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Differential Transport and Processing of Variant Mouse Mammary Tumor Virus Glycoproteins

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Abstract The transport and proteolytic processing of two individual gene isolates of the mouse mammary tumor virus (MMTV) glycoprotein were compared in transfected rat HTC hepatoma cells. Plasmids were constructed such that the MMTV glycoprotein genes were constitutively expressed from the promoter within the Rous Sarcoma Virus 5′ Long Terminal Repeat in the absence of other MMTV proteins. An isolate of the GR strain MMTV glycoprotein was efficiently transported and processed resulting in the localization of MMTV glycoproteins at the cell surface and in the extracellular environment. Moreover, the kinetics of acquisition of endoglycosidase H resistant oligosaccharide side chains and the rate of endoproteolytic cleavage of the glycosylated polyprotein expressed in transfected cells were virtually identical to that observed in viral-infected rat hepatoma cells. In contrast, a natural variant of the C3H strain MMTV glycoprotein expressed in transfected cells was retained in an intracellular compartment by a heavy chain binding protein (BiP)-independent pathway in an endoglycosidase H sensitive and uncleaved form. This MMTV glycoprotein isolate was retained early in the exocytic pathway and displayed a half-life of approximately 45 min in transfected cells. Only a minor fraction of the expressed C3H variant glycoprotein was detected at the cell surface but was not externalized. Our results suggest that the variant C3H MMTV glycoprotein contains one or more mutations that preclude its efficient transport through the exocytic pathway. © 1992 Wiley-Liss, Inc.

Key words: cell surface localization, retrovirus glycoproteins, polyprotein cleavage, RER retention, membrane protein trafficking

The envelope glycoproteins of retroviruses utilize host cell exocytic machinery for proper synthesis, processing, and transport to the cell surface. Typically, the envelope glycoprotein is initially translated on RER-associated ribosomes as a single glycosylated polyprotein [Klenk and Rott, 1980; Sabatini et al., 1982], and in some cell types, viral glycoproteins appear to oligomerize prior to their exit from the RER [Copeland et al., 1986, 1988; Hurtley and Helenius, 1989; Rose and Doms, 1988]. During transport into and through the Golgi, the oligosaccharide side chains are matured into complex forms [Dunphy and Rothman, 1985; Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985; Presper and Heath, 1985] and the polyprotein undergoes endoproteolytic cleavage, generally at di- and tetrabasic amino acid motifs, to generate several stable maturation products [Bosch and Pawlita, 1990; Jain et al., 1991; Klenk and Rott, 1980; Perez and Hunter, 1987; Sarkar and Racevskis, 1983; Stein and Engleman, 1990; Wold, 1981]. The endoproteolytic cleavage reaction has been postulated to be important for assembly of viral particles and for virion infectivity [Bosch and Pawlita, 1990; Klenk and Rott, 1980; McCune et al., 1988]. After the release of biologically active virions, the viral envelope glycoproteins participate in cell surface recognition and membrane fusion events which can contribute to the host-range and tissue tropism. Conceivably, specific structural features within an individual retroviral glycoprotein may dictate the selective transport within the exocytic pathway and/or modulate assembly into functional virions.

We have utilized mouse mammary tumor virus (MMTV) glycoproteins to examine the exocytic trafficking of proteolytically processed membrane glycoproteins. The MMTV genome encodes the glycosylated envelope glycoproteins, gag proteins which form the viral core, and a polymerase with reverse transcriptase and inte-

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grase function. A variety of tumor cell lines derived from different tissues, such as rat HTC hepatoma cells, can be infected with MMTV [Ringold et al., 1977, 1979]. Therefore, viral-infected rat hepatoma cells have been particularly useful in examining the regulation of MMTV gene expression [Ringold, 1979; Yamamoto, 1985] and of the cellular requirements for MMTV protein trafficking in a cell type that does not contain secretory granules [Amacher et al., 1989; Bravo et al., 1991; Firestone, 1988; Firestone et al., 1982, 1986; Haffar et al., 1987, 1988; John et al., 1988; Winguth and Firestone, 1987]. In the presence of glucocorticoids, the exocytic pathway for the MMTV glycoproteins in the viral-infected HTC cell line, M1.54, is generally similar to that observed in virion producing mammary carcinoma cells [Firestone, 1988; Sarkar and Racevskis, 1983]. A 70 kDa MMTV glycoprotein precursor is initially co-translationally modified by the addition of five high-mannose glycosylamine-linked oligosaccharide side chains. During transport to the plasma membrane, a portion of the glycosylated polyprotein is cleaved at a Arg-Ala-Lys-Arg dibasic amino acid recognition motif by a cellular encoded endoprotease [Firestone, 1988; Haffar et al., 1988; Majors and Varmus, 1983] to yield two cleavage products, an aminoterminal portion of 50 kDa (gp50) and a carboxyterminal transmembrane fragment of 32 kDa (gp32). Our recent evidence suggests that this endoproteolytic cleavage occurs in the trans Golgi [L.J. Goodman, S.R. Kain, and G.L. Firestone, submitted]. In glucocorticoid-treated M1.54 cells, both non-sialylated (gp70) and sialylated (gp78) forms of the glycosylated polyprotein as well as the carboxyterminal fragment, gp32, are stably expressed at the cell surface as integral membrane glycoproteins [Firestone et al., 1986; Haffar et al., 1987, 1988]. M1.54 cells produce a small number of viral particles, although a 70 kDa polyprotein and the gp50 aminoterminal fragment can be externalized without an associated viral particle [Firestone, 1988]. We have documented that primarily constitutive trafficking events, and at least one glucocorticoid-regulated trafficking reaction, act on proviral-encoded MMTV glycoproteins during transport to the cell surface and extracellular environment in viral-infected and transfected HTC cells [Platt et al., 1991; Platt and Firestone, 1991].

Similar to other cellular encoded membrane glycoproteins, individual structural regions within the MMTV glycoprotein may be required for proper folding, oligomerization, and transport dependent upon the host cell trafficking machinery. By expression of MMTV glycoprotein carboxyterminal truncations, we have recently shown that the hydrophobic region of the ectodomain (amino acids 457–483) and a short contiguous hydrophilic region within the carboxyterminal maturation product, gp32, may modulate MMTV glycoprotein folding and subsequent transport [Platt et al., 1991]. As part of our studies on the regulation of MMTV glycoprotein trafficking, natural MMTV glycoprotein variants encoded by individual isolates of the C3H or GR strains of the virus were linked to the constitutive Rous Sarcoma Virus promoter and expressed in transfected rat HTC hepatoma cells. Previous DNA sequencing information has shown that these MMTV glycoprotein gene variants diverge by 30 single base pair changes, including single base insertions and deletions which result in several amino acid differences [Majors and Varmus, 1983; Redmond and Dickson, 1983]. Analysis of MMTV glycoprotein trafficking revealed that the MMTV glycoprotein encoded by a natural C3H variant remained primarily in an intracellular compartment in an endo H sensitive and uncleaved form, whereas the MMTV glycoprotein expressed from a GR isolate was efficiently processed, transported, and externalized. These results suggest that the variant C3H MMTV glycoprotein is not competent for exocytic transport to the cell surface and proteolytic processing.

