Glucocorticoid-dependent complementation of a hepatoma cell variant defective in viral glycoprotein sorting

(protein-trafficking activity/mouse mammary tumor virus/cell surface glycoprotein/cell fusion/immunofluorescence)

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ABSTRACT We have utilized the rat hepatoma (HTC) cell sorting variant CR4 to examine the glucocorticoid-regulated pathways that localize mouse mammary tumor virus glycoproteins to the cell surface. The defective sorting of cell surface mouse mammary tumor virus glycoproteins in CR4 cells was complemented after fusion with either normal rat hepatocytes or uninfected HTC cells. Indirect immunofluorescence of transient heterokaryons revealed that the regulated localization of mouse mammary tumor virus glycoproteins was dependent upon glucocorticoid treatment and required de novo RNA and protein synthesis. Thus, a glucocorticoid-regulated trafficking activity, unrelated to mouse mammary tumor virus sequences, which is induced in both adult rat liver and cultured hepatoma cells, can act in trans to mediate an intracellular sorting pathway for membrane glycoproteins.

Glycoproteins destined to be membrane-associated or secreted undergo a series of specific compartmentalization and processing reactions during transit from their site of synthesis in the rough endoplasmic reticulum to their final cellular sites of function (1–6). Relatively little is known about the reactions in the rough endoplasmic reticulum or Golgi that control the sorting of membrane-associated glycoproteins in cells that lack secretory granules. The existence of multiple intracellular sorting routes in these cells is suggested by individual membrane-associated glycoproteins that are transported with varied kinetics (7) or with a rate-limiting step in rough endoplasmic reticulum to Golgi translocation (8, 9). Conceivably, these transport pathways may include regulatory steps or branch points that specify alternate processing or localization fates that are necessary to ensure delivery of properly processed and biologically active glycoproteins to the cell surface; however, the cellular mechanism and transport machinery has not been fully defined.

To pursue this general question, we are utilizing a genetically manipulatable experimental system in which the posttranslational maturation and localization of specific cell surface glycoproteins are under the control of selective hormonal signals. Previous studies have shown that glucocorticoids regulate the maturation and sorting of mouse mammary tumor virus (MMTV) glycoproteins in M1.54 cells, an MMTV-infected rat hepatoma cell line (10–13). A single M1.54, 74,000 glycosylated precursor polyprotein (Pr74env) is initially synthesized in the rough endoplasmic reticulum in both hormone induced and uninduced cells. The predominant Pr74env-derived maturation product in uninduced cells is an extracellular M1.54, 50,000 glycoprotein (gp50). In contrast, exposure to dexamethasone, a synthetic glucocorticoid, redirects the posttranslational expression of this viral encoded precursor resulting in the localization of three new cell surface viral glycoproteins (gp78, gp70, and gp32) as well as an extracellular form of gp70. Both gp78 and gp70 represent different glycosylated forms of the same polyprotein, whereas gp50 represents the amino-terminal domain and gp32 the carboxyl-terminal domain of a proteolytically cleaved polyprotein (12, 13). We have recovered and characterized a M1.54-derived variant (designated CR4) that is selectively defective in the glucocorticoid-regulated sorting pathway but retains general competence for glucocorticoid responsiveness (11, 12). In this study, we have used variant CR4 cells to demonstrate that de novo synthesized glucocorticoid-inducible components encoded by rat hepatoma and normal liver cells can act in trans to regulate the sorting of MMTV glycoprotein substrates.

MATERIALS AND METHODS

Cells and Method of Culture. The origin and characterization of M1.54 cells, a cloned cell line derived from MMTV-infected rat hepatoma (HTC) cells, and the immunoselected glycoprotein-trafficking variant CR4 have been described elsewhere (11, 12, 14). Rat hepatocytes isolated from juvenile Sprague–Dawley rats according to the method of Moldes et al. (15) were a generous gift from M. S. Sandy, D. DiMasto, and M. T. Smith (School of Public Health, University of California, Berkeley). Hepatoma cells were propagated as monolayers in Dulbecco’s modified Eagle’s medium (DMEM; University of California Tissue Culture Facility, San Francisco) supplemented with 10% horse serum, whereas rat hepatocytes were incubated in Ham’s nutrient mixture F-12/DMEM, 1:1, supplemented with 10% fetal calf serum (FCS); both cell types were incubated at 37°C in a humid atmosphere of 5% CO2/95% air. Radiolabeling with [35S]methionine was accomplished in methionine-free medium as described (13); where appropriate, incubations contained 1 μM dexamethasone (Sigma).

Fluorescence-Activated Cell Sorting. Monolayer cultured cells were incubated with 1 μM dexamethasone overnight, harvested in TE/PBS (10 mM Tris, pH 7.4/1 mM EDTA/10 mM sodium phosphate/150 mM NaCl), washed once with DMEM/1% FCS, and resuspended in