Disruptions in Intracellular Membrane Trafficking and Structure Preclude the Glucocorticoid-dependent Maturation of Mouse Mammary Tumor Virus Proteins in Rat Hepatoma Cells*

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We have previously shown that glucocorticoids regulate the trafficking and processing of mouse mammary tumor virus (MMTV) proteins in viral-infected M1.54 rat hepatoma cells. To examine the role of intracellular membrane integrity on MMTV protein maturation, brefeldin A (BFA) was utilized to disrupt membrane flow between the endoplasmic reticulum and Golgi. Immunoprecipitation and immunofluorescence microscopy revealed that in the presence of dexamethasone, BFA inhibited the proteolytic processing, cell surface delivery, and externalization of MMTV glycoproteins. Glycosidase digestion and inhibitors of protein glycosylation confirmed that the observed differences in apparent sizes of MMTV glycoprotein products are due to BFA-induced changes in oligosaccharide processing. BFA treatment inhibited the proteolytic processing of the MMTV phosphoprotein precursor, which normally associates with the cytoplasmic face of intracellular membranes. Similarities in salt extraction efficiency revealed that BFA did not affect the membrane affinity of the uncleaved phosphorylated precursor. In a complementary approach, proteolytic processing of the phosphorylated polyprotein did not occur in glucocorticoid-treated HTC cells transfected with a mutant MMTV provirus encoding a normal phosphorylated precursor, but which express a truncated MMTV glycoprotein missing its transmembrane domain and cytoplasmic tail. These results suggest that the MMTV glycoproteins and phosphoproteins may interact at a late step in the transport pathway in a manner required for their mutual processing in response to glucocorticoids and establishes the importance of functional interactions with intracellular membranes for maturation of the cytoplasmic MMTV phosphoproteins.

Glucocorticoid hormones elicit their biological effects in a variety of target tissues by selectively modulating the transcription of glucocorticoid-responsive genes (1-5). Conceivably, regulatory factors under the direct transcriptional control of this steroid could modulate the expression, synthesis, transport, or processing of other biologically active proteins. By utilizing the expression of mouse mammary tumor virus (MMTV) polypeptides in viral-infected M1.54 rat hepatoma cells as traceable molecular probes, we have uncovered two distinct posttranslational regulatory circuits under the control of glucocorticoids (6-12). The core MMTV phosphoproteins (encoded by the gag gene) and the MMTV envelope glycoproteins (encoded by the env gene) are each produced as precursor polyproteins that require a series of processing steps to yield stable maturation products. Treatment of M1.54 cells with dexamethasone, a synthetic glucocorticoid, selectively stimulates the glycosylated precursor to enter a sorting route resulting in the generation of three new cell surface-associated glycoproteins (two uncleaved forms of the precursor, gp78 and gp70, and the gp32 carboxy-terminal fragment), and an extracellular 70-kDa glycoprotein (gp70) (reviewed in Ref. 8). The amino-terminal fragment, gp50, is detected in the presence or absence of dexamethasone. Glucocorticoids also regulate maturation of the MMTV phosphorylated precursor, yielding two new proteolytic products designated p35 and p24 (6, 8, 9, 12). Only steroid hormones with glucocorticoid biological activity elicit these two responses in M1.54 cells; each posttranslational response is abrogated by the glucocorticoid antagonist RU38486 (10, 12). Additionally, the glucocorticoid-regulated trafficking and processing events require a functional receptor protein (13) and de novo synthesis of cellular proteins (14). Detailed analysis of oligosaccharide-processing kinetics and subcellular fractionation in M1.54 cells have shown that glucocorticoids exert their regulatory effects on MMTV glycoprotein trafficking within the medial to trans-Golgi network of the Golgi (11, 15, 16), suggesting that hormone-modulated cellular and/or viral regulatory factors function within or target components to this organelle.

Although the glucocorticoid-dependent maturation of the viral phosphoproteins is generally observed under the same conditions as those required for trafficking of integral membrane-associated MMTV glycoproteins, virtually nothing is known about the relationship between membrane components and the regulated processing of the cytoplasmic-residing MMTV phosphoproteins. As one test of the role of membrane integrity in MMTV protein maturation, we have utilized the