METHODS

Materials

All media and sera were purchased from the University of California, San Francisco Tissue Culture Facility. L-[^{35}S]Methionine (1,000 Ci/mmole) was obtained from Amersham Corporation (Arlington Heights, IL). Dexamethasone and tunicamycin were obtained from Sigma Chemical Co. (St. Louis, MO). Endoglycosidase H and Peptide-N-glycosidase F were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Pansorbin was acquired from Calbiochem-Behring Corporation (San Diego, CA). The goat anti-gp52 antiserum used in the immunofluorescence studies was obtained from the NIH repository (Bethesda, MD), and the fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of swine anti-goat IgG was purchased from Organon Teknika-Cappel (Malvern, PA).

The anti-MMTV and preimmune sera used for immunoprecipitations [Cardiff et al., 1978] were generous gifts of L.J.T. Young and R.D. Cardiff (Department of Pathology, University of California, Davis). The anti-BiP monoclonal antibody was a generous gift of David G. Bole [Bole et al., 1986]. The plasmid pGR21, containing a GR strain MMTV provirus, was a generous gift of Helmut Ponta [Salmons et al., 1985] and pLEL, which contains the C3H strain MMTV glycoprotein, was a generous gift of Ken Zaret (Division of Biology and Medicine, Brown University, Providence, RI). All other reagents were of the highest available purity.

Cells and Method of Culture

M1.54 is a cloned cell line isolated from MMTV-infected populations of rat HTC hepatoma cells [Ringold et al., 1977]. Hrel3 cells are rat HTC hepatoma cells stably transfected with an isolate of the exogenous milkborn C3H strain MMTV glycoprotein and WT cells [Platt and Firestone, 1991] are transfected with an isolate of the exogenous GR strain MMTV glycoprotein. All cell lines were propagated as monolayers on Corning Tissue Culture plates in DME medium supplemented with 10% horse serum in a humidified atmosphere of air:CO_2 (95:5). Where appropriate, the culture medium contained 1 mg/ml of the neomycin analog G418. For tunicamycin treatment, 2 h prior to radiolabeling, monolayers were exposed to tunicamycin at a final concentration of 5 μg/ml. Tunicamycin was made as a stock solution of 1 mg/ml in 0.1 N NaOH and stored at −20°C in the dark.

Construction of pRSVEL in Which an Exogenous Isolate of the C3H Strain MMTV Glycoproteins Is Driven by the Constitutive RSV LTR in the Absence of Other Viral Products

The pLEL plasmid encodes a C3H isolate of the MMTV glycoprotein gene [Majors and Varmus, 1983] under control of the glucocorticoid regulatable MMTV LTR. To place the MMTV glycoprotein gene under transcriptional control of the RSV 5′ LTR, the PvuII/SacI env containing fragment was excised from pLEL which eliminates the 5′ MMTV LTR. This fragment was gel purified, and the protruding ends were filled in with klenow fragment of DNA polymerase yielding blunt ends onto which were ligated Hind III linkers. Hind III compatible ends were generated by digestion of the linkers with Hind III restriction endonuclease and the fragment sticky-end ligated to a Hind III linearized and phosphatased plasmid (pRSV-poly) containing the RSV 5′ LTR immediately preceeding the unique Hind III restriction enzyme site. The generated plasmid, pRSVEL, encodes the C3H MMTV glycoprotein gene under transcriptional control of the constitutive promoter RSV LTR.

Construction of pR22 in Which an Exogenous Isolate of the GR Strain MMTV Glycoprotein Is Driven by the Constitutive RSV LTR in the Absence of Other Viral Products

Standard recombinant DNA technology was used to construct a plasmid expressing a *GR* isolate of the MMTV glycoprotein gene in the absence of gag and pol genes. The gag and pol genes were first excised away from a provirus-containing plasmid pGR21 [Salmons et al., 1985] at the Xho I and Hind III sites, which leaves the RNA splice donor and splice acceptor sites intact for efficient MMTV glycoprotein expression. After the 5′ protruding ends were filled in and Hind III linkers blunt-end ligated, the plasmid pGR22 was formed by ligation at the new Hind III sites; this plasmid contains the *glucocorticoid* regulated 5′ LTR. To eliminate the MMTV 5′ LTR, the Pvu II site just 3′ of the 5′ MMTV LTR and upstream of the splice donor site and the Aat II site in the pBR322 sequences just 3′ of the 3′ MMTV LTR were used to excise the MMTV glycoprotein gene. To generate a plasmid in which expression of wild-type MMTV glycoprotein would be driven constitutively by the RSV LTR (pR22), the fragment containing the MMTV glycoprotein gene and 3′ LTR sequences was sticky-end ligated to an RSV LTR–containing plasmid at the SmaI and AatII sites. Thus, pR22 contains the wild-type MMTV glycoprotein gene, including the 3′ LTR, driven by the constitutive promoter within the 5′ RSV LTR.

Transfection and Expression of MMTV Glycoprotein Genes

The plasmid pRSVEL was cotransfected along with pSV2-neo [Southern and Berg, 1982] into rat hepatoma cells using calcium phosphate mediated transfection [Graham and van der Eb, 1973]. Two days after transfection, cells were replated into selective media containing 1 mg/ml G418. After 3 weeks, individual cell colonies were picked, expanded in culture, and tested for expression of MMTV RNA and protein. Single cell derived colonies were picked and expanded after growth in selective media for 2 weeks. Cell

clones were initially screened for MMTV glycoprotein gene expression by RNA cytoblot analysis [White and Bancroft, 1982]. Clones expressing high amounts of MMTV-specific RNA were further screened for the production of MMTV glycoproteins by immunoprecipitation of [^{35}S]methionine-labeled cell extracts with MMTV specific antibodies. The plasmids containing the GR strain MMTV glycoprotein driven by the RSV LTR (pR22) were introduced into cells using the lipofectin technique [Felgner et al., 1987]. Lipofection was performed according to the manufacturer's recommendations except for the following: DNA (100 ng of pSV2-neo and 650 ng of each construct) and lipofectin reagent were each diluted into 0.5 ml of Opti-mem medium (Bethesda Research Labs); the two were mixed and allowed to stand at room temperature for 10 min. The mixture was added to cells and allowed to incubate for 8 h at 37°C. The reaction was stopped by aspirating the mixture and then adding DME supplemented with 10% horse serum. After 36 to 48 h, cells were passaged into serum supplemented medium containing 1.2 mg/ml G418.

