Stable Secretion of a Soluble, Oligomeric Form of Rabies Virus Glycoprotein: Influence of *N*-Glycan Processing on Secretion†

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ABSTRACT: Rabies virus glycoprotein (RGP) is a 505 amino acid type I transmembrane glycoprotein that is important in the pathogenesis of rabies virus infection. RGP also stimulates the development of neutralizing antibodies by the host. N-Linked glycosylation is required for both cell surface expression and immunogenicity of RGP. In the current study, a soluble form of RGP, constructed by insertion of a stop codon external to the transmembrane domain, was expressed in transfected Chinese hamster ovary cells. The soluble form of RGP was found to be appropriately antigenic and immunogenic. Similar to full-length RGP, the soluble form was assembled into homodimers and homotrimers. Core glycosylation was required for secretion of soluble RGP and cell surface expression of full-length RGP. In addition, initial glucose trimming of the *N*-glycans was necessary and sufficient for secretion of soluble RGP and cell surface expression of full-length RGP. Further *N*-glycan processing was not required for secretion or cell surface expression of soluble or full-length RGP, respectively.

Rabies virus glycoprotein (RGP)[1] from the Evelyn−Rokitnicki−Abelseth (ERA) strain is a 505 amino acid type I membrane glycoprotein containing a 22 amino acid transmembrane domain and a 44 amino acid cytoplasmic domain (Fishbein & Robinson, 1993; Wunner et al., 1988). The extracellular domain has three potential N-linked glycosylation sites (Anilionis et al., 1981), only two of which are efficiently core glycosylated (Shakin-Eshleman et al., 1992). As the only glycoprotein on the viral surface, it plays a critical role in rabies virus invasion of host cells and is the single immunogenic protein capable of stimulating production of virus-neutralizing antibodies during infection. RGP is also effective as a subunit vaccine (Kieny et al., 1984), provided that it is glycosylated (Yelverton et al., 1983; Lathe et al., 1984). A soluble, secreted form of RGP, G_s, containing 447 amino acids, is also produced in infected cells by proteolytic cleavage of full-length RGP (Dietzschold et al., 1983). G_s consists of the entire extracellular domain of RGP, lacks the cytoplasmic tail and most of the transmembrane domain, and retains full antigenicity. Purified G_s was not highly immunogenic, perhaps due to its inability to aggregate; however, it did induce production of virus-neutralizing antibodies (Dietzschold et al., 1983).

The availability of cloned RGP cDNA has allowed expression of this transmembrane protein in bacterial (Yelverton et al., 1983; Lathe et al., 1984), yeast (Klepfer et al., 1993), insect (Prehaud et al., 1989; Tuchiya et al., 1992), and mammalian cells (Burger et al., 1991; Morimoto et al., 1992; Shakin-Eshleman et al., 1992). In the current study, a soluble form of RGP, constructed by insertion of a stop codon just external to the transmembrane domain (Shakin-Eshleman et al., 1993), was expressed in transfected Chinese hamster ovary (CHO) fibroblasts. The assembly, antigenicity, and immunogenicity of this truncated form of RGP were analyzed. In addition, the roles that core glycosylation and subsequent *N*-glycan processing play in its secretion were examined. Finally, the influence of *N*-glycan processing on cell surface expression of full-length RGP was examined and compared to that found with the truncated form.

EXPERIMENTAL PROCEDURES

Description of the RGP Termination Mutant. The construction of plasmid pRGP(WT)T434, containing the RGP termination mutant, was described previously (Shakin-Eshleman et al., 1993). This plasmid directs the synthesis of a 433 amino acid polypeptide encompassing the extracellular domain of full-length RGP.

Cell Lines and Tissue Culture. The wild-type CHO cell line, Pro-5 (Stanley et al., 1975), was obtained from the American Type Culture Collection (Rockville, MD). The transfected Pro-5 CHO cell line expressing full-length recombinant RGP was described previously (Burger et al., 1991; Shakin-Eshleman et al., 1992). Cells were routinely

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[1] Abbreviations: RGP, rabies virus glycoprotein; ERA, Evelyn−Rokitnicki−Abelseth; CHO, Chinese hamster ovary; α-MEM, α-modified minimal essential medium; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; ERA-BPL, β-propriolactone-inactivated rabies virus (ERA strain); PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Endo H, endoglycosidase H; PNGase F, peptide *N*-glycosidase F; EGS, ethylene glycol bis(succinimidylsuccinate); DMSO, dimethyl sulfoxide; DSP, dithiobis(succinimidylpropionate); IL, interleukin; VSV G protein, vesicular stomatitis virus glycoprotein.

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cultured in complete medium: α-modified minimal essential medium (α-MEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 international units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL of amphotericin B.

Monoclonal and Polyclonal Antibodies. Rabbit polyclonal anti-rabies virus antiserum was produced by immunizing New Zealand White rabbits (East Acres Biologicals, Southbridge, MA) intramuscularly with 100 μg of β-propriolactone-inactivated rabies virus (ERA strain) vaccine (ERA-BPL) in complete Freund's adjuvant using a previously described protocol (Wiktor et al., 1973). Four booster intramuscular immunizations were performed at weekly intervals in incomplete Freund's adjuvant using the same concentration of vaccine. Antiserum collected 1 week following the last booster immunization was titered using immunoprecipitation and SDS−PAGE (see below).

The following mouse monoclonal antibodies specific for RGP were used as ascites, were produced at The Wistar Institute, and were described previously (Flamand et al., 1980; Lafon et al., 1983; Seif et al., 1985; Prehaud et al., 1988; Dietzschold et al., 1988): 523-11, 719-3, and 509-6. Culture supernatants containing anti-RGP mouse monoclonal antibodies 61-105-2, 62-114-6, 62-111-6, and 62-36-7 were provided by J. Smith (Centers for Disease Control, Atlanta, GA; Smith et al., 1984). The hybridoma cell line producing the 62-80-6 anti-RGP mouse monoclonal antibody (Smith et al., 1984) was also provided by J. Smith; ascites was used in the studies described below.

Transfection. CHO cells were co-transfected with 10 μg of pRGP(WT)T434 and 1 μg of pSV2neo (Southern & Berg, 1982) with a 2-min glycerol shock at 4 h (Burger et al., 1991). After 48 h the medium was replaced with fresh complete α-MEM containing 1 mg/mL active G418 (Gibco, Grand Island, NY), and thereafter the medium was replaced every 2 days with fresh complete α-MEM containing 1 mg/mL active G418. After 10 days, G418-resistant colonies were isolated, amplified, and screened for secretion of metabolically labeled RGP(WT)T434 (see below). Clonal cell lines were isolated by repetitive subcloning by limiting dilution. Transfected clonal cell lines were maintained in complete α-MEM containing 0.5 mg/mL active G418.

Metabolic Labeling. Petri dishes (100 mm) of transfected cells were washed twice with Hanks' balanced salt solution (HBSS; Sigma, St. Louis, MO). Five milliliters of methionine-free complete Dulbecco's modified Eagle's medium (DMEM), supplemented exactly like complete α-MEM and also containing 50 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL), was added. Following a 4-h incubation at 37 °C, the cells were washed twice with ice-cold phosphate-buffered saline (PBS: 100 mM Na_2HPO_4/NaH_2PO_4 and 150 mM NaCl, pH 7.4) containing 200 μg/mL phenylmethanesulfonyl fluoride (PMSF) (PBS/PMSF), scraped, and transferred to 1.5-mL Eppendorf tubes on ice. Cells were washed once with ice-cold PBS/PMSF, resuspended in 100 μL of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM EDTA, pH 7.4, containing 0.5% Nonidet P-40 and 200 μg/mL PMSF), incubated on ice for 20 min, and clarified by centrifugation at 12000*g* for 20 min at 4 °C.