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The abbreviations used are: MMTV, mouse mammary tumor virus; BFA, brefeldin A; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; endo H, endoglycosidase H; endo F, N-glycosidase F; DMM, 1-deoxymannojirimycin; SDS, sodium dodecyl sulfate.
fungal metabolite brefeldin A (BFA) to reversibly disrupt protein trafficking past the endoplasmic reticulum (ER) transmembrane elements in viral-infected cells. BFA has been shown to rapidly induce vesiculation of the Golgi cisternae (17, 18), leading to the selective disassembly of this organelle and movement of Golgi membrane components to the ER via a microtubule-dependent retrograde transport pathway (19, 20). When BFA is removed from cells, this process is reversed, leading to reformation of the Golgi apparatus and restoration of ER to Golgi protein transport (17, 21). This reagent has therefore allowed us to investigate whether a global disruption in membrane trafficking affects the glucocorticoid-dependent maturation of the cytoplasmic MMTV phosphoproteins and the membrane-associated MMTV glycoproteins. In a complementary approach that modifies only the MMTV glycoprotein component of the membrane, HTC cells were transfected with a mutated MMTV provirus that encodes a wild type glycoprotein precursor but a truncated glycoprotein precursor missing its transmembrane domain and cytoplasmic tail (23). In cells transfected with this mutant provirus, the phosphoprotein precursor, despite having a wild type sequence, is not processed in the presence of glucocorticoids. Our results suggest that global and specific disruptions in intracellular membrane trafficking and structure can preclude the hormone-dependent processing of cytoplasmic MMTV phosphoproteins in rat hepatoma cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—All media and sera used for tissue culture were purchased from Whittaker M. A. Bioproducts (Walkersville, MD). [35S]Methionine (1000 Ci/mmol) and [32P]orthophosphate (8500 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA). Deoxycholate, sodium deoxycholate, and 1-deoxymannojirimycin (DMM) were purchased from Sigma. Endo glycocidase H, N-glycosidase F, and octyl glucoside were obtained from Boehringer Mannheim. Staphylococcus aureus cells (Pansorbin) were obtained from Calbiochem. The anti-p52 antisera used in the immunofluorescence studies was obtained from the National Institutes of Health repository (Bethesda, MD), and the fluorescein isothiocyanate-conjugated F(ab′)2 fragment of anti-goat IgG was purchased from Organon Teknika-Cappel (Malvern, PA). Total anti-MMTV (24) and preimmune sera used for immunoprecipitations were generously provided by L. J. T. Young and D. Cardif Departent of Pathology, University of California, Davis). The placid pGR16, containing an MMTV provirus with a premature termination codon in the env gene (22), was provided by H. Ponto (Institute for Genetics and Toxicology, Karlsruhe, Germany). Brefeldin A was a generous gift of D. Roemer (Sandos LTD, Basel, Switzerland). A stock solution of 1.0 mg/ml BFA in methanol was prepared and stored at -20°C. All additional reagents were of the highest available quality.

Cells and Method of Culture—M1.54 cells were a clonal cell line derived from MMTV-infected rat HTC hepatoma cells and contains 10 stably integrated MMTV proviruses (6, 23). HGR16.5 cells are single cell-derived clones selected from rat HTC hepatoma cells that have been transfected with the plasmid pGR16 encoding a truncated MMTV glycoprotein (23, 26). Each cell line was propagated as monolayer cultures at 37°C on Corning Tissue Culture plates in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum in a 5% CO2 atmosphere in 95% air. HGR16.5 cells were additionally supplemented with 1 mg/ml Geneticin (G418; Gibco Laboratories, Santa Clara, CA).

**Indirect Immunofluorescence**—Immunofluorescence was performed essentially as described previously (14, 27). Briefly, M1.54 cells were seeded on glass coverslips and cultured for 24 h in the presence of 1 μM dexamethasone alone or in addition to 2.0 μg/ml BFA. The cells were washed with phosphate-buffered saline (PBS) and fixed by incubation with 3% formaldehyde in PBS containing 0.1 M glycine (PBS/glycine). To stain intracellular proteins, cells were permeabilized with 0.5% Triton X-100, 0.5% sodium deoxycholate in PBS for 10 min at room temperature. This step was omitted for immunofluorescence. The cells were then washed extensively with PBS/glycine prior to incubation with 1:350 serum dilution of polyclonal anti-p52 IgG for 30 min at room temperature. After washing in PBS/glycine, the cells were incubated with a 1:400 dilution of fluorescein isothiocyanate-conjugated F(ab′)2 fragment of anti-goat IgG. The cells were washed a final time in PBS/glycine and mounted on microscope slides in 90% glycerol, 10% 100 mM Tris-HCl, pH 7.5. Slides were sealed with Permount (Fisher Scientific Co., Pittsburgh, PA) and viewed using a Zeiss fluorescent microscope.