Indirect Immunofluorescence

Transfected and untransfected HTC cells were seeded on glass coverslips and cultured overnight. The cells were washed 3 times with phosphate buffered saline (PBS) and fixed by incubation with 3.7% formaldehyde in PBS containing 0.1 M glycine (PBS-glycine). To stain intracellularly, cells were permeabilized with 0.5% Triton X-100, 300 mM sucrose, PBS for 10 min at room temperature [John et al., 1987; Murname and Painter, 1982], while for cell surface staining, this step was omitted. The cells were then washed 3 times with PBS-glycine containing 1% bovine serum albumin (BSA) prior to incubation with a 1:350 dilution of polyclonal anti-gp52 IgG for 30 min at room temperature. After washing 3 times with PBS-glycine-BSA, cells were incubated for 30 min at room temperature with a 1:400 dilution of fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of swine anti-goat IgG. The cells were then washed 3 times in PBS-glycine-BSA and mounted on slides in 90% glycerol:10% 100 mM Tris-HCl, pH 7.5. The slides were stored at −20°C until visualized and photographed using a Zeiss fluorescent microscope.

Steady State Radiolabeling and Harvesting of Cellular and Secreted Fractions

Prior to radiolabeling, monolayer cultures were incubated for 30 min in methionine-free labeling media; during the last 4 h of appropriate hormonal treatment, the cells were radiolabeled with 25 μCi/ml of [^{35}S]methionine. The secreted fraction was harvested from radiolabeled cells by centrifugation at 2,000*g* for 10 min. The supernatant fractions, containing the externalized MMTV glycoproteins, were brought to 1% Triton X-100, 0.5% deoxycholate, and 5 mM EDTA just prior to immunoprecipitation. For the cellular fractions, radiolabeled monolayers were washed 3 times in ice-cold phosphate buffered saline (PBS) and cells released from the culture plate in 1 mM EDTA in PBS, centrifuged at 1,000*g* for 10 min and then solubilized in immunoprecipitation buffer (1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl, pH 7.5). The solubilized material was then centrifuged at 15,000 rpm (Sorvall SE12 rotor) for 15 min. The total level of radiolabeled protein in each sample was determined by precipitation with 10% trichloroacetic acid and scintillation counting to normalize the amount of secreted and cellular fractions to be immunoprecipitated.

Pulse-Chase Radiolabeling

Cells were placed in methionine-free labeling medium for 30 min before addition of 50 μCi/ml [^{35}S]methionine. After a 10 min pulse, cells were washed 3 times in PBS, then chased in medium containing excess unlabeled methione for the indicated time points. Cell extacts were harvested and prepared for immunoprecipitation as described below.

Immunoprecipitation, SDS Polyacylamide Gel Electrophoresis, and Flouroradiography

Immunoprecipitations of the cellular and secreted MMTV glycoproteins using total anti-MMTV antibodies were performed as described previously [Firestone and Winguth, 1990; Platt et al., 1986]. The immunoprecipitation of cell surface [^{35}S]methionine-labeled MMTV glycoproteins was accomplished by a modification of a method [Krangel et al., 1979] as we have previously described [Haffar et al., 1987]. SDS polyacrylamide gel electrophoresis of immunoprecipitated material and subsequent flourorad-

iography were also accomplished as previously described [Haffar et al., 1987] except with the modification in the fluorographic procedure in which the gels were soaked in 100 mM salicyclic acid for 1 h prior to drying.

Endoglycosidase H Digestion

Staph A pellets resulting from the immunoprecipitations were resuspended in 50 μl 1% SDS in 100 mM sodium citrate (pH 5.5), boiled, and the Staph A fixed cells removed by centrifugation. The supernatant was added to 100 μl of 100 mM sodium citrate containing 0.8 mU/μl endoglycosidase H and incubated overnight at 37°C. The reaction was terminated by adding the 150 μl reaction mixture to eppendorf tubes containing 400 μl immunoprecipitation buffer and 100 μl 50 mg/ml BSA. Immunoprecipitation was continued by adding MMTV antibodies and Staph A.

Peptide-N-Glycosidase F Digestion

Final Staph A pellets were resuspended in 45 μl 1% SDS in phosphate buffered saline (PBS), boiled for 3 min, then centrifuged to pellet away the Staph A. The resulting supernatant fraction was divided into two 20 μl portions; a 20 μl reaction mixture composed of 1 μl (0.2 units) Peptide-N-glycosidase F (PNGase F), 4 μl 25% n-octylglucoside, and 15 μl PBS was added to one portion while the second control portion received 4 μl 25% n-octylglucoside and 16 μl PBS. After an overnight incubation at 37°C, the reactions were terminated by evaporation in a savant vacuum centrifuge and resuspended in SDS-PAGE sample buffer and 80 mM DTT prior to electrophoresis as described above.

Coprecipitation With Anti-BiP Monoclonal Antibodies

The coprecipitation for BiP-binding proteins using anti-BiP monoclonal antibodies was performed as a modification of previously described procedures [Hurtley et al., 1989; Machamer et al., 1990]. Radiolabeled cells were lysed on ice with 1 ml of 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 0.15 μ/ml aprotinin, 10 mM glucose, and 5 μ/ml hexokinase. Lysed cells were scraped into a 1.5 ml Eppendorf tube on ice, vortexed, spun in an Eppendorf centrifuge at 4°C for 10 min, and the resulting supernant fraction transferred to an ice-cold Eppendorf tube. Trichloroacetic acid precipitation was performed to determine incorporation of [^{35}S]methionine into macromolecular material in order to normalize the immunoprecipitation to similar protein content. To initiate the BiP immunoprecipitation, 60 μl of the culture supernate harvested from hybridomas secreting the BiP monoclonal antibody (Mab) were incubated with 30 μl of recombinant Protein A conjugated to cross-linked agarose beads (Repligen, Cambridge, MA) and allowed to incubate for 60 min while rotating at room temperature. During this incubation, harvested radiolabeled cell extracts were pre-absorbed with Pansorbin for 15 min on ice. The radiolabeled cell extracts were spun in an Eppendorf centrifuge for 3 min at 4°C and the supernant fractions transferred to ice-cold Eppendorf tubes. After the BiP Mab/protein A–agarose incubation was complete, the immunocomplex was pelleted and washed 3 times with a washing buffer (150 mM NaCl, 0.1% Triton X-100, 10 mM Tris, pH 7.4). The preabsorbed cell extracts were then added to the washed BiP Mab/protein A–agarose pellets, the pellets resuspended by vortexing, and the mixture allowed to incubate while rotating for 1 h at 4°C. The mixture was then centrifuged at 4°C and the pellet washed thrice with ice-cold washing buffer. All centrifugations for the washing procedure were performed at 4°C. SDS gel sample buffer containing 80 mM DTT was added to the final pellets and the mixture boiled and then spun in an Eppendorf centrifuge. The supernates were collected and fractionated in SDS polyacrylamide gels as described earlier.