Pulse−Chase Experiments. Sixty-millimeter dishes of transfected cells were washed twice with HBSS. Prior to labeling, the cells were preincubated with one of the following soluble glycosylation inhibitors [for a review, see Elbein (1991)] in 1 mL of complete α-MEM: 50 μg/mL castanospermine, 5 μg/mL tunicamycin, 5 μg/mL swainsonine, 20 μM australine, 1 mM bromoconduritol, 1 mM 1-deoxymannojirimycin, or 1 mM 1-deoxynojirimycin. Following incubation at 37 °C for 2 h, the cells were washed twice with HBSS. One milliliter of methionine-free complete DMEM was then added, containing both 18 μCi of [^{35}S]-methionine and the appropriate glycosylation inhibitor at the concentration described above. After labeling at 37 °C for a 20-min pulse, the cells were washed twice with HBSS. Then 1 mL of methionine-free complete DMEM, containing an excess of cold methionine (1 mM) and the appropriate glycosylation inhibitor, was added. At chase times of 0, 0.5, 1, 2, and 4 h after the pulse, the conditioned media were collected, centrifuged at 12000*g* at 4 °C for 5 min, and maintained at 4 °C. The remaining cells were washed once with ice-cold PBS/PMSF, resuspended in 100 μL of lysis buffer, incubated on ice for 20 min, and clarified by centrifugation at 12000*g* for 20 min at 4 °C (cell lysate).

Immunoprecipitation. From one dish of transfected cells expressing RGP(WT)T434, 800 μL of conditioned medium diluted with 200 μL of 5X RIPA Buffer (100 mM Tris pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.3 M NaCl, 0.1% SDS, 1 mM PMSF) and 100 μL of cell lysate was prepared. Rabbit polyclonal anti-rabies virus antiserum at a final dilution of 1:100 was added to the cell lysate (for the cell-associated form) or the conditioned medium (for the secreted form) and incubated with gentle agitation for 16 h at 4 °C. Immune complexes were isolated with protein A beads (Gibco BRL, Gaithersburg, MD) after a 3-h incubation at 4 °C with gentle agitation (20 or 40 μL of a 50% slurry of beads for cell lysate or conditioned medium, respectively). Protein A beads were then washed three times for 10 min each at 4 °C with gentle agitation in wash buffer (15 mM Tris, pH 7.5, 0.5 M NaCl, 5 mM EDTA, and 1% Nonidet P-40). Immunoprecipitated truncated RGP was eluted from the beads by boiling for 5 min in 40 μL of sample buffer (62 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) containing 5% β-mercaptoethanol or in 40 μL of an endoglycosidase buffer (see below), and either analyzed directly by SDS−polyacrylamide gel electrophoresis (PAGE) or subjected to endoglycosidase digestion.

Transfected CHO cells expressing full-length RGP (Burger et al., 1991; Shakin-Eshleman et al., 1992) were metabolically labeled, as above. Cell surface full-length RGP was immunoprecipitated from the labeled cells by incubating the washed, intact monolayer (100-mm dish) with 2 mL of rabbit polyclonal anti-rabies virus antiserum (1:8 dilution) for 20 min at 4 °C. The monolayer was lysed with lysis buffer, and the immune complexes were isolated, as above. Fresh polyclonal antibody was then added to the immunodepleted lysate to immunoprecipitate intracellular full-length RGP using the approach described above.

Endoglycosidase Digestion. Immunoprecipitated radiolabeled full-length or truncated RGP was eluted from Protein A beads by boiling for 5 min in 40 μL of either endoglycosidase H (Endo H) buffer (60 mM Na_2HPO_4/NaH_2PO_4, pH 5.5, containing 1% SDS, 200 μg/mL PMSF) or peptide *N*-glycosidase F (PNGase F) buffer (30 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 20 mM EDTA). Samples were divided, supplemented with 4 μL of Endo H (4 milliunits; Boehringer-Mannheim, Indianapolis, IN) or PNGase F (0.8 units; Boehringer-Mannheim) in the corresponding buffer, or with

the same volume of buffer alone, and incubated for 24 h at 37 °C. An additional volume (4 μL) of Endo H or PNGase F was added after 16 h of incubation. The samples were then mixed with 12 μL of 5× sample buffer, boiled, and analyzed by SDS−PAGE.

Oligomerization Analysis. To each 800-μL aliquot of conditioned medium from metabolically labeled cells was added 10, 30, or 40 μL of a stock solution of the covalent cross-linker ethylene glycol bis(succinimidylsuccinate) (EGS; Pierce Chemical Co. Rockford, IL) dissolved in dimethyl sulfoxide (DMSO; 18.4 mg of EGS/250 μL of DMSO) and each was incubated for 30 min at room temperature (Doms et al., 1987). The final concentrations of EGS were 2, 6, and 8 mM. The reaction was quenched by incubation with 30 mM glycine for 30 min at room temperature. The cross-linked, secreted form of RGP(WT)T434 was immunoprecipitated and analyzed by SDS−PAGE on nonreducing 4−12% gradient gels. In some experiments, the cleavable cross-linker dithiobis(succinimidylpropionate) (DSP; Pierce) was used (1−10 mM) and the immunoprecipitates were analyzed by electrophoresis under reducing and nonreducing conditions.

For sedimentation analysis (Doms et al., 1987), 14-mL linear sucrose gradients were prepared from stocks of 5 and 20% sucrose (w/w) in MNT buffer [20 mM 2-(*N*-morpholino)ethanesulfonic acid, 100 mM NaCl, and 30 mM Tris] at either pH 7.4 or 5.5. The metabolically labeled conditioned medium (200 μL) diluted with 200 μL of MNT buffer at pH 7.4 or 5.5 was loaded onto prepared gradients and centrifuged at 40 000 rpm for 16 h at 4 °C using an SW 40Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). Fractions (800 μL) were collected from the bottom of each tube, mixed with 200 μL of 5× RIPA buffer, immunoprecipitated, and analyzed by SDS−PAGE.

Analysis of Radiolabeled Proteins by SDS−PAGE. Proteins suspended in sample buffer were reduced with 5% β-mercaptoethanol, boiled for 5 min, and separated by SDS−PAGE (10% gels), as described (Shakin-Eshleman et al., 1992). After electrophoresis, gels were fixed, incubated with Amplify (Amersham Corp.), dried, and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY). The relative molecular masses of radiolabeled proteins were determined using the following prestained markers (Gibco-BRL): myosin H-chain (215 kDa), phosphorylase B (105 kDa), bovine serum albumin (70 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18 kDa). The same protein markers were also used in a ^{14}C-labeled form (Gibco-BRL) with the following relative molecular masses: 200, 97.4, 68, 43, 29, and 18 kDa. Autoradiographs were analyzed by densitometry using a Personal Densitometer (Molecular Dynamics; Sunnyvale, CA). Gels were also analyzed directly using a Molecular Dynamics PhosphorImager and Image-quant version 3.22 software.

Animal Immunization. Female C3H/He mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intramuscularly with 100 μg of pRGP(WT) or pRGP(WT)T434. Positive control mice were inoculated intraperitoneally with 5 μg of ERA-BPL-inactivated viral vaccine.

