein (Figs. 5e and 5f). The G2 proteins sometime appeared as a doublet, as seen previously when expressed by VV-G or a Hantaan virus M gene recombinant, which was believed to be due to heterogeneous glycosylation of the proteins (Matsuoka *et al.*, 1988; Pensiero *et al.*, 1988). Furthermore, the G2 protein expressed by pGEM-G2 slowly became partially endo H resistant during longer chases (Fig. 6B). Thus, our results indicate that the full-length G2 protein expressed by the pGEM-G2 recombinant is slowly transported out of the ER and expressed on the cell surface.

Secretion of a truncated G2 glycoprotein

To determine the requirement of the hydrophobic domain near the G2 C-terminus for membrane anchorage, and its effect on localization of the glycoprotein, a truncated G2 protein was constructed in which the hydrophobic and C-terminal domains were deleted (Fig. 1). The truncated G2 protein contains 25 cysteine residues from a total of 26 residues in the G2 protein (lacking one in the hydrophobic domain) and all four potential glycosylation sites, and was thus expected to maintain a conformation similar to the native N-external domain. HeLa T4$^+$ cells infected with VV-T7 were transfected with the recombinant pGEM-G2(A-), pulse-labeled, and chased for indicated times. A protein band with an estimated mol. wt. of 53 kDa, consistent with the truncated G2 protein (32 amino acids were deleted), was found in the cell lysates (Fig. 7). The truncated protein was detected in the culture medium after a 120-min chase and the fraction of the protein in the medium increased with time of chase (Fig. 7). The mobility of the protein in the medium was slightly slower than that in the cell lysates, suggesting that the protein was terminally glycosylated before secretion into the medium. The $T_{1/2}$ for secretion of the molecules was about 4 hr as analyzed by densitometry scanning of the autoradiograms. Thus, the secretion of the truncated G2 protein into the medium indicates that the hydrophobic domain near the C-terminus serves as a membrane anchor, and the glycosylated N-external domain does not contain signals for intracellular retention.

Expression and localization of a truncated G1-G2 glycoprotein

To determine whether interaction between the G1 and G2 glycoprotein occurs and affects G2 protein localization, we constructed a recombinant pGEM-G(A-), containing the intact G1 sequence and the truncated G2 sequence in which the hydrophobic and C-terminal domains of the G2 protein (45 amino acids) were deleted (Fig. 1). To identify the protein products, HeLa T4$^+$ cells infected with VV-T7 were transfected with the recombinant plasmid DNA, labeled for 15 min, and then chased for the indicated times. As shown in Fig. 8, a 63 kDa molecular weight band corresponding to native G1, and a 52-kDa molecular weight band smaller than the native G2 protein, were found in the cell lysates by SDS–PAGE. No PTV-specific protein was found on the cell surface or in the medium for up to a 12-hr chase. Endo H treatment showed that a fraction of the G1-truncated G2 protein was incompletely processed to endo H-resistant forms as the chase continued, suggesting that these molecules reached the Golgi complex (Fig. 6C). The localization of the truncated G1–G2 proteins was further examined by immunofluorescence staining. No specific fluorescence was detected on the cell surface (Figs. 9e and 9f). In permeabilized cells, the intracellular localization of both the G1 and truncated G2 proteins appeared predominantly in a perinuclear Golgi pattern after the cell culture was treated with cycloheximide for 4 hr (Figs. 9a and 9b). The Golgi localization of the glycoproteins was further indicated by BFA treatment, which caused the perinuclear distribution pattern of the G1 and truncated G2 proteins to change into a dispersed ER-like pattern (Figs. 9c and 9d), as seen for resident Golgi proteins in response to BFA treatment (Lippincott-Schwartz *et al.*, 1989). Thus, in contrast to the secre-

to fixation, then BFA (10 μg/ml) was added to the culture medium for 20 min. After fixation, cells were stained with Mabs against G1 or G2, followed with fluorescein-labeled goat anti-mouse IgG. a. G1 glycoprotein after cycloheximide treatment. b. Truncated G2 glycoprotein after cycloheximide treatment. c. G1 glycoprotein in cells after BFA treatment. d. Truncated G2 glycoprotein in cells after BFA treatment. e. Cell surface staining for the G1 glycoprotein. f. Cell surface staining for the truncated G2 glycoprotein. (Bar = 3.0 μm.)