RESULTS

Differential Localization of Variant MMTV Glycoproteins Constitutively Expressed in Transfected HTC Cells

As one approach to define structural domains that may be important for the trafficking of membrane-associated glycoproteins that undergo endoproteolytic cleavage, the transport and processing of two natural MMTV glycoprotein variants were compared in transfected rat HTC hepatoma cells. Plasmids were constructed such that the C3H [Majors and Varmus, 1983] and the GR [Salmons et al., 1986] MMTV glycoprotein genes were constitutively *expressed from* the RSV promoter in the absence of the nonglycosylated viral proteins but containing the 3′ MMTV long terminal repeat. Each plasmid was

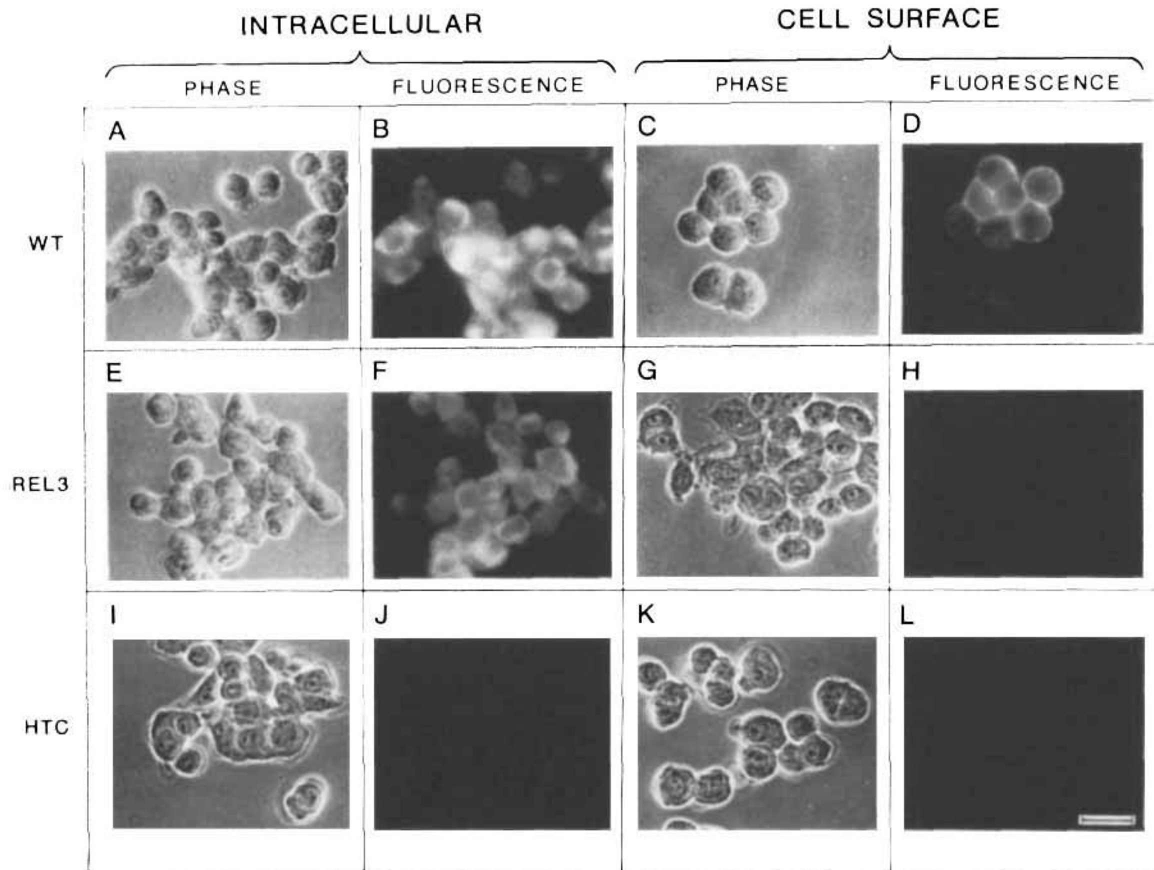

Fig. 1. Localization of cell surface and intracellular MMTV glycoproteins by indirect immunofluorescence. WT cells (**A–D**; transfected with the exogenous GR strain MMTV glycoprotein gene), Hrel3 cells (**E–H**; transfected with the exogenous C3H strain MMTV glycoprotein gene), and untransfected HTC cells (**I–L**) were seeded on glass coverslips for 24 h. Indirect immunofluorescence of permeabilized (A,B,E,F,I,J) and unpermeabilized (C,D,G,H,K,L) cells using goat anti-MMTV glycoprotein primary antibodies and fluorescein-conjugated goat swine anti-goat IgG secondary antibodies was carried out as described in the text. The cells were visualized by both phase and fluorescent microscopy; the bar represents 60 μm.

cotransfected into rat HTC hepatoma cells along with the neomycin resistance gene. Single cell derived populations expressing the highest level of transcripts for the C3H MMTV glycoprotein were designated Hrel3, while those expressing the GR MMTV glycoprotein transcripts were designated WT. The localization of intracellular and cell surface MMTV glycoproteins was initially examined by indirect immunofluorescence. In unpermeabilized fixed cells, WT cells displayed a bright immunofluorescent pattern on the cell surface, which is specific for cell-surface-associated GR MMTV glycoproteins, whereas the C3H variant MMTV glycoproteins were not detected at the cell surface in Hrel3 cells (Fig. 1, D vs. H). In contrast, when the total MMTV glycoproteins were examined by indirect immunofluorescence of fixed permeabilized cells, the intracellular viral glycoproteins produced in both Hrel3 and WT cells produced similar intracellular staining patterns (Fig. 1, B vs. F). This fluorescent signal was specific for MMTV glycoproteins since untransfected HTC cells did not express any immunostaining material (Fig. 1, J,L). The distribution of intracellular and cell surface staining of the GR MMTV glycoprotein was similar to that observed in viral-infected M1.54 rat hepatoma cells [Bravo et al., 1991; John et al., 1988], whereas the C3H glycoprotein variant appears to be retained in an intracellular compartment.