Lymphokine Release Assay. Splenocytes (4×10^6) were cultured in 24-well plates in 1.6 mL of DMEM supplemented with 2% fetal calf serum and 1 μM β-mercaptoethanol. The splenocytes were cultured in medium either containing or lacking 5 μg/mL of ERA-BPL virus. Cell-free supernatants

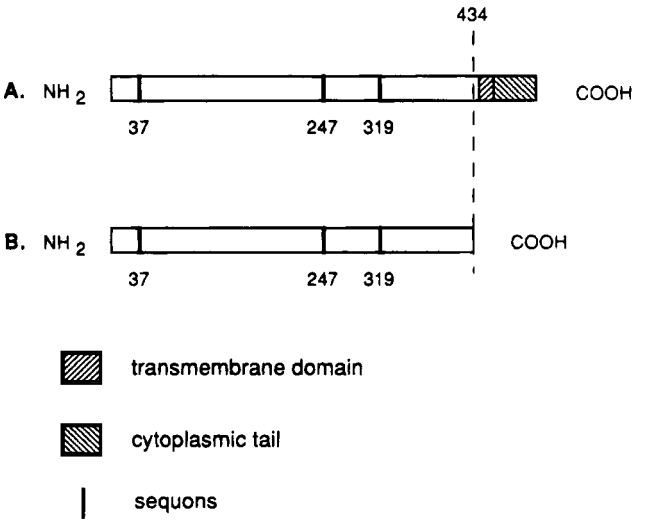

FIGURE 1: Structure of full-length RGP and the RGP termination mutant. (A) Structure of full-length RGP. The Asn-X-Ser/Thr potential N-linked glycosylation sites at Asn^{37}, Asn^{247}, and Asn^{319} are indicated by dark bars. The transmembrane domain, which extends from amino acid 440 to amino acid 461, and the C-terminal cytoplasmic tail, which extends from amino acid 462 to amino acid 505, are hatched. The position of amino acid 434, which represents the site of the termination codon in the RGP termination mutant, is indicated with a vertical dashed line. (B) Structure of RGP(WT)-T434, the RGP termination mutant.

(75 μL) obtained 24 h later were incubated with 2×10^3 HT-2 cells, a cell line that proliferates in the presence of interleukin-2 (IL-2) or IL-4. Proliferation was measured 72 h later by a 6-h [^3H]thymidine pulse (0.35 μCi/well). The assay was performed in triplicate, and the means and standard deviations were calculated. Although this assay measures production by T cells of both IL-2 and IL-4, previous studies indicated that immunization with ERA-BPL and pRGP(WT) only induced production of IL-2 (Xiang et al., 1994).

ELISA. Microtiter plates (96-well) were coated overnight at 4 °C with 0.2 mg/well ERA-BPL virus in 100 μL of 15 mM sodium carbonate buffer, pH 9.6, containing 3 mM sodium azide. The wells were then washed twice and blocked overnight at 4 °C with 2% bovine serum albumin in PBS. After washing, 100 μL of various dilutions of serum was added to individual wells and incubated at 4 °C for 1 h. Normal mouse serum was used as a negative control. After washing, 60 μL of goat anti-mouse immunoglobulin coupled to alkaline phosphatase (1:100 dilution in 2% bovine serum albumin in PBS) was added to each well and incubated for 1 h at 4 °C. After washing, 100 μL of substrate (10 mg of *p*-nitrophenyl phosphate dissolved in 10 mL of 1 mM $MgCl_2$ and 900 mM diethanolamine, pH 9.8, containing 3 mM sodium azide) was added to each well, and the optical density at 405 nm was determined. The assay was performed in triplicate, and the means and standard deviations were calculated.

RESULTS

Expression of the RGP Termination Mutant. The RGP-(WT)T434 termination mutant was generated from plasmid pRGP(WT) by introducing a termination codon at amino acid 434 (Figure 1), as described (Shakin-Eshleman et al., 1993). Thus, RGP(WT)T434 contains all three glycosylation sites, but lacks the transmembrane domain, the cytoplasmic domain, and the six amino acids N-terminal to the trans-

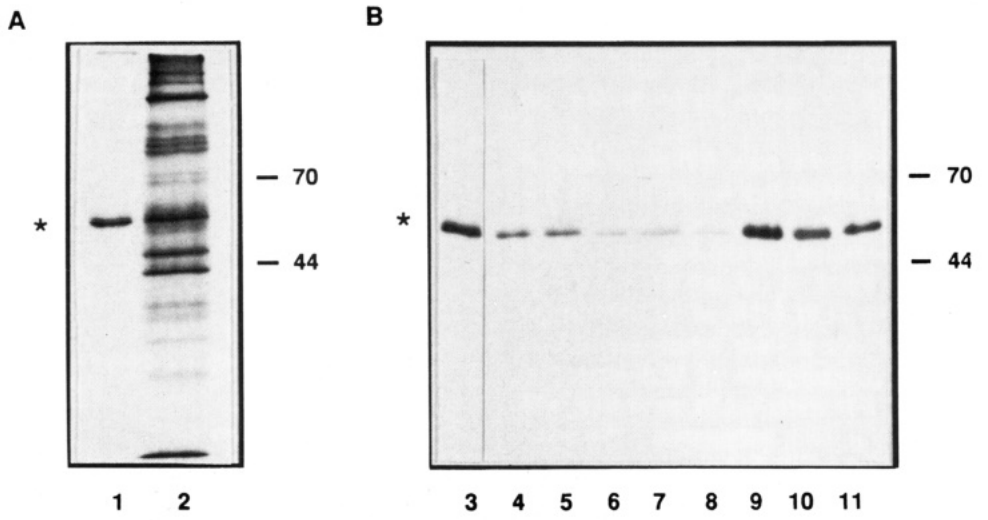

FIGURE 2: Secretion of RGP(WT)T434 by transfected CHO cells. pRGP(WT)T434-transfected CHO cells were metabolically labeled with [^{35}S]methionine, and immunoprecipitates were analyzed by SDS−PAGE and autoradiography, as described in Experimental Procedures. The migration positions of the prestained electrophoretic standards are indicated. The migration position of RGP(WT)T434 is indicated with an asterisk. (A) Conditioned medium from cells cultured in serum-free medium was analyzed before (lane 2) or after (lane 1) immunoprecipitation with polyclonal rabbit anti-rabies virus antiserum. (B) Conditioned medium from cells cultured in complete medium was immunoprecipitated with either rabbit polyclonal anti-rabies virus antiserum (lane 3) or monoclonal antibodies 62-80-6, 523-11, 719-3, 509-6, 61-105-2, 62-114-6, 62-111-6, and 62-36-7 (lanes 4−11, respectively).

membrane domain (Figure 1). pRGP(WT)T434 was co-transfected with pSV2neo into CHO cells. After selection with G418, 24 colonies were isolated. Seventeen colonies exhibited long-term growth and were screened for secretion of metabolically labeled RGP(WT)T434 by immunoprecipitation of the conditioned medium with rabbit polyclonal antiserum and subsequent analysis by SDS−PAGE. Each colony secreted RGP(WT)T434 to some degree. The colony demonstrating the highest level of secretion was expanded and cloned by a single round of limiting dilution to isolate a subclone expressing high levels of the recombinant protein. Figure 2A shows the results with this clone cultured in serum-free medium before (lane 2) and after immunoprecipitation (lane 1). This demonstrates that RGP(WT)T434 is approximately 55 kDa and is one of the major soluble proteins secreted by the transfected CHO cells.

To evaluate the immunological similarity of this secreted form of RGP to the external domain of authentic full-length RGP produced during viral infection, secreted RGP(WT)-T434 was tested by immunoprecipitation with a panel of eight conformation-dependent monoclonal antibodies recognizing several antigenic determinants. RGP(WT)T434 was recognized by each monoclonal antibody (Figure 2B). Similar results were found with full-length RGP (data not shown). In addition, a naked DNA vaccine approach was used to evaluate the immunogenicity of RGP(WT)T434 and compare it with full-length RGP (Xiang et al., 1994). Previous studies demonstrated that pRGP(WT) functioned as an effective vaccine in a mouse model using this approach. The current results demonstrated that both pRGP(WT)T434 and pRGP(WT) similarly induced T cell-mediated (Figure 3) and B cell-mediated (Figure 4) immune responses to rabies virus. Thus, sequences on the extracellular domain of RGP are appropriately antigenic and immunogenic.