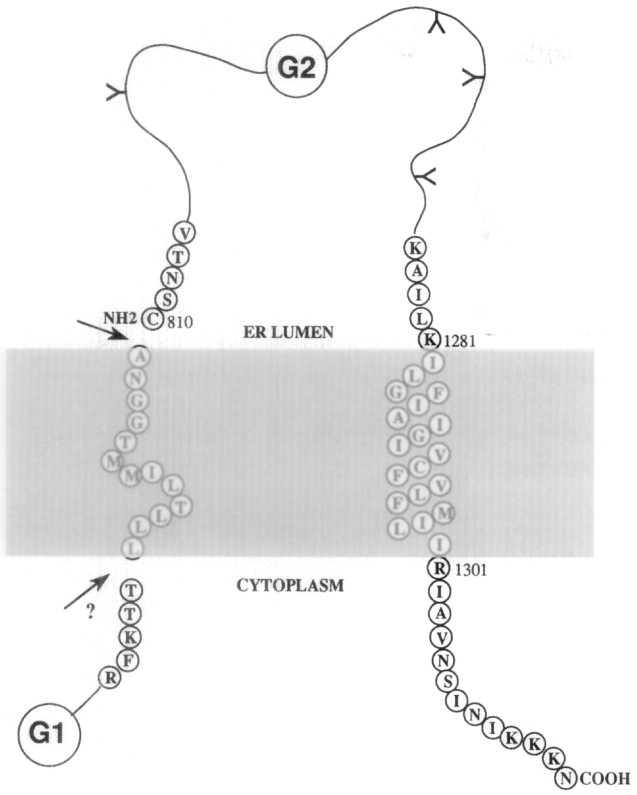

FIG. 10. Schematic representation of the proposed transmembrane topology of the G2 glycoprotein of PTV. Possible sites of cleavage are indicated by arrows. Most of the luminal portion of the G2 glycoprotein is not drawn to scale (solid line). The N-terminus of the G2 glycoprotein was determined by Ihara *et al.* (1985); the C-terminus of the G1 protein is undetermined as yet. The positions of the amino acids in the polyprotein are marked. (Y) indicates the potential N-linked oligosaccharide chains.

tion into the medium of the truncated G2 protein when expressed on it own, the G1 and truncated G2 glycoproteins are transported out of the ER and retained in the Golgi complex when expressed together using the pGEM-G(A-) recombinant. This result indicates that the Golgi retention of the truncated G2 glycoprotein occurs because of its association with the G1 glycoprotein.

DISCUSSION

Topogenic sequences for the G2 glycoprotein

We investigated the signal sequence for the second glycoprotein encoded in a bunyavirus polyprotein precursor (G2 in PTV) by analysis of two different G2 constructs. One construct (G2(HH)), containing a partial deletion of the presumptive signal peptide, was transcribed and translated into a G2-specific protein *in vitro,* but no protein product was detected in cells infected with the corresponding vaccinia recombinant virus. In contrast, using a construct containing the full hydrophobic sequence preceding G2, which has all the characteristics of a signal peptide as described by von Heijne (1984), the G2 protein was found to be stably produced and glycosylated *in vivo.* Newly synthesized membrane proteins that fail to undergo translocation into the ER have been reported to be extremely unstable in the cytoplasm (Gething and Sambrook, 1982; Sekikwa and Lai, 1983). Thus, a likely reason for not detecting a protein product in VV-G2(HH)-transfected cells is rapid proteolysis of the untranslocated protein. These results provide evidence that translocation of the G2 protein into the ER can be mediated by a thirteen amino acid hydrophobic sequence, preceding the G2 protein, as a signal peptide. The internally located signal sequences of the PTV polyproteins are similar to those of the Semliki Forest virus structural polyprotein, although the translocational signal peptide of the E1 protein is located in the C-terminus of a 6-kDa protein rather than the structural protein p62 (Liljestrom and Garoff, 1991; Melancon and Garoff, 1986).

The results obtained by comparison of truncated vs full-length G2 proteins indicate that a hydrophobic domain near the C-terminus of the G2 protein serves as a membrane anchor. A truncated G2 protein in which the hydrophobic and C-terminal domains were deleted was found to be secreted into the medium, whereas the intact G2 protein remained membrane-associated. This conclusion is consistent with the membrane topology analysis predicted from the deduced amino acid sequence. Since all potential glycosylation sites in the G2 are located in the N-terminal side of the membrane anchor domain, and the G2 protein is glycosylated, we conclude that the G2 glycoprotein is a class I membrane protein with the topology depicted in Fig. 10.