The production of individual intracellular, cell surface or extracellular MMTV glycoproteins were examined in [^{35}S]methionine-labeled cells by immunoprecipitation with anti-MMTV antibodies. SDS polyacrylamide gel analysis revealed that WT cells expressed two forms of the uncleaved viral glycoprotein, gp78 and gp70, as

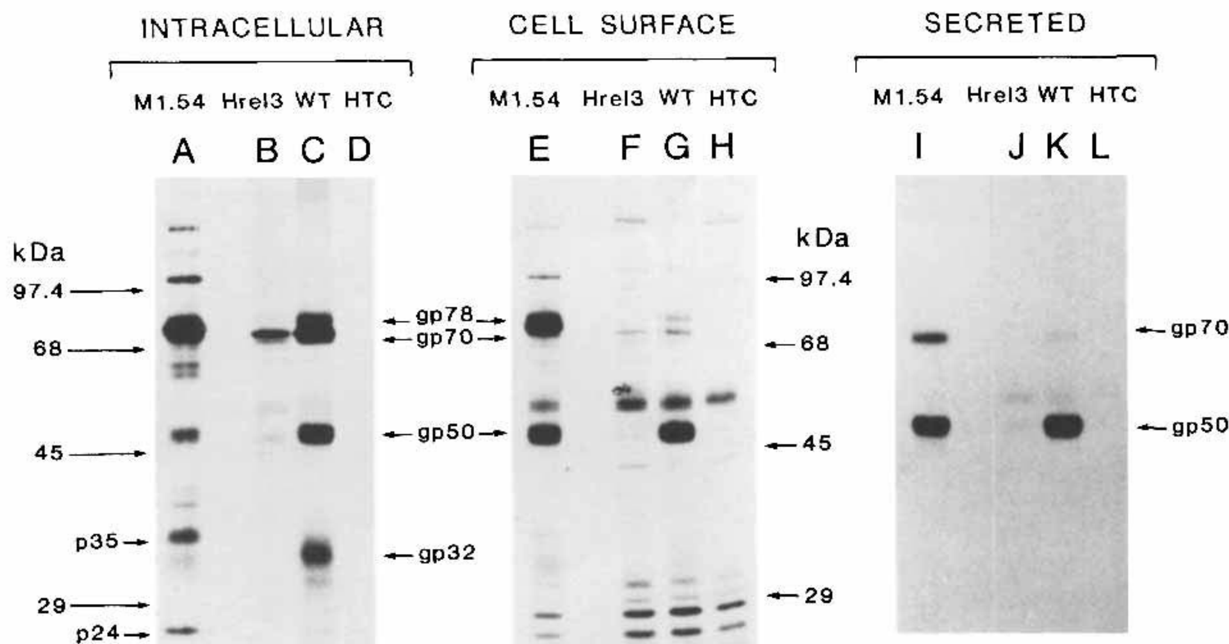

Fig. 2. Localization of MMTV glycoproteins expressed from transfected and viral-infected HTC cells. Cultures of viral-infected M1.54 cells, transfected Hrel3 and WT cells, as well as untransfected HTC cells were radiolabeled with [^{35}S]methionine for 4 h and the secreted MMTV glycoproteins immunoprecipitated from culture medium (**lanes I–L**). The cell surface (**lanes E–H**) and intracellular (**lanes A–D**) MMTV proteins were immunoprecipitated from the remaining cells as described in the text. The immunoprecipitated MMTV products were electrophoretically fractionated in SDS polyacrylamide gels and radioactive protein bands visualized by fluorography. The molecular weight standards are phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

well as stable forms of the aminoterminal (gp50) and carboyxterminal (gp32) endoproteolytic fragments (Fig. 2, lanes C,G,K). Cell surface immunoprecipitations showed that all four of the GR isolate MMTV glycoproteins were localized to the cell surface (lane G), whereas a 70 kDa and a 50 kDa fragment were externalized (lane K). We have previously shown that gp78, gp70, and gp32 are transmembrane forms of the MMTV glycoprotein, while externalized gp50 appears to be noncovalently associated with gp32 on the extracellular side of the plasma membrane [Firestone, 1988; Haffar et al., 1988]. The localization and processing pattern of the GR isolate glycoprotein was essentially identical to that of the MMTV glycoprotein expressed in viral-infected M1.54 cells which also express the MMTV gag phosphoproteins (Fig. 2, lanes A,E,I). Viral-infected M1.54 cells appeared to externalize a larger fraction of the 70 kDa MMTV glycoprotein (Fig. 2, lane I vs. K). In contrast, Hrel3 cells produced an intracellular 70 kDa C3H MMTV glycoprotein which was inefficiently transported to the cell surface and not externalized (Fig. 2, lanes G,F,J). The majority of the C3H gp70 was retained intracellularly. Moreover, this MMTV glycoprotein variant remained in an uncleaved form since neither of the proteolytic cleavage products gp50 and gp32 was detected. Immunoprecipitation of the culture medium from radiolabeled cells revealed that both Hrel3 and WT cells efficiently secrete both alpha$_1$-acid glycoprotein and plasminogen activator inhibitor (data not shown) which indicates that the altered trafficking and processing of MMTV glycoproteins in Hrel3 cells cannot be explained by a fortuitous defect in the general exocytosis of glycoproteins.

Electrophoretic Migration of the Nonglycosylated Forms of Expressed MMTV Glycoproteins

To verify that the C3H and GR MMTV glycoprotein precursors, gp70, are in fact glycosylated, and to determine the sizes of the corresponding nonglycosylated polypeptide backbones, cells were radiolabeled in the presence or in the absence of tunicamycin, an antibiotic which inhibits N-linked core glycosylation by preventing transfer of the dolichol linked sugar to asparagine residues [Hubbard and Ivatt, 1981; Pres-

per and Heath, 1985]. Immunoprecipitation of [^{35}S]methionine-labeled cell extracts and subsequent fractionation in SDS polyacrylamide gels revealed that the nonglycosylated forms of both MMTV glycoprotein variants are similar in size (Fig. 3B). Treatment with tunicamycin resulted in the appearance of a novel band of approximately 62 kDa (lanes G,H) corresponding to the nonglycosylated MMTV glycoprotein precursor. Immunoprecipitated MMTV glycoproteins were also deglycosylated with PNGase F, an enzyme which removes all N-linked oligosaccharide side chains. As also shown in Figure 3 (A, lanes C,D), the deglycosylated C3H and GR MMTV polyproteins migrated with similar apparent mobilities in SDS polyacrylamide gels. Thus, the lack of transport of the C3H MMTV glycoprotein variant to the cell surface cannot be explained by major alterations in oligosaccharide side chain addition or size of the polypeptide backbone.