N-Linked Glycosylation of RGP(WT)T434. Core glycosylation and *N*-glycan processing of RGP(WT)T434 synthesized by transfected CHO cells were examined by comparing the electrophoretic migration before and after digestion with Endo H and PNGase F. PNGase F releases any type of

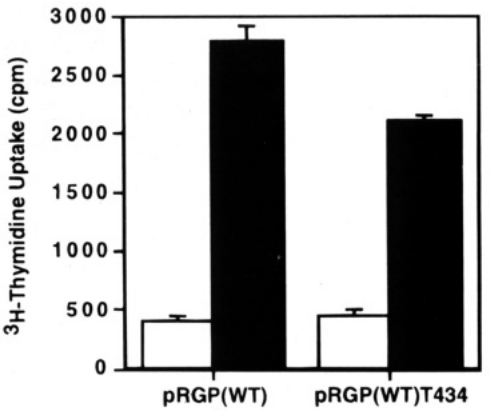

FIGURE 3: Murine T cell response following immunization with plasmid vectors expressing full-length or soluble RGP. Mice were immunized twice with 100 μg of either pRGP(WT) or pRGP(WT)-T434 . Splenocytes were obtained 15 days later and were tested for lymphokine secretion, as described in Experimental Procedures, upon coculture with medium containing (filled bars) or lacking (open bars) 5 μg/mL ERA-BPL-inactivated rabies virus. The means ± 1 SD are shown.

N-glycan (e.g., high mannose or complex) from glycoproteins, whereas Endo H only releases high mannose type *N*-glycans.

Metabolically labeled RGP(WT)T434 was immunoprecipitated with polyclonal antiserum from conditioned medium (secreted form) and from cell lysate (cell-associated form), digested with endoglycosidases, and analyzed by SDS−PAGE. When secreted RGP(WT)T434 was treated with Endo H (Figure 5, lane 2), the resulting protein comigrated with untreated RGP(WT)T434 (Figure 5, lane 1), whereas the PNGase F-treated form migrated faster (Figure 5, lane 3). This demonstrates that the secreted form of RGP(WT)-T434 is glycosylated by Pro-5 cells; since the *N*-glycans are completely Endo H resistant, they are of complex type. In contrast, when the cell-associated form of RGP(WT)T434 was treated with Endo H (Figure 5, lane 5), the resulting protein comigrated with the PNGase F-treated cell-associated form of RGP(WT)T434 (Figure 5, lane 6). This demon-

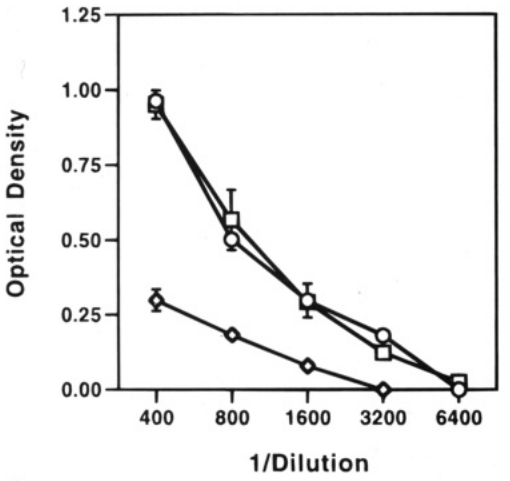

FIGURE 4: Murine antibody response following immunization with plasmid vectors expressing full-length or truncated RGP. Mice were immunized once with 100 μg of either pRGP(WT) (○) or pRGP-(WT)T434 (□), and blood samples were obtained 10 weeks later. Antibodies to rabies virus were identified using the ELISA method described in Experimental Procedures. Normal mouse serum (◇) was used as a negative control. The means ± 1 SD are shown.

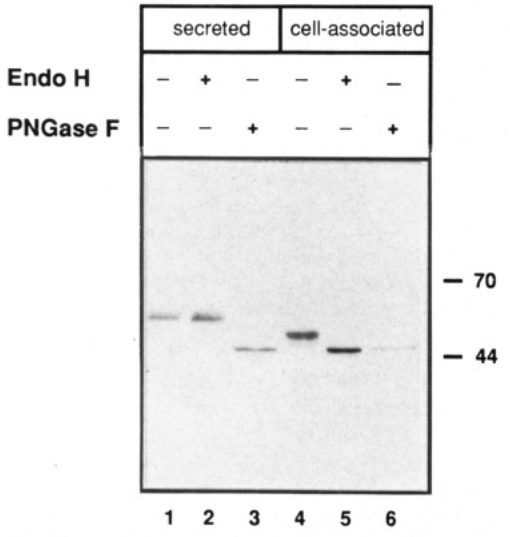

FIGURE 5: Glycosylation of RGP(WT)T434 synthesized by transfected CHO cells. pRGP(WT)T434-transfected CHO cells were metabolically labeled with [^{35}S]methionine, and conditioned medium (lanes 1−3) and cell lysates (lanes 4−6) were immunoprecipitated with rabbit polyclonal anti-rabies virus antiserum. The immunoprecipitates were incubated with Endo H (lanes 2 and 5), PNGase F (lanes 3 and 6), or buffer alone (lane 1 and 4) and then analyzed by SDS−PAGE and autoradiography, as described in Experimental Procedures. The positions of the prestained electrophoretic standards are indicated.

strates that the cell-associated form possesses only high mannose type *N*-glycans.

Oligomerization of RGP(WT)T434. To determine whether secreted RGP(WT)T434 is oligomeric, both chemical cross-linking experiments and sucrose density gradient centrifugation were performed. Metabolically labeled conditioned medium was treated with several different cross-linking reagents separately and in combination. Similar results were obtained with each cross-linker. This approach identified cross-linked species having mobilities on SDS−PAGE consistent with those expected for both dimers and trimers of RGP(WT)T434 (Figure 6A). Only the monomer was observed when conditioned medium was incubated in the absence of cross-linker (Figure 6A). Experiments under reducing and nonreducing conditions using DSP, a reversible cross-linker that has an internal disulfide bond, also demonstrated that the oligomers were homodimers and homotrimers (data not shown).

Conditioned medium obtained from metabolically labeled transfected CHO cells expressing RGP(WT)434 was also analyzed by sucrose density gradient centrifugation. Conditions were used which result in the stabilization of trimers of vesicular stomatitis virus glycoprotein (VSV G protein), which is homologous to RGP (Rose et al., 1982). These conditions also separate VSV G protein monomers from trimers (Doms et al., 1987). This approach identified secreted RGP(WT)T434 in fractions 12−14 (Figure 6B), the same position as found with VSV G protein monomers (data not shown). Identical results were obtained when centrifugation was performed at either pH 7.4 or pH 5.5 (data not shown). Taken together, these results demonstrated that secreted RGP(WT)T434 forms weakly associated dimers and trimers that can be identified by chemical cross-linking, but that are not stable under the conditions used for sucrose density gradient centrifugation.

Influence of Glycosylation Inhibitors on RGP(WT)T434 Secretion. Transfected CHO cells were pulse-labeled with [^{35}S]methionine and then chased for defined times in the presence of several inhibitors of N-linked glycosylation. Radiolabeled RGP was then immunoprecipitated from cell lysates and conditioned media and analyzed by SDS−PAGE.

Tunicamycin, which blocks core glycosylation (Figure 7), was used to test whether any glycosylation of RGP(WT)-T434 is required for secretion. In the presence of tunicamycin, both full-length RGP and cell-associated RGP(WT)-T434 migrated faster (data not shown), consistent with the lack of *N*-glycans. In addition, secreted RGP(WT)T434 was not detected in the conditioned medium (Table 1). These results demonstrate that secretion of this protein is dependent on core glycosylation.

To allow core glycosylation but then block the initial step in *N*-glycan processing, pulse−chase experiments were performed in the presence of castanospermine (Figure 7). In the presence of this inhibitor, the electrophoretic migration of cell-associated RGP(WT)T434 decreased, secretion of RGP(WT)T434 was almost completely blocked, and cell-associated RGP(WT)T434 was retained within the cells for a significantly longer time (Figure 8 and Table 1). In accord with previous findings with other proteins [e.g., Gross et al. (1983)], RGP(WT)434 secreted in the presence of castanospermine was partially Endo H resistant (Figure 9), indicating that this inhibitor did not completely block the processing pathway, even when a concentration of 2 mM (378 μg/mL) was used (data not shown). To confirm the importance of glucose removal for secretion of RGP(WT)T434, three other inhibitors were used (Figure 7). 1-Deoxynojirimycin inhibits both α-glucosidase I and II, australine inhibits the action of α-glucosidase I, and bromoconduritol inhibits α-glucosidase II. The effects of these three inhibitors on RGP(WT)T434 secretion were very similar to those obtained with castanospermine (Table 1), suggesting that initial removal of all three glucose residues is required for efficient secretion of RGP-(WT)T434.