The G2 glycoprotein does not contain a signal for Golgi retention

The cDNA sequence information for the M genomic segment from members of the bunyavirus family reveals that the predicted C-terminal amino acid sequence of the second glycoprotein in the polypeptide in several genera (G2 in PTV) shows a common feature which includes multiple positively charged residues: –KHKKS in Hantaan virus (Schmaljohn *et al.,* 1987) and in SR-11 viruses (Arikawa *et al.,* 1990), –KYKKS in Uukuniemi virus (Ronnholm and Petterson, 1987), and –IKKKN in PTV (Ihara *et al.,* 1985). We investigated the possibility that this C-terminal sequence of the short cytoplasmic tail may serve as a retention signal, since the C-terminal tetrapeptides K/HDEL of the luminal ER proteins have been well defined as an ER retention signal (Munro and Pelham, 1987; Pelham, 1988) and a

C-terminal consensus sequence in ER membrane proteins was also identified as a signal for their ER retention (Jackson *et al.*, 1990; Nilsson *et al.*, 1989). Interestingly, the G2 C-terminal sequence corresponds to this consensus ER-retention signal, with lysines positioned three and four or five residues from the C-terminus (Jackson *et al.*, 1990). In contrast to our expectation, a full-length G2 protein was found to be transported to the cell surface. Further, a truncated G1–G2 protein, in which the G2 C-terminal sequence was deleted, was transported out of the ER, and still targeted to the Golgi complex. Thus, the C-terminal sequence of the G2 glycoprotein does not serve as an ER retention signal or function to maintain a conformation needed for Golgi retention of the G1 and G2 protein. These results indicate that the consensus motif of basic residues at the C-terminus of transmembrane proteins (Jackson *et al.*, 1990) does not always represent an ER-retention signal, since the PTV glycoproteins with or without this consensus motif are transported out of the ER.

The intracellular retention of the G2 glycoprotein occurs by its association with the G1 glycoprotein

The G2 glycoprotein expressed in the absence of the G1 protein was found to be transported out of the ER and expressed on the cell surface, although its transport was less efficient than transport of the vesicular stomatitis virus (VSV)-G or influenza hemagglutinin (HA) proteins (Rose and Doms, 1988). Furthermore, the anchor-minus G2 protein was found to be secreted into the medium. Thus, we conclude that the G2 glycoprotein does not contain a specific signal for Golgi retention. In contrast to misfolded or unassembled proteins which fail to exit the ER (Copeland *et al.*, 1988; Doms *et al.*, 1988; Gething *et al.*, 1986; Rose and Doms, 1988), the PTV membrane glycoproteins are transported out of the ER, but specifically retained in the Golgi complex. Our results indicate that Golgi localization of the G2 glycoprotein requires its interaction with the G1 glycoprotein. This conclusion is supported by the following evidence: (1) The anchor minus G2 protein is secreted into medium, whereas it is retained in the Golgi complex when coexpressed with the G1 protein; (2) The G2 protein when expressed individually is transported to the cell surface, whereas it is retained in the Golgi complex when expressed together with the G1 protein. Thus, it seems likely that a Golgi retention signal is present on the G1 protein. Studies to identify a possible retention signal in the G1 protein are in progress. Alternatively, a functional retention signal might require formation of a complex between the G1 and G2 proteins. We recently observed that the majority of the G1 and G2 glycoprotein are assembled into noncovalently linked G1–G2 heterodimers, and that the G2 C-terminal and transmembrane domains are not required for dimerization (S.Y. Chen and R. W. Compans, manuscript in preparation). The dimerization of the G1 and G2 glycoproteins was also recently reported in Uukuniemi virus-infected cells (Persson and Pettersson, 1991).

Except for the G2 C-terminal sequence, the bunyavirus G1 and G2 proteins have no significant sequence homology among different genera, but the hydropathy profiles of the glycoproteins of bunyaviruses reveal a striking similarity, suggesting a common transmembrane topology (Elliott, 1990; Schmaljohn and Patterson, 1990). Similarly, glycosyltransferases, which are resident membrane proteins of the Golgi complex and the ER, show virtually no sequence homology in their deduced amino acid sequences, but they have a common transmembrane topology with a short NH_2-terminal cytoplasmic tail, a 16- to 20-amino acid signal-anchor domain, and an extended stem region which is followed by the large COOH-terminal catalytic domain (Paulson and Colley, 1989; Shaper *et al.*, 1988; Weinstein *et al.*, 1987). When various Golgi resident membrane proteins, the coronavirus E1 glycoprotein (a class III membrane protein), bunyavirus G1 and G2 glycoproteins (class I membrane proteins), and glycosyltransferases (class II membrane proteins) are compared, no sequence homology or similarity of hydropathy profiles among these proteins was found. We believe that these membrane proteins do not employ the same strategy as luminal ER proteins which use a short linear C-terminal sequence as a retention signal. Alternatively, all of these proteins may have a similar signal patch as proposed for lysosomal enzymes specifically recognized by *N*-acetylglucosaminylphosphotransferase (Dahms *et al.*, 1989; Kornfeld, 1990), which cannot be observed by analysis of the linear sequence.

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