Comparison of Intracellular Transport Kinetics of the MMTV Glycoprotein Variants Expressed in HTC Cells

The kinetics of transport from the RER to the medial Golgi was examined by the rate of acquisition of endoglycosidase H (endo H) resistant oligosaccharide side chains. Cells were pulse-labeled for 10 min with [^{35}S]methionine and then chased for the indicated times in excess unlabeled methionine. Immunoprecipitated MMTV glycoproteins at each pulse and chase time point were incubated in the presence or the absence of endo H and the resulting MMTV glycoproteins analyzed by SDS polyacrylamide gels. As shown in Figure 4 (lower panel), the 70 kDa C3H MMTV glycoprotein variant failed to acquire endo H resistant oligosaccharide side chains as indicated by the recovery of a 62 kDa protein. The lack of oligosaccharide maturation suggests that the MMTV glycoprotein is being retained within the cell at an early point in the exocytotic pathway. Also, the pulse-chase analysis indicates that C3H gp70 is somewhat unstable, exhibiting a half-life of approximately 45 min. The endo H sensitivity indicates that most of the synthesized C3H gp70 was retained in the ER and is perhaps turned over by the ER degradative pathway [Klausner and Sitia, 1990; Stafford and Bonifacino, 1991]. In contrast, the GR MMTV glycoprotein is efficiently transported into and through the Golgi and acquires endo H resistant carbohydrate side chains with a half-

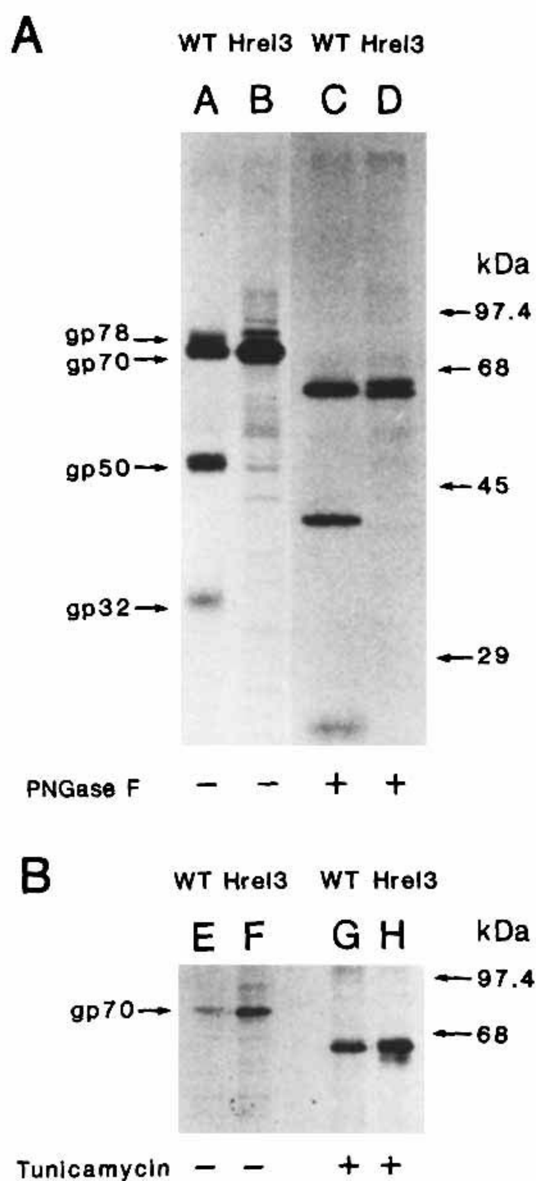

Fig. 3. Electrophoretic mobilities of deglycosylated and nonglycosylated MMTV glycoproteins. **A:** WT (**lanes A,C**) and Hrel3 (**lanes B,D**) cells were radiolabeled with [^{35}S]methionine for 4 h, cellular MMTV glycoproteins immunoprecipitated with anti-MMTV antibodies, and the final immunoprecipitated material treated overnight without (lanes A,B) or with (lanes C,D) PNGase F as described in the text. The samples were evaporated by vacuum centrifugation, resuspended in sample buffer, and electrophoretically fractionated in SDS polyacrylamide gels. **B:** WT and Hrel3 cells were treated in the presence (**lanes G,H**) or in the absence (**lanes E,F**) of 5 μg/ml tunicamycin for 1 h before and during the 4 h labeling period with [^{35}S]methionine. Immunoprecipitated glycosylated and nonglycosylated MMTV glycoproteins were fractionated in SDS polyacrylamide gels as described above. Molecular weight standards are described in Fig. 2.