To examine the importance of further *N*-glycan processing in secretion of RGP(WT)T434, 1-deoxymannojirimycin was used (Figure 7). With this inhibitor the electrophoretic migration of the cell-associated form of RGP(WT)T434 was

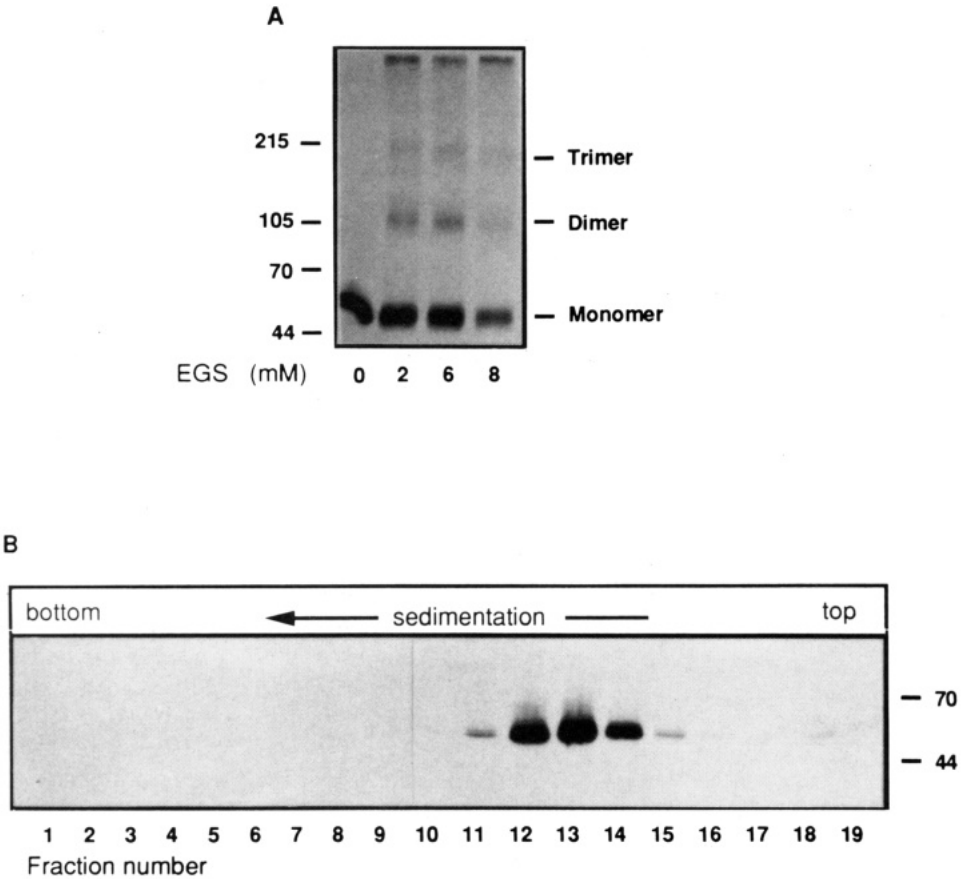

FIGURE 6: Oligomerization of RGP(WT)T434. (A) Analysis of oligomerization by chemical cross-linking. pRGP(WT)T434-transfected CHO cells were metabolically labeled with [^{35}S]methionine, and conditioned medium was incubated with or without the chemical cross-linker EGS, as described in Experimental Procedures. After cross-linking, the proteins were immunoprecipitated with polyclonal rabbit anti-rabies virus antiserum, separated by nonreducing SDS−PAGE (4−12% gradient gel), and visualized by autoradiography. The concentration of EGS is indicated below each lane. The positions of RGP(WT)T434 trimers, dimers, and monomers are indicated. The positions of the prestained electrophoretic standards are also shown. (B) Analysis of oligomerization by sucrose gradient centrifugation. pRGP(WT)T434-transfected CHO cells were metabolically labeled with [^{35}S]methionine. The conditioned medium, diluted with MNT buffer at pH 7.4, was layered onto 5−20% sucrose gradients. The samples were centrifuged, and fractions were collected, as described in Experimental Procedures. RGP(WT)T434 was immunoprecipitated with polyclonal rabbit anti-rabies virus antiserum, separated by nonreducing SDS−PAGE, and visualized by autoradiography. The positions of the prestained electrophoretic standards are shown.

slightly slower, and that of the secreted form was significantly faster (Figure 10). However, the level of secretion of RGP(WT)T434 was not changed. In addition, the secreted RGP(WT)T434 was completely Endo H sensitive (Figure 9). Thus, 1-deoxymannojirimycin affects processing of RGP(WT)T434, but does not inhibit the level or kinetics of its secretion. Swainsonine, another inhibitor of later *N*-glycan processing (Figure 7), affected *N*-glycan processing of the recombinant glycoprotein (Figure 9), but did not block its secretion (Table 1).

The effects of all the inhibitors tested on secretion of RGP(WT)T434 are summarized in Table 1. Taken together, these results establish that (i) core glycosylation of RGP(WT)T434 is necessary for its secretion, (ii) the removal of all three glucose residues from the *N*-glycans is necessary and sufficient for secretion, and (iii) further processing from high mannose to complex type *N*-glycans is not necessary for secretion. The latter is interesting because RGP(WT)T434 secreted in the absence of glycosylation inhibitors possesses complex type *N*-glycans (Figure 5).

Influence of Glycosylation Inhibitors on Cell Surface Expression of Full-Length RGP. The influence of core glycosylation and *N*-glycan processing on cell surface expression of full-length RGP was examined in order to compare the results to those obtained with the secreted form of the protein. The current study used a method which specifically immunoprecipitates cell surface proteins (Figure 11). This approach demonstrated that cell surface full-length RGP contained complex type *N*-glycans (Figure 11A, lanes 2 and 3), whereas the cell-associated form contained high mannose type *N*-glycans (Figure 11B, lanes 2 and 3). These results are similar to those found with RGP(WT)T434 (Figure 5) where the secreted and cell-associated forms contained complex and high mannose type *N*-glycans, respectively.

Tunicamycin specifically blocked cell surface expression (Figure 11A, lane 4) but not intracellular synthesis (Figure 11B, lane 4) of full-length RGP, in contrast to cells incubated in the absence of inhibitor (Figure 11A,B, lane 2). Castanospermine significantly inhibited cell surface expression of full-length RGP (Figure 11A, lane 6). In addition, the small amount of full-length RGP which did reach the cell surface was partially Endo H resistant (Figure 11A, lane 7), again indicating that castanospermine did not completely inhibit glucosidase function. In contrast, 1-deoxymannojirimycin had no effect on the level of cell surface expression of full-length RGP (Figure 11A, lane 8), demonstrating that mannose trimming is not required for cell surface transport of this glycoprotein.