**Transfection and Expression of a Plasmid Containing a Truncated MMTV Glycoprotein Gene**—The plasmid pGR16 was cotransfected with pSV2neo into rat HTC hepatoma cells using the calcium phosphate procedure (28); 48 h after transfection, the cells were replated into selective media containing 1 mg/ml G418. After 3 weeks, individual cell colonies were harvested, expanded in culture, and tested for expression of MMTV RNA by cytoplasmic dot blot analysis (29). Cell lines expressing high amounts of MMTV-specific RNA were further screened for the production of MMTV protein by immunoblot analysis of total cellular lysates. The labeled cell lines were cotransfected with total anti-MMTV sera as described below. One such clone, designated HGR16.5, was used for the experiments described in this study.

**Radicolabeling and Collection of Cellular and Secreted Fractions**—Monolayer cultures of M1.54 cells were treated with dexamethasone in the presence or absence of BFA, as described in appropriate figures. Prior to radiolabeling, the cells were incubated with methionine-free or phosphate-free Dulbecco’s modified Eagle’s medium in the absence of serum for 30 min, followed by the addition of 50 μCi/ml [35S]methionine or 200 μCi/ml [32P]orthophosphate in the appropriate medium for an additional 4 h. For cultures with transfected M1.54 cells, cultures were preincubated with either 1 μM DMM or 20 μg/ml swainsonine in methionine-free Dulbecco’s modified Eagle’s medium for 1 h at 37°C prior to the addition of [35S]methionine. The secreted fractions were harvested from radiolabeled cells by centrifugation of the culture medium at 2000 × g for 10 min. The supernatant fractions from the secreted MMTV proteins were then solubilized in immunoprecipitation buffer (1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-HCl, pH 7.5) and centrifuged in an Eppendorf microcentrifuge at maximum speed for 10 min at 4°C. The supernatant fraction was then used for subsequent analysis. The total level of radiolabeled protein was determined by precipitation onto filter discs using 10% trichloroacetic acid and the values used to normalize samples for immunoprecipitation.

**Immunoprecipitation and Electrophoresis of MMTV Proteins**—Immunoprecipitations of cellular and secreted MMTV proteins using either total anti-MMTV or preimmune sera was performed as described previously (14). In all cases, the immunoprecipitates were solubilized in immunoprecipitation buffer containing 6% SDS, 200 mM NaCl, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 0.1% SDS. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (33), using a 10% resolving gel to separate MMTV proteins. After electrophoresis, the gels were prepared for fluorography by soaking in 100 mM salicylic acid for 1 h prior to drying. The dried gels were exposed to Kodak X-Omat AR film (Eastman Kodak) at -80°C.

**Sodium Chloride Extraction of Peripheral Membrane Proteins**—Salt extraction of peripheral membrane proteins was performed essentially as described previously (34). The cell fraction was radiolabeled monolayers were obtained as described above, and the cells were resuspended in ice-cold hypotonic buffer containing 20 mM PBS, pH 7.5, 5 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.3 μg/ml aprotinin. The cells were then allowed to swell on ice for 30 min and then homogenized using a TE-16 teflon-glass homogenizer. The samples were centrifuged in an Eppendorf microcentrifuge for 10 min, and supernatant fractions were adjusted to the appropriate salt concentration using 5 mM NaCl in hypotonic buffer. The samples were then incubated on ice for 20 min and then homogenized using a TE-16 teflon-glass homogenizer. The resulting high speed pellet and supernatant fractions were solubilized, and the samples were immunoprecipitated using total anti-MMTV antibodies as described above.
Digestion with Endoglycosidase H and N-Glycosidase F—For endoglycosidase H (endo H) digestions, the final Pansorbin pellets from immunoprecipitation of cell extracts were resuspended in 45 µl of 100 mM sodium citrate buffer, pH 5.5, containing 1% SDS, boiled for 3 min, and then centrifuged in an Eppendorf microcentrifuge for 10 min. The supernatant fractions were collected and divided into 20-µl aliquots, and 20 µl of sodium citrate alone or 26 µl of sodium citrate containing 0.2 milliunits of endo H added to each sample. The reaction mixture was incubated for 18 h at 37 °C, and then terminated by evaporation in a Savant vacuum centrifuge. N-Glycosidase F (endo F) digestions were performed by the same protocol, except Pansorbin pellets were resuspended in PBS containing 1% SDS, and the reaction mixture consisted of 20 µl of sample supernatant, 15 µl of PBS, 4 µl of N-acetyl glucosamine, in the presence or absence of 0.1 units of endo F. The samples from each digestion were resuspended in electrophoresis sample buffer and resolved on 10% gels as described above.