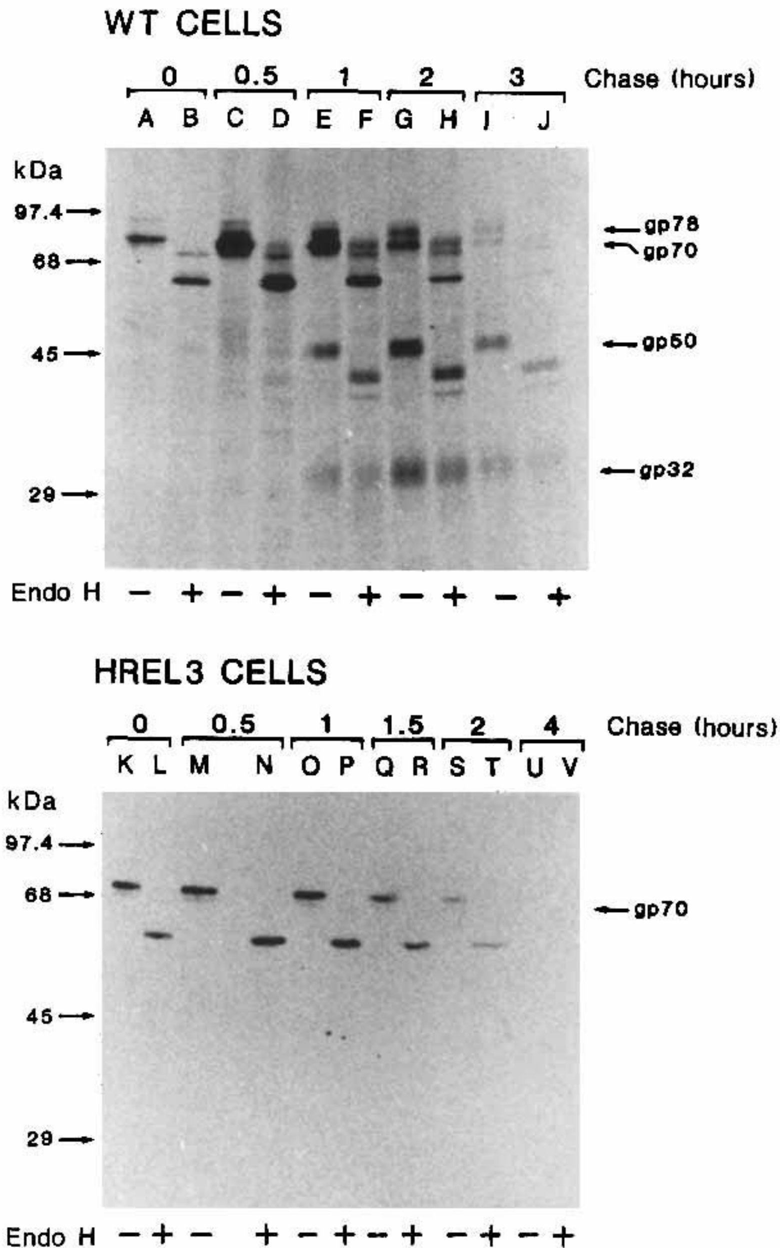

Fig. 4. Pulse-chase analysis of the intracellular transport of MMTV glycoproteins. WT and Hrel3 cells were pulse-labeled with [^{35}S]methionine for 10 min, washed 3 times in phosphate buffered saline, and then incubated with excess unlabeled methionine. At the indicated chase times, cells were harvested, solubilized, immunoprecipitated with anti-MMTV antibodies, and then digested with (**lanes B,D,F,H,J,L,N,P,R,T,V**) or without (**lanes A,C,E,G,I,K,M, O,Q,S,U**) endoglycosidase H as described in the text. The endo H digestions were terminated by re-immunoprecipitated with anti-MMTV antibodies. The final immunoprecipitated material was fractionated by SDS polyacrylamide gel electrophoresis and radioactive bands visualized by fluorography. The arrows at the right side of the gel denote the migration of undigested MMTV glycoproteins. The molecular weight standards are described in Fig. 2.

maximal rate of approximately 60 min (Fig. 4, top panel). As also shown in Figure 4 (top panel), the pulse-labeled gp70 was completely sensitive to endo H digestion since a lower molecular weight (62 kDa) protein band was detected after incubation with this glycosidase. At later times in the chase period, higher *molecular weight* forms of the endo H treated polyprotein were recovered from the glycosidase incubations which represent the acquisition of endo H resistant oligosaccharides (for example, lane F vs. E, H vs. G). Also based on their molecular weights

after glycosidase treatment, virtually all of the gp50 appears to contain endo H sensitive oligosaccharides, whereas gp32 contains primarily endo H resistant carbohydrate side chains. Densitometric analysis revealed that in transfected HTC cells, the half time of transport of the GR strain MMTV glycoprotein variant from RER to the medial Golgi was approximately 60 min.

Intracellular Retention of MMTV Glycoproteins in Hrel3 Cells Is Not Due to Binding to the Endogenous BiP Protein

As judged by their sensitivity to endo H, most of the MMTV gp70 expressed in Hrel3 cells appeared to be retained intracellularly at an early step in the exocytic pathway. Association with the heavy chain binding protein, BiP (a member of the hsp70 gene family), has been observed for many misfolded or mutant proteins retained intracellularly [Pelham, 1989]. Therefore, BiP binding to the two different MMTV glycoprotein variants was assessed by co-immunoprecipitation with anti-BiP antibodies. Parallel immunoprecipitations were performed using anti-MMTV antibodies under conditions (ATP depleting at 4°C) which would stabilize the association of BiP with bound proteins [Hurtley et al., 1989; Machamer et al., 1990]. Since the molecular weight of the MMTV glycoprotein precursor, gp70, is approximately the same as BiP, a second set of co-precipitations were incubated with endo H to visualize any MMTV glycoproteins that associate with BiP. The mobility of MMTV glycoproteins should increase after endo H digestion (due to the loss of endo H sensitive oligosaccharide sidechains), whereas BiP will maintain its usual mobility since it is not a glycoprotein and therefore resistant to endo H digestion. As expected, the electrophoretic migration of MMTV gp70 approximates that of BiP prior to endo H treatment (Fig. 5, top panel, lane A vs. B, E vs. F). When the co-immunoprecipitated proteins were treated with endo H prior to their fractionation in SDS polyacrylamide gels, the MMTV gp70s expressed in Hrel3 and WT cells can be unambiguously shown not to co-precipitate with anti-BiP antibodies (Fig. 5, lane I vs. J, M vs. N). As a positive control for BiP binding, the trgp58 truncated form of the GR isolate MMTV glycoprotein (designated LM445), which we have shown to be tightly associated with BiP and presumably misfolded [Platt and Firestone, 1991], efficiently co-precipitated with the anti-BiP antibodies (Fig. 5, lanes C vs. D). In untransfected HTC cells, BiP is expressed in the absence of MMTV glycoproteins (lane G vs. H, K vs. L). Thus, the MMTV glycoprotein variant expressed in Hrel3 cells was retained intracellularly in an endo H sensitive form by a BiP-independent pathway.

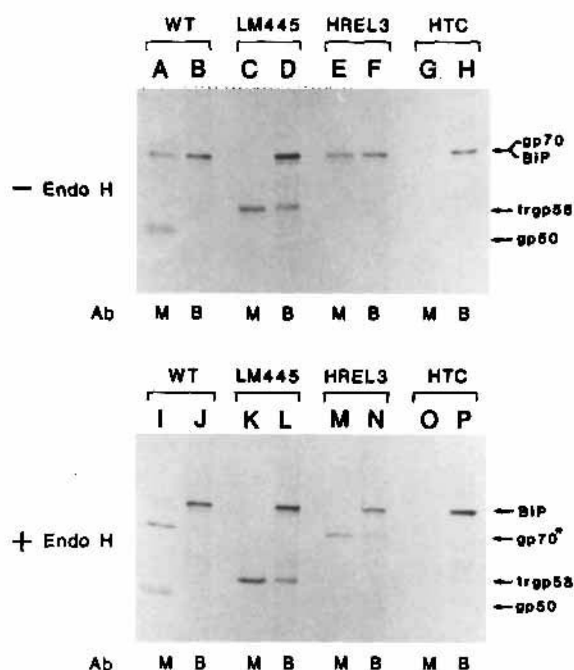

Fig. 5. *Co-immunoprecipitation of MMTV glycoproteins and BiP.* [^{35}S]Methionine-labeled WT, LM445, Hrel3, and HTC cells were immunoprecipitated with either anti-MMTV antibodies (designated M; **lanes A,C,E,G,I,K,M,O**) or anti-BiP monoclonal antibodies (designated B; **lanes B,D,F,H,J,L,N,P**) and immunoprecipitated material digested with (lanes I–P) or without (lanes A–H) endoglycosidase H as described in the text. Double immunoprecipitations were carried out with anti-MMTV antibodies to eliminate noncovalently bound proteins. The immunoprecipitated and digested material was electrophoretically fractionated in SDS polyacrylamide gels and radiolabeled protein bands visualized by fluorography. The molecular weight standards are described in Fig. 2. The migration of endo H treated gp70 is denoted by gp70*.