Taken together, these results show that initial trimming of the glucose residues on *N*-glycans is a necessary and

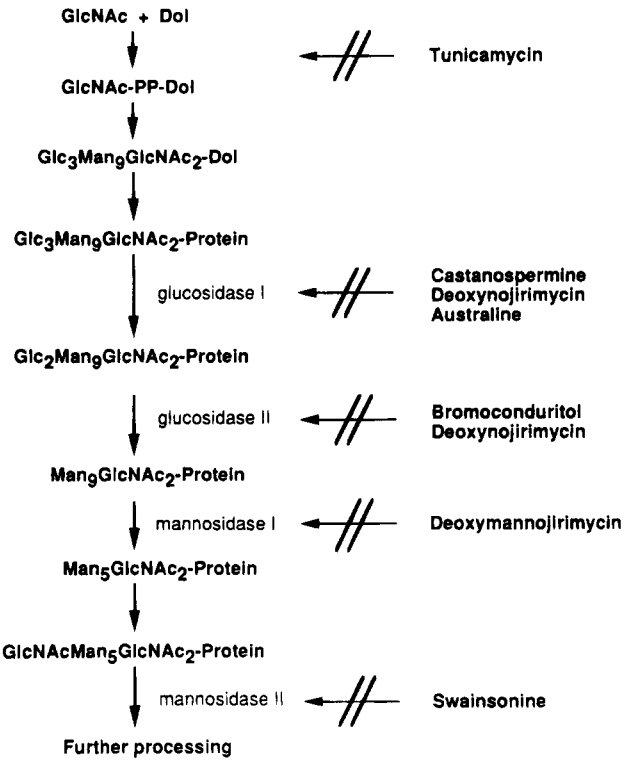

FIGURE 7: Pathway for the processing of N-linked oligosaccharides. This diagram outlines the initial steps in the processing of N-linked oligosaccharides. Core glycosylation is blocked by tunicamycin. *N*-Glycan processing can be limited by the different α-glucosidase or α-mannosidase inhibitors that block the pathway at the indicated steps.

Table 1: Influence of Glycosidase Inhibitors on Secretion of RGP /T)T434

inhibitor	concn	inhibition of secretion[a] (%)
tunicamycin	6 μM	92
castanospermine	260 μM	86
1-deoxynojirimycin	1 mM	90
australine	4 mM	77
bromoconduritol	1 mM	46
1-deoxymannojirimycin	1 mM	0
swainsonine	30 μM	1

[a] pRGP(WT)T434-transfected CHO cells were metabolically labeled for 4 h with [^{35}S]methionine in the presence or absence of inhibitor, as described in Experimental Procedures. The conditioned media were separately immunoprecipitated with rabbit polyclonal anti-rabies virus antiserum, and the immunoprecipitates were separated by SDS−PAGE and visualized by autoradiography. The amount of RGP(WT)T434 secreted under each condition was quantified by densitometry. Inhibition of secretion of RGP(WT)T434 was calculated as {1 − [(amount secreted in the presence of inhibitor)/(amount secreted in the absence of inhibitor)]} × 100. Similar results were found when secretion was quantified by a phosphorimaging technique (data not shown).

sufficient condition for both secretion of RGP(WT)T434 and cell surface expression of membrane-anchored, full-length RGP.

DISCUSSION

RGP is the only glycoprotein on the viral surface and plays a critical role in rabies virus infection (Fishbein & Robinson, 1993; Wunner et al., 1988). It stimulates production of virus-neutralizing antibodies during infection and is effective as a subunit vaccine (Kieny et al., 1984), provided that it is glycosylated (Yelverton et al., 1983; Lathe et al., 1984). To study RGP structure and biosynthesis in more detail, we produced a secreted form of this glycoprotein, RGP(WT)-T434 (Figure 1). CHO cells stably transfected with RGP-(WT)T434 cDNA secreted the truncated product into conditioned medium (Figure 2). The antigenicity, immunogenicity, assembly, and glycosylation of this truncated form of RGP were studied. The influence of core glycosylation and *N*-glycan processing on RGP(WT)T434 secretion were also compared to those obtained with full-length RGP.

Immunological methods detect minor conformational differences that can discriminate between highly homologous proteins. To assess the antigenic similarity of recombinant RGP(WT)T434 secreted by transfected CHO cells and authentic RGP synthesized during viral infection, eight monoclonal antibodies were used. These antibodies, which recognize conformational epitopes on the extracellular domain of full-length RGP, all recognized RGP(WT)T434 (Figure 2B). In addition, previous studies using a naked DNA vaccine approach showed that pRGP(WT), encoding full-length RGP cDNA, functioned as a protective vaccine in mice (Xiang et al., 1994). Current studies using this method showed that pRGP(WT)T434 induced T cell (Figure 3) and B cell (Figure 4) immune responses that were very similar to those induced by pRGP(WT). The differences between these results with pRGP(WT)T434 and those reported for G_s (Dietzschold et al., 1983) may be due to issues such as differences in the method of immunization, aggregation of purified G_s, and possible denaturation or modification of G_s during purification. Nonetheless, the current results show that both RGP(WT) and RGP(WT)T434 are immunogenic and that both induce a T cell response and antibodies to rabies virus. In addition, the current results indicate that the transmembrane and cytoplasmic domains of RGP are not required for the extracellular domain to be appropriately antigenic or immunogenic.

We next examined assembly of RGP(WT)T434. Previous studies showed that oligomerization is required for intracellular transport and cell surface expression of many viral glycoproteins [for a review, see Doms et al. (1993)]. Misfolding, failure to assemble, or assembly of misfolded monomers precludes exit of glycoproteins from the endoplasmic reticulum. Sucrose density gradient sedimentation studies previously revealed that full-length RGP sedimented as a monomer (Gaudin et al., 1992; Whitt et al., 1991), even under conditions in which the homologous glycoprotein, VSV G protein (Rose et al., 1982), sedimented as a trimer (Doms et al., 1987). Similarly, secreted RGP(WT)T434 only sedimented as a monomer (Figure 6). In contrast, chemical cross-linking identified dimers and trimers of secreted RGP-(WT)T434 (Figure 6), similar to those found previously with full-length RGP (Whitt et al., 1991) and VSV G protein (Doms et al., 1987). Thus, the extracellular domain of RGP possesses sufficient structural information to induce appropriate oligomerization and secretion of RGP(WT)T434. The ability of RGP to assemble into multimers in the absence of the transmembrane and cytoplasmic domains is similar to results obtained with other glycoproteins [for example, see Navarro et al. (1993)]. However, in other cases the transmembrane and cytoplasmic domains contribute significantly to proper assembly [for example, see Cosson and Bonifacino (1992)].

Since appropriate glycosylation is important for the proper folding, assembly, transport, antigenicity, and function of