RESULTS

BFA Disrupts the Glucocorticoid-dependent Processing, Cell Surface Delivery, and Externalization of MMTV Glycoproteins—Along with the rapid changes in Golgi morphology brought about by BFA, there is a coincidental arrest of protein transport to this organelle (17, 18, 21, 35, 36). To verify that BFA appropriately prevents the transport of MMTV glycoproteins in viral-infected M1.54 hepatoma cells, the localization of the cell surface viral glycoproteins in glucocorticoid-treated cells was examined by immunofluorescence microscopy. Monolayer cultures of M1.54 cells were treated with either 1 µM dexamethasone alone or in combination with 2.0 µg/ml BFA for 16 h and incubated with primary total anti-MMTV antiserum and secondary fluorescein isothiocyanate-conjugated anti-goat IgG. As shown in Fig. 1, cells treated with dexamethasone in the absence of BFA display a prominent immunofluorescence capping pattern indicative of cell surface-localized antigens (panel D). In contrast, when cells were incubated with both dexamethasone and BFA, no detectable cell surface immunofluorescent staining was evident (panel H). Intracellular viral glycoproteins were observed in detergent-permeabilized, fixed cells. The immunofluorescence staining of intracellular MMTV glycoproteins was generally similar in both control and BFA-treated cells (panel B versus panel F), indicating that BFA does not alter the concentration of glycoprotein gene products, nor is the accessibility of intracellular compartments to antisera compromised. The intracellular staining in control cells (panel B) is juxtanuclear, suggesting an association with the Golgi, whereas in BFA-treated cells, the staining is more diffuse and spread throughout the cytoplasm (panel F). Such dispersion of intracellular MMTV glycoproteins is consistent with the actions of BFA in other cell types (17, 18, 21). These results indicate that BFA is acting much like in other systems to prevent delivery of immunoreactive MMTV glycoproteins to the cell surface of M1.54 cells.

To test whether the observed BFA-mediated block in transport of MMTV glycoproteins was accompanied by alterations in proteolytic processing, a time course of BFA effects on MMTV protein maturation was examined by immunoprecipitation of [35S]methionine-labeled cell extracts. As shown in Fig. 2, the addition of BFA to dexamethasone-treated M1.54 cells resulted in both qualitative and quantitative changes in the overall pattern of MMTV maturation products. During shorter incubations with BFA, the 70-kDa MMTV glycoprotein precursor (gp70), and the 50-kDa (gp50) and 32-kDa (gp32) proteolytic products were each shifted to an apparent lower molecular mass following treatment with BFA (referred to as gp70*, gp50*, and gp32*, respectively). Additionally, the sialylated glycosylated polyprotein, gp78, was absent from

Fig. 1. Localization of cell surface and intracellular MMTV glycoproteins by indirect immunofluorescence. M1.54 cells were cultured on glass coverslips and incubated with 1 µM dexamethasone in the presence (panels E-H) or absence (panels A-D) of 2 µg/ml BFA for 16 h. Direct immunofluorescence of permeabilized (panels A, B, E, and F) and unpermeabilized (panels C, D, G, and H) cells using goat anti-MMTV glycoprotein primary antibodies and fluorescent-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. IC, intracellular staining; CS, cell surface staining.

Fig. 2. Time course of effects of BFA on MMTV processing and externalization. Dexamethasone (DEX)-treated M1.54 cells were preincubated with 2 µg/ml BFA for the indicated times, radiolabeled with [35S]methionine for 4 h in the continued presence of BFA, and the cellular (lanes A-J) and secreted (lanes J-M) fractions were harvested as described in the text. One set of cells was not treated with either DEX or BFA (lanes A and J), and one cellular fraction was immunoprecipitated with preimmune antibodies (lane I). The immunoprecipitated MMTV products were electrophoretically fractionated in SDS-polyacrylamide gels and radioactive protein bands visualized by fluorography. The molecular mass standards are phosphorylase a (92 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

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BFA-treated cells. The change in the molecular mass of these glycoproteins was maintained throughout the BFA time course up to approximately 8 h of treatment and was accompanied by a progressive decrease in the cellular concentration of each protein. The cellular loss of these proteins could not be accounted for by their externalization into the culture medium, as immunoreactive MMTV proteins were completely absent from this fraction under all conditions of BFA treatment up to 16 h (Fig. 2, lanes L and M). Similar to the 1-h time point (lane M), the culture medium from cells treated with BFA from 2 up to 16 h lacked any immunoprecipitable MMTV glycoproteins (data not shown). After 16 h of exposure to BFA, the processing of gp70* to form gp50* and gp32* was completely inhibited, and the low levels of remaining precursor suggested that gp70* is rapidly turned over. When cells were released from the inhibitory effects of BFA by incubating in the absence of this compound, the hormone-dependent trafficking of MMTV glycoproteins and processing of viral phosphoproteins were restored, along with a complete recovery of total secretory capacity (data not shown). This recovery suggests that the BFA-mediated inhibition of MMTV protein processing resumes as the intracellular membranes reform into their functional structures.

**Effects of BFA on MMTV Glycoprotein Oligosaccharide Processing**—To test whether BFA disrupts the maturation of oligosaccharide side chains attached to MMTV glycoproteins, radiolabeled extracts from BFA-treated and untreated cells were immunoprecipitated with anti-MMTV antibodies, and the resultant proteins digested with either endo H or endo F. Endo H cleaves N-linked oligosaccharide side chains of the high mannose type characteristic of glycoproteins in transit between the ER and Golgi apparatus (37); glycoproteins acquire resistance to endo H only after reaching the medial Golgi. MMTV glycoproteins expressed from glucocorticoid-treated cells contain a mixture of endo H sensitivities. The carboxyl-terminal fragment, gp32, was completely resistant to endo H, whereas gp50 contains both endo H-resistant and -sensitive sugar side chains (Fig. 3, lanes A and B). In the presence of BFA, each of these glycoprotein products is rendered completely sensitive to endo H digestion (Fig. 3, lanes C and D). Production of the gp50* and gp32* maturation products suggests that the normally Golgi-associated proteolytic enzymes redistribute into the ER in the presence of BFA. Alternatively, it is formally possible that the proteases newly synthesized in the ER are responsible for the generation of gp50* and gp32* in BFA-treated cells. However, digestion of immunoprecipitated proteins with endo F to remove all the oligosaccharide side chains from MMTV glycoproteins yielded an equivalent array of proteins in the presence or absence of BFA (Fig. 3, lane F versus lane H), demonstrating that BFA does not alter the structure of the polypeptide backbone of the glycoprotein derivatives.

To further confirm that the BFA-mediated alteration in electrophoretic mobilities of MMTV glycoproteins can be accounted for by an alteration in carbohydrate side chain maturation, M1.54 cells were treated with known inhibitors of oligosaccharide processing within the Golgi. Glucocorticoid-treated cells were exposed to either DMM, which inhibits the Golgi mannosidase I and results in the formation of oligosaccharide side chains with a Man6GlcNAc2 high mannose structure (38), or to swainsonine, an inhibitor of Golgi mannosidase II (39), which causes the formation of carbohydrate side chains with a GlcNAc2Man6GlcNAc2 structure. Intracellular and cell surface MMTV proteins were immunoprecipitated from [35S]methionine-labeled cells and analyzed by electrophoretic fractionation. In the presence of either oligosaccharide processing inhibitor, both of the glycosylated proteolytic maturation products were stably produced. The carboxy-terminal maturation product, gp32, migrated with the same apparent molecular mass as observed for gp32* in BFA-treated cells (Fig. 4, lanes C-F), which is consistent with BFA preventing the MMTV glycoproteins from being accessible to the Golgi oligosaccharide-processing enzyme. The gp32 produced in DMM-treated cells also migrated as a sharper band in the gel, most likely reflecting a decreased heterogeneity of the carbohydrate side chains. As expected, all of the oligosaccharide side chains on gp32 were resistant to endo H digestion, while gp50 contained a mixture of high mannose and complex structures.
The presence or absence of the indicated combinations of 1 or 2 μg/ml BFA for 16 h were radiolabeled with [35S]methionine. The molecular mass markers are described in the legend to Fig. 2.

Effects of BFA on MMTV Phosphoprotein Maturation—The BFA-mediated disruption of the Golgi was used to examine the role of membrane trafficking on the glucocorticoid-dependent maturation of the cytoplasmic viral phosphoproteins. Immunoprecipitations were carried out with extracts isolated from cells radiolabeled for 4 h with either [35S]methionine or [32P]orthophosphate; the 32P-labeled extracts and untreated cells to a similar extent. Efficient extraction of MMTV phosphoprotein precursor, resulting in diminished levels of p35 and the total absence of p24 (Fig. 5, lane G and I). As expected, no processing of MMTV precursors was evident in the absence of dexamethasone. These results demonstrate that the BFA disruption of the Golgi causes a concomitant inhibition of the glucocorticoid-dependent maturation of MMTV phosphoproteins.

The Membrane Association of MMTV gag Phosphoproteins Is Not Impaired by BFA—Our previous results suggest that the MMTV phosphoproteins expressed in hepatoma cells reside as cytoplasmic peripheral membrane proteins (8). To determine whether BFA inhibits MMTV phosphoprotein maturation by disruption of membrane-phosphoprotein interactions, microsomes isolated from radiolabeled cells treated with and without BFA were salt-extracted at differing concentrations of NaCl to remove peripheral membrane proteins. Pretreatment conditions that allow detection of salt-extracted processing products were chosen based on the results shown in Fig. 2, which indicate that 0.5 h of pretreatment with BFA results in altered electrophoretic mobilities of the MMTV glycoproteins and detectable phosphoproteins (lanes G-L). Sixteen hours of pretreatment largely eliminates all processing, which allowed the selective monitoring of the salt extractability of the phosphoprotein precursor Pr74gag (Fig. 2, lanes M-R). The pellets and supernatant fractions after a 100,000 × g centrifugation were immunoprecipitated with anti-MMTV antibodies. In the absence of NaCl, all of the MMTV proteins expressed in BFA-treated and untreated cells reside predominantly in the membrane pellet (Fig. 6, lane A versus lane G versus lane M). In microsomes isolated from cells not treated with BFA, the addition of NaCl prior to centrifugation resulted in the elution of Pr74gag, as well as the phosphoprotein maturation products, p35 and p24, from the membrane pellet into the soluble fraction (Fig. 6, lane B versus D versus F). In microsomes isolated from BFA-treated cells, Pr74gag and p35 can be salt-extracted into the soluble fraction in the absence of detectable p24. The efficiency of salt extraction of individual MMTV proteins was quantitated by densitometry of the autoradiogram displayed in Fig. 6. As shown in Table I, the MMTV phosphoprotein precursor Pr74gag was salt-extracted from microsomes isolated from BFA-treated and untreated cells to a similar extent. Efficient extraction of Pr74gag occurred in both microsome samples at 0.2 M NaCl, which indicated that these viral proteins are weakly associated with membranes. Interestingly, p35 appeared to be extracted somewhat more efficiently in the absence of BFA, suggesting that this MMTV protein is more tightly associated with the membrane. 

FIG. 5. Effects of BFA on the glucocorticoid-regulated processing of MMTV phosphoproteins. M1.54 cells pretreated with the indicated combinations of 1 μM dexamethasone (DEX) and 2 μg/ml BFA for 16 h were radiolabeled with [35S]methionine (lanes A–E) or [32P]orthophosphate (lanes F–J) for an additional 4 h in the continued presence or absence of DEX and BFA. The cellular fractions were detergent-solubilized and immunoprecipitated with either anti-MMTV (lanes A–D and F–I) or preimmune (lanes E and J) antibodies. The immunoprecipitated MMTV proteins were electrophoretically fractionated in SDS-polyacrylamide gels, and radioactive protein bands were visualized by fluorography. The molecular mass markers are described in the legend to Fig. 2.

FIG. 6. Membrane association of the MMTV phosphoproteins in BFA-treated and untreated hepatoma cells. Dexamethasone-treated M1.54 cells were preincubated with BFA for the indicated times and radiolabeled with [35S]methionine for 4 h in the presence of BFA, and isolated microsomes were incubated with the indicated concentrations of NaCl as described in the text. Membrane-associated (P, pelleted material) and salt-extracted (S, supernatant fractions) proteins were isolated by centrifugation, immunoprecipitated with anti-MMTV antibodies, and electrophoretically fractionated in SDS-polyacrylamide gels, and radioactive protein bands were visualized by fluorography. The molecular mass markers are described in the legend to Fig. 2.

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membranes in the presence of BFA (Table I). As expected for integral membrane proteins, the MMTV glycoproteins remained exclusively associated with the high speed pellet in the presence or in the absence of BFA (Table I) by virtue of their transmembrane domain (23, 40, 41). These results demonstrate that in the presence of BFA, binding of the MMTV phosphoprotein precursor to the intracellular membranes occurs with approximately the same affinity as in untreated cells, suggesting that a disruption of the inherent ability of this viral phosphoprotein to bind to membranes cannot account for its altered proteolytic processing in the BFA-treated cells.

### Proteolytic Processing of Both MMTV Polyproteins Is Absent in Cells Expressing a Truncated Viral Glycoprotein—The BFA-mediated inhibition of MMTV phosphoprotein maturation suggests that overall membrane integrity is a requirement for this regulated processing reaction. To determine whether specific structural domains of the MMTV glycoprotein also need to be accessible for MMTV phosphoprotein maturation, HTC hepatoma cells were transfected with a mutant provirus encoding a wild type phosphoprotein precursor (Pr74\(^{trg}\)) but containing a point mutation in the viral glycoprotein gene that results in a premature termination codon (22). The resulting truncated MMTV glycoprotein (trgp67) is missing its transmembrane domain and cytoplasmic tail and remains completely within the lumen of the microsomes (23). Transfected glucocorticoid-treated and untreated hepatoma cells (designated HGR16.5) were radiolabeled with \(^{35}\)S-methionine and immunoprecipitated with anti-MMTV antibodies. As shown in Fig. 7, in contrast to viral-infected M1.54 cells (lane B), both the viral phosphorylated precursor and the truncated MMTV glycoprotein remained unprocessed in dexamethasone-treated HTC cells transfected with the mutant provirus (Fig. 7, lane D). This result suggests that the transmembrane or cytoplasmic tail of the MMTV glycoprotein may be necessary for maturation of both classes of MMTV proteins. Thus, subtle interactions between the MMTV phosphoproteins and glycoproteins may be important for efficient polyprotein processing in the presence of glucocorticoids.

### DISCUSSION

We have previously established that glucocorticoids concurrently regulate the trafficking of MMTV glycoproteins and the proteolytic maturation of MMTV phosphoproteins in viral-infected rat hepatoma cells (6-12). Salt extractions revealed that the cytoplasmic-residing viral phosphoproteins are loosely associated with the intracellular membranes, suggesting that a specific interaction could occur with the membrane-associated viral glycoproteins that may be required for their mutual transport and/or maturation. Consistent with this notion, our current results have demonstrated that proteolytic processing of both the MMTV glycoproteins and phosphoproteins observed in glucocorticoid-treated hepatoma cells were inhibited after global and selective disruptions in MMTV glycoprotein trafficking. The fungal metabolite BFA was used to inhibit general protein trafficking from the ER to the Golgi complex by inducing the disassembly of the Golgi cisternae (17-20). In a second approach, transfection of a mutant MMTV provirus, which encodes a truncated MMTV glycoprotein lacking its transmembrane domain and cytoplasmic tail, expresses MMTV glycoproteins and phosphoproteins that reside in different cellular compartments. These complementary strategies have provided the first evidence that proteolytic processing of the MMTV phosphopolyprotein is dependent on the integrity of intracellular membranes and the accessibility of the MMTV glycoprotein. These results further suggest that specific glycoprotein/phosphoprotein interactions may be required for intracellular trafficking and processing of MMTV proteins in viral-infected cells.

Several studies have established that the MMTV glycoproteins and phosphoproteins interact with each other in the virion (42, 43). For example, chemical cross-linking studies have suggested that a heterodimer composed of the amino-terminal gag protein region p10 and gp36 (equivalent to gp32 expressed in hepatoma cells) exists in mature MMTV virions (42). Furthermore, p10 has been shown to have an affinity for the MMTV envelope, most likely by binding to the viral glycoproteins (44, 45). This interaction has been suggested to occur between the p10 portion of the uncleaved MMTV phosphoprotein and hydrophilic membrane component prior to the endoproteolytic cleavage of the phosphoprotein (45). In general, however, the role of viral glycoprotein-phosphoprotein interactions in exocytic trafficking is poorly understood. It is intriguing to consider that since the relative orientations of the MMTV glycoproteins and phosphoproteins are maintained during their exocytic trafficking and viral assembly, certain contact points between these viral components may be responsible for their mutual processing and/or transport. Consistent with our results, other investigators have postulated a role for the cytoplasmic-associated nonglycosylated viral proteins in promoting the transport of HIV glycoproteins in transfected CHO cells (46). There are also several examples of mutant viral glycoproteins inhibiting virion formation and/or phosphoprotein proteolysis (22, 47), suggesting the possibility that specific structural domains within the glycoproteins interact with the phosphoprotein precursors. To determine the precise MMTV glycoprotein-phosphoprotein interactions involved in exocytic trafficking and processing, we are currently constructing and expressing a series of mutant MMTV proviral sequences that contain specific mutations in either the viral phosphoprotein or glycoprotein genes.

BFA has been utilized to examine the effects of blocking

### TABLE I

| Concentration of added NaCl | Percent of MMTV protein in soluble fraction* |
|-----------------------------|---------------------------------------------|
|                            | Pr74\(^{trg}\) | p35 | gp70 |
| No additions                | %             | %   | %    |
| -BFA                        | <5            | <5  | <5   |
| +BFA                        | <5            | <5  | <5   |
| 0.2 M                       | 60            | 75  | <5   |
| +BFA                        | 65            | 50  | <5   |
| 0.4 M                       | 85            | 80  | <5   |
| +BFA                        | 87            | 52  | <5   |

* Appropriate protein bands in the autoradiogram shown in Fig. 6 were scanned with a soft laser densitometer, and the percentages of individual salt-extracted viral proteins were calculated from the ratio of soluble MMTV protein to the total protein in both the membrane and supernatant fractions. The values are the mean of duplicate determinations.
Polyprotein Processing and Membrane Trafficking

Fig. 7. Expression and processing of MMTV glycoproteins and phosphoproteins encoded by pGR16. The illustrations show the wild type and truncated MMTV glycoproteins encoded in viral-infected M1.54 cells and transfected HGR16.5 cells. pGR16 encodes a mutant MMTV proviral genome with a premature stop codon (TAG) at the start of the transmembrane sequences of the glycoprotein gene. Dexamethasone (DEX)-treated and untreated M1.54 (lanes A and B) and HGR16.5 (lanes C and D) cells were radiolabeled with [35S]methionine for 4 h, immunoprecipitated with anti-MMTV antibodies, and electrophoretically fractionated in SDS-polyacrylamide gels, and radioactive protein bands were visualized by fluorography. The molecular mass markers are described in the legend to Fig. 2.

ER to cis-Golgi movement on the posttranslational trafficking of a variety of viral and cellular glycoproteins. For example, BFA prevents the transport and secretion of vesicular stomatitis virus VSV G protein (21), E1 and E2 envelope proteins of sindbis virus (48), and the pseudorabies virus glycoproteins (49), as well as inhibiting the release of thyrotropin from pituitary cells (50). BFA has also been shown to prevent the processing of viral envelope glycoproteins in murine erythroblastosis virus VSV G protein (21), El and E2 envelope proteins of a variety of viral and cellular glycoproteins. For example, leukemia cells (51) and to prevent maturation of glycophorins (49), virtually all the BFA studies have examined integral membrane proteins. The unique feature of our results, therefore, is that the proteolytic processing of a protein (MMTV phosphoprotein) that resides in equilibrium between the cytosol and the cytoplasmic face of the membranes can be concurrently inhibited by a global disruption in membrane structure induced by BFA. Salt extractions further revealed that the peripheral association of MMTV phosphoprotein precursor with isolated microsomes was only minimally affected by BFA treatment, suggesting that more subtle interactions are affected in a way that precludes the proteolytic processing of the phosphorylated MMTV polyprotein.

Exposure to BFA has been shown to dissociate a 110-kDa peripheral membrane protein from the Golgi stacks referred to as β-COP (35, 52). The displacement of this protein from Golgi membrane is very rapid following BFA treatment and precedes all morphological changes in the distribution of Golgi membrane components (53). We hypothesize that the BFA-mediated disruption in membrane structure may be masking a critical MMTV glycoprotein-phosphoprotein interaction that occurs relatively late in the exocytic pathway. It is intriguing to consider that the gag-encoded MMTV phosphopolyprotein, either alone or in a complex with the viral core, may be transported as a peripheral membrane protein via vesicular transport and proteolytically processed only when in the cellular location that activates or releases the viral protease to yield stable derivatives such as p24. Consistent with this notion, BFA disrupts transport from the ER, whereas the formation of p24-like products in other retroviral systems appears to be a late event in the exocytic pathway leading to or during viral assembly and budding (54, 55). Moreover, other investigators have found that a significant portion of Moloney murine leukemia virus-myristylated gag proteins are transported by vesicles to the plasma membrane in a process that requires specific matrix proteins and the myristic acid modification (56). It is also possible that the retroviral proteases contained within the intracellular viral A particles reside in an inappropriate physical environment that precludes their ability to act on the viral polyprotein substrates. For example, pH and ionic conditions greatly influence viral polyprotein processing (57). Alternatively, it is conceivable that in BFA-treated cells, the MMTV viral core with associated uncleaved phosphopolyproteins reaches the cytoplasmic side of the plasma membrane, whereas the viral glycoproteins remain in the ER. The final viral phosphoprotein maturation events may presumably fail to occur due to a lack of glycoproteins associated with the viral core proteins at the plasma membrane.

Exposure to BFA causes a drastic rec compartmentalization of membrane-associated processing enzymes and cellular transport machinery (58). This suggests that the inhibition of MMTV phosphoprotein maturation by BFA may be mechanistically distinct from the lack of processing observed in cells expressing the mutant MMTV glycoprotein precursor. However, regardless of the precise mechanism by which BFA inhibits MMTV phosphoprotein maturation, the concurrent disruption of MMTV glycoprotein trafficking and phosphoprotein processing suggests that the glucocorticoid-regulated events affecting each viral component may be functionally interrelated. In this regard, we have recently observed that glucocorticoids exert a significantly reduced effect on MMTV glycoprotein trafficking in rat hepatoma cells transfected with the glycoprotein gene in the absence of expressed viral phosphoproteins (59). We are currently extending our use of BFA as an important reagent to examine the relative contributions of the MMTV phosphoproteins and glycoproteins and membrane remodeling to the glucocorticoid-regulated trafficking and processing reactions in hepatoma cells transfected with both wild type and mutated viral genes.

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