DISCUSSION

Two natural variants of the MMTV glycoprotein gene were expressed in rat HTC hepatoma cells to examine their trafficking in the absence of the other nonglycosylated viral proteins. The GR variant glycoprotein was efficiently transported to the cell surface and extracellular environment, and similar to viral-infected HTC cells [Firestone, 1988; Platt et al., 1991], undergoes endoproteolytic cleavage to produce stable aminoterminal and carboxyterminal maturation products. In contrast, the C3H variant glycopro-

tein was retained at an early step in the exocytic pathway in an endoglycosidase H sensitive and uncleaved form. The intracellular retention of the C3H MMTV glycoprotein was also accompanied by a relatively rapid turnover of the glycoprotein. Conceivably, the C3H variant glycoprotein is conformationally altered, due to changes in its primary amino acid sequence, which precludes its exocytosis. This altered folding may affect the oligomerization required for exit from the RER. Also, many types of misfolded proteins are retained in the RER due to their interactions with the BiP, which is a member of the heat shock protein gene family [Pelham, 1989; Rose and Doms, 1988]. However, the intracellular retention of the C3H MMTV glycoprotein cannot be explained by a stable association with BiP since anti-BiP antibodies failed to co-immunoprecipitate the MMTV glycoprotein. It is possible, however, that BiP interacts transiently with this variant MMTV glycoprotein. BiP-independent mechanisms for retaining and/or degrading misfolded proteins have been observed in other systems [Klausner and Sitia, 1990; Machamer et al., 1990; Stafford and Bonifacino, 1991], suggesting that the C3H variant glycoprotein may be retained by a similar process. Whatever the precise mechanism, the C3H variant glycoprotein also fails to be transported and processed in other transfected cell types, such as CHO Cells (data not shown), further suggesting that the defect is specific to this MMTV glycoprotein variant.

Sequence analysis predicts that the MMTV glycoprotein has several distinct structural features which may be potentially important for its final tertiary structure and efficient intracellular transport [Redmond and Dickson, 1983; Majors and Varmus, 1983; Henderson et al., 1983]. For example, the MMTV glycoprotein encodes five N-linked glycosylation sites, an endoproteolytic cleavage site, a large hydrophobic region in the ectodomain (amino acids 457–483), and several acidic regions and can be lipid modified [Firestone et al., 1986]. Little is known about the functional role that these regions play in the modulation of transport, oligomerization, and/or processing of the MMTV glycoprotein. The C3H variant MMTV glycoprotein is deficient in its ability to exit the rough endoplasmic reticulum, suggesting that amino acids altered in this glycoprotein may affect folding and/or transport. The most prominent difference of the MMTV glycoprotein between the GR and C3H variants used in our study is a divergent stretch of seven amino acids (between numbers 79 and 87) in the aminoterminal portion of glycosylated polyprotein, although several other single amino acid changes exist through the polypeptide backbone [Majors and Varmus, 1983; Redmond and Dickson, 1983]. It is possible that the cloning procedure to obtain the C3H isolate MMTV glycoprotein may have generated several mutations. Rat XC cells were infected with the C3H strain of exogenous MMTV and individual cell clones screened for expression of MMTV specific sequences. One clone expressed a truncated provirus which, upon further characterization, was found to lack the gag, pro, and pol genes, while possessing an intact env gene [Majors and Varmus, 1983]. The investigators speculated that this clone arose from a rare reverse transcription of the spliced form of the viral mRNA. Given that the error rate is much higher for reverse transcriptase vs. DNA polymerases (10^{-5} vs. 10^{-9} error rate) it is possible that this reverse transcript contains subtle point mutations which allow synthesis of an MMTV glycoprotein of the correct size and antigenicity, but which are incapable of efficient transport thru the exocytic pathway due to its inability to properly oligomerize or to fold correctly. Consistent with the difference between the C3H and GR variant MMTV glycoproteins being only a few amino acids, the size and number of fragments generated upon restriction mapping of each glycoprotein gene were identical (data not shown). In addition, treatment of cells with tunicamycin or deglycosylation with PNGase F revealed polypeptide backbones of similar apparent molecular masses.

Similar to most cellular glycoproteins, the 70 kDa glycosylated MMTV precursor is transported into and through the Golgi where the oligosaccharide side chains are matured into an endo H resistant 78 kDa species. Analysis of an endoproteolytic site mutant has revealed that the polypeptide backbone of the MMTV glycoprotein is cleaved at an Arg-Ala-Lys-Arg dibasic amino acid motif which generates the gp50 aminoterminal fragment and the gp32 carboxyterminal transmembrane fragment [L.J. Goodman, S.R. Kain and G.L. Firestone, submitted]. The proteolytic maturation products of the GR variant have oligosaccharides that are endo H resistant and contain sialic acid, indicating that the endoproteolytic event occurs no earlier in the exocytic pathway than the trans Golgi. Recent evidence suggests that the HIV-1 [Stein and

Engleman, 1990], Rous Sarcoma Virus [Perez and Hunter, 1987] and murine leukemia virus [Fitting and Kabat, 1982] envelope precursors are most likely cleaved in the trans compartment of the Golgi. Both the C3H and GR variant MMTV glycoproteins genes encode the identical Arg-Ala-Lys-Arg dibasic endoproteolytic site suggesting that the C3H isolate glycoprotein fails to be cleaved due to retention in a cellular compartment that precludes accessibility to the endoproteases and/or as a result of its conformational altered structure. The nature of the enzymes involved in the endoproteolytic cleavage of the MMTV polyprotein remains largely unknown, although the proteolytic recognition site within the MMTV glycoprotein is similar to that of a variety of prohormones and retroviral envelope glycoproteins [Docherty and Steiner, 1982; Douglass et al., 1984; Fisher and Scheller, 1988; Stein and Engleman, 1990].

We have previously documented that glucocorticoids regulate the trafficking of the GR variant MMTV glycoprotein in viral-infected rat hepatoma cells [Amacher et al., 1989; Bravo et al., 1991; Firestone, 1988; Firestone et al., 1982, 1986; Haffar et al., 1987, 1988; John et al., 1988]. In HTC cells transfected with a constitutively expressed MMTV glycoprotein, transport and processing of the viral glycoprotein are not affected by glucocorticoids although steroid treatment stimulates a moderate increase in the final level of cell-surface-associated gp78 [Platt et al., 1991]. The analysis of transport and processing of the GR and C3H variant MMTV glycoproteins suggest that specific MMTV glycoproteins can display inherent differences in their folding, processing, and intracellular transport. Therefore, any interpretation of results concerning MMTV glycoprotein trafficking must take into account the individual MMTV glycoprotein gene, the viral context, and cell type used for a given study.

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