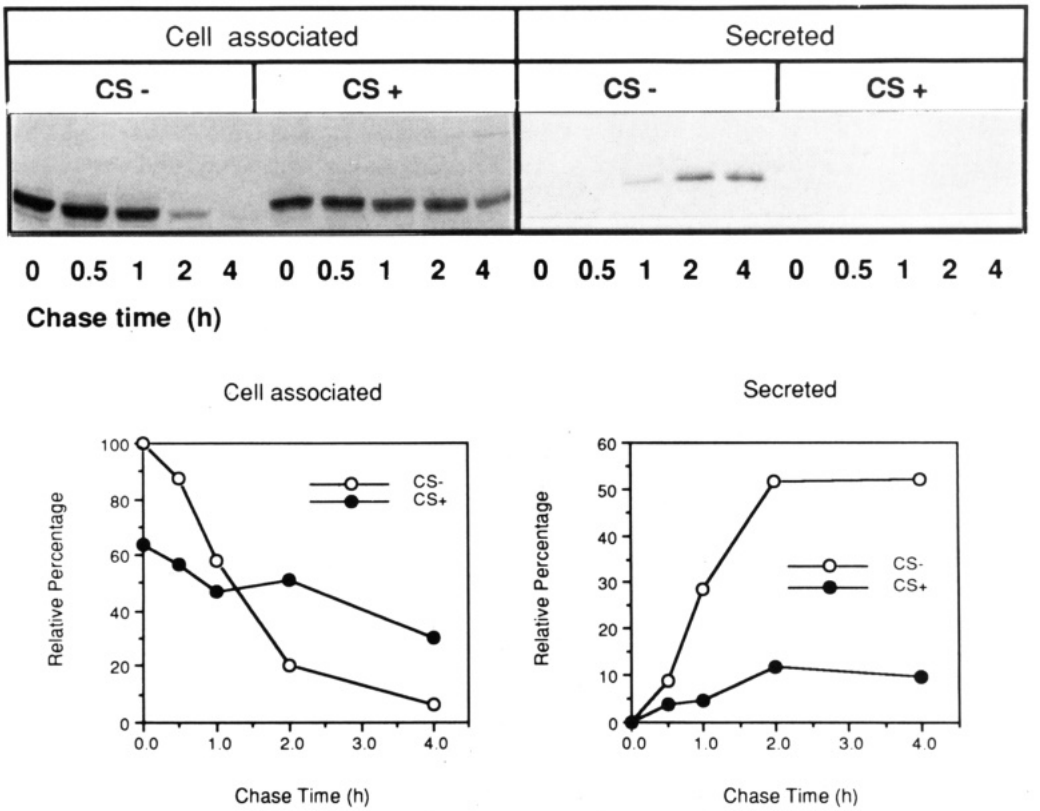

FIGURE 8: Expression of RGP(WT)T434 by CHO cells grown in the presence of castanospermine. pRGP(WT)T434-transfected CHO cells were grown in the presence (CS+) or the absence (CS−) of castanospermine (50 μg/mL) for 2 h and then pulse-labeled for 20 min with [^{35}S]methionine and chased with excess cold methionine in the presence or the absence of inhibitor. In the top panel, the cell lysates and conditioned media were immunoprecipitated with rabbit polyclonal anti-rabies virus antiserum and analyzed by SDS−PAGE and autoradiography. Chase intervals are indicated. In the bottom panel, the gels were analyzed using a phosphorimaging technique. The amounts of RGP(WT)T434 that were synthesized and secreted were calculated as a relative percentage normalized to the results at 0 chase time in the absence of inhibitor. Thus, the 100% value was defined as the sum of the values of both the cell-associated and secreted forms at 0 chase time in the absence of inhibitor.

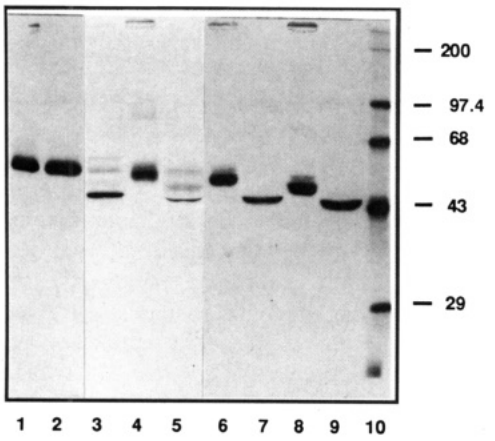

FIGURE 9: Endoglycosidase sensitivity of RGP(WT)T434 secreted in the presence of processing inhibitors. pRGP(WT)T434-transfected CHO cells were metabolically labeled for 4 h with [^{35}S]-methionine in the absence of inhibitors (lanes 1−3) or in the presence of castanospermine (lanes 4 and 5), swainsonine (lanes 6 and 7), or 1-deoxymannojirimycin (lanes 8 and 9). The conditioned media were immunoprecipitated with rabbit polyclonal anti-rabies virus antiserum and incubated with Endo H (lanes 2, 5, 7, and 9), with PNGase F (lane 3), or in buffer alone (lanes 1, 4, 6, and 8). The immunoprecipitates were then analyzed by SDS−PAGE and autoradiography. ^{14}C-labeled electrophoretic standards are shown in lane 10. The results were visualized by autoradiography following exposure for 24 h (lanes 1, 2, and 6−10) or 72 h (lanes 3−5).

many glycoproteins [for reviews, see Doms et al. (1993) and Opdenakker et al. (1993)], the role of glycosylation in RGP-(WT)T434 secretion was examined. Analysis of RGP(WT)-T434 produced by transfected cells showed that the cell-associated form was completely Endo H sensitive, whereas the secreted form was completely Endo H resistant (Figure 5). Thus, the cell-associated form represented the biosynthetically immature form of the glycoprotein containing only high mannose type *N*-glycans, whereas the secreted, mature form contained only complex type *N*-glycans. The current studies also showed that cell surface and cell-associated full-length RGP were completely Endo H resistant and Endo H sensitive, respectively (Figure 11). Therefore, core glycosylation and processing of RGP(WT)T434 and full-length RGP are similar. Further detailed biochemical studies are required to determine whether the *N*-glycans on secreted RGP(WT)T434 and cell surface RGP are identical.

Tunicamycin was used to study the role of core glycosylation in secretion and cell surface expression of the truncated and full-length forms of RGP, respectively. Since tunicamycin prevented both cell surface expression of full-length RGP [Figure 11 and Burger et al. (1991)] and secretion of RGP(WT)T434 (Table 1), some degree of N-linked glycosylation is crucial for appropriate intracellular transport of both proteins. Thus, removal of the transmembrane and cytoplasmic domains of RGP did not alter the importance of *N*-glycans in intracellular transport of this protein.

To examine the role of *N*-glycan processing on RGP(WT)-T434 secretion, other inhibitors were used (Table 1 and Figure 7). Inhibitors that allow core glycosylation but block

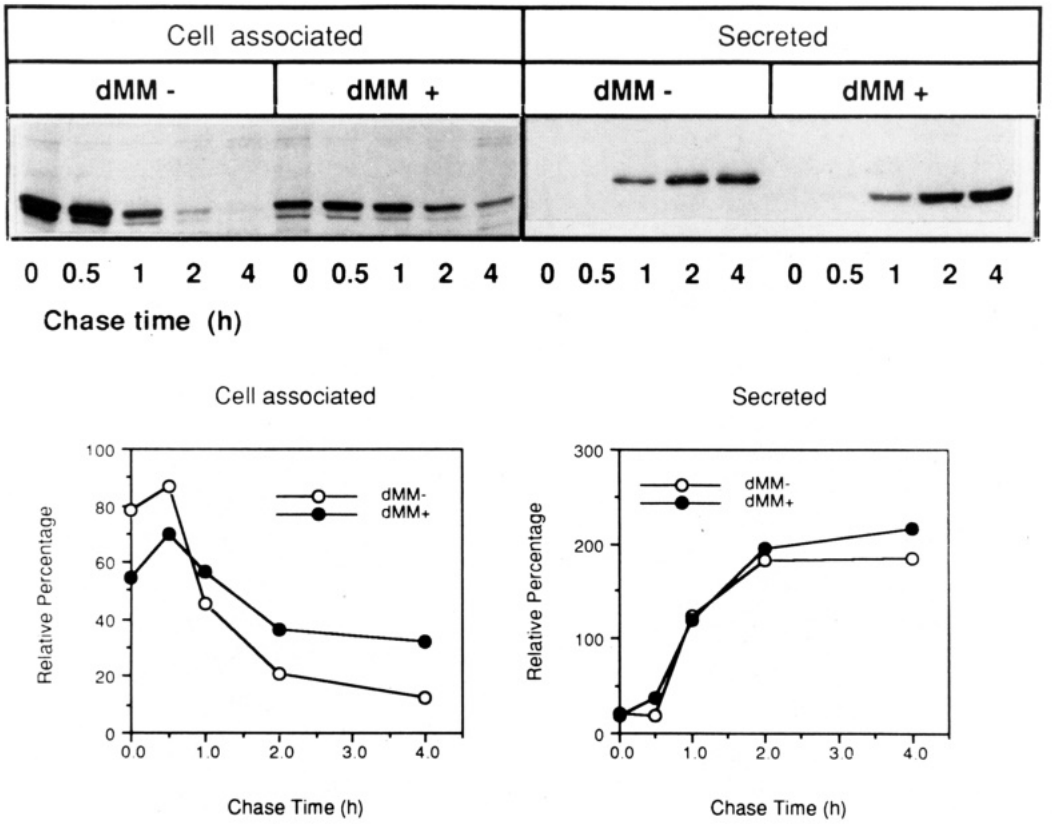

FIGURE 10: Expression of RGP(WT)T434 by CHO cells grown in the presence of 1-deoxymannojirimycin. pRGP(WT)T434-transfected CHO cells were grown in the presence (dMM+) or the absence (dMM−) of 1-deoxymannojirimycin (1 mM) for 2 h and then pulse-labeled for 20 min with [^{35}S]methionine and chased with excess cold methionine in the presence or the absence of inhibitor. Cell lysates and conditioned media were immunoprecipitated with rabbit polyclonal anti-rabies virus antiserum and analyzed by SDS−PAGE and autoradiography (top panel). Chase intervals are indicated. The gels were also analyzed by a phosphorimaging technique, and the amounts of cell-associated and secreted RGP(WT)T434 were quantified (bottom panel) as described in Figure 8.

removal of glucose residues (e.g., castanospermine, 1-deoxynojirimycin, australine, and bromoconduritol) significantly blocked secretion of RGP(WT)T434 (Figure 8 and Table 1). These inhibitors resulted in the intracellular accumulation (Figure 8), presumably in the endoplasmic reticulum, of RGP(WT)T434 containing glucosylated *N*-glycans. Although an endomannosidase has been described that can deglucosylate $Glc_3Man_9GlcNAc_2$ by releasing Glc_3Man (Moore & Spiro, 1992), this enzyme is not found in CHO cells (Ray et al., 1991; Hiraizumi et al., 1993). In addition, if the glucose residues were removed from RGP(WT)T434 by another mechanism, the glycoprotein containing $Man_9GlcNAc_2$ *N*-glycans would have been secreted (see the results with 1-deoxymannojirimycin, Figures 9 and 10).

Inhibition of glucosidase activity also blocks secretion or cell surface expression of other glycoproteins [for example, see Gross et al. (1983); Moore and Spiro (1993), Schlesinger et al. (1984), and Simsolo et al. (1992)]. In several cases glucosylated proteins were rapidly degraded [for example, see Moore and Spiro (1993)] or retained intracellularly (Simsolo et al., 1992), suggesting that the glucosylated *N*-glycan functions as a specific signal for glycoprotein retention and/or degradation in the endoplasmic reticulum. However, since glucosidase inhibitors do not inhibit secretion or cell surface expression of other glycoproteins [for example, Collet and Fielding (1991)], it is unlikely that the glucosylated *N*-glycan is recognized as a signal by a specific receptor, but rather that it plays a more subtle role, such as being involved in protein folding. In some cases, glucose residues on *N*-glycans have a temperature-dependent effect on glycoprotein transport (Schlesinger et al., 1984), suggesting that the persistence of the glucose residues may prevent completion of the folding and/or assembly of particular glycoproteins, leading to retention and/or degradation in the endoplasmic reticulum.

The role of mannose trimming on RGP(WT)T434 secretion was examined next. Exposure to mannosidase inhibitors resulted in secretion of an Endo H-sensitive form of RGP(WT)T434 (Figure 9 and Table 1). Therefore, RGP(WT)T434 secretion only depends on trimming of glucose residues and does not require further *N*-glycan processing, demonstrating that complex type *N*-glycans are not necessary for secretion. Others found similar results concerning secretion or cell surface expression of specific glycoproteins [for example, see Simsolo et al. (1992)].

The availability of full-length and truncated forms of RGP allowed a comparison of the effects of glycosylation inhibitors on expression of membrane-anchored and soluble forms of the same glycoprotein. These results showed that core glycosylation and subsequent glucose trimming are necessary and sufficient for both cell surface expression of full-length RGP and secretion of RGP(WT)T434 (Figures 8−11; Table 1; Burger et al., 1991). Few previous reports examined this issue. Tunicamycin blocked cell surface expression of the full-length form (Hurtley et al., 1989) and secretion of the soluble form (Singh et al., 1990) of influenza virus hemagglutinin. Although addition of truncated *N*-glycans to the full-length form led to a temperature-sensitive defect in folding, assembly, and transport (Hearing et al., 1989), analogous studies with the soluble form were not described.

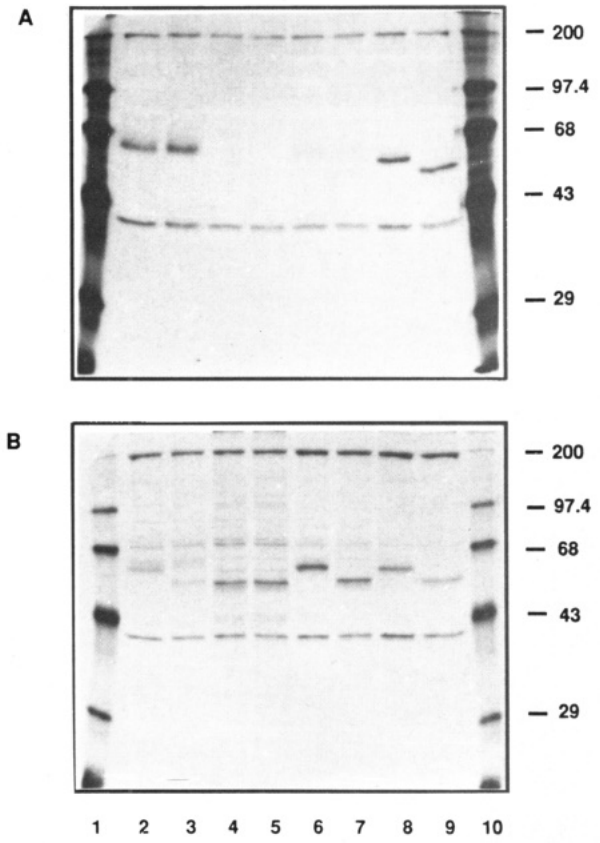

FIGURE 11: Influence of processing inhibitors on cell surface expression of full-length RGP. pRGP(WT)-transfected CHO cells were metabolically labeled for 4 h with [^{35}S]methionine in the absence of inhibitors (lanes 2 and 3) or in the presence of tunicamycin (lanes 4 and 5), castanospermine (lanes 6 and 7), or 1-deoxymannojirimycin (lanes 8 and 9). Cell surface full-length RGP was immunoprecipitated with rabbit polyclonal anti-rabies virus antiserum (panel A), as described in Experimental Procedures. Intracellular full-length RGP was subsequently immunoprecipitated from the immunodepleted detergent lysate (panel B), as described. Immunoprecipitated full-length RGP was incubated with Endo H (lanes 3, 5, 7, and 9) or in buffer alone (lanes 2, 4, 6, and 8). The immunoprecipitates were then separated by SDS−PAGE and visualized by autoradiography. ^{14}C-Labeled electrophoretic standards are shown in lanes 1 and 10. The autoradiographs were exposed for 24 h (panel B) or 72 h (panel A).

Studies with the α subunit of human chorionic gonadotropin, normally a secreted product, demonstrated that the soluble glycoprotein and a recombinant membrane-anchored form behaved differently with respect to glycosylation. When N-linked glycosylation was blocked, the soluble form was still secreted, but the membrane-anchored form was not expressed at the cell surface (Guan et al., 1988). Additional studies with the membrane-anchored form showed that although tunicamycin blocked cell surface expression, mannosidase inhibition had no effect (Fukuda et al., 1988); no results with glucosidase inhibitors were reported.

In summary, RGP(WT)T434 is appropriately glycosylated, folded, assembled, and transported. It also retains the antigenicity and immunogenicity of authentic full-length RGP. In addition, since secreted RGP(WT)T434 is soluble in aqueous solutions, it may be more amenable than the full-length glycoprotein to structural analysis by methods such as X-ray crystallography. In particular, since RGP(WT)-T434 was abundantly secreted by CHO cells in the presence of 1-deoxymannojirimycin, it will be possible to purify an aqueous soluble form of RGP with homogeneous *N*-glycans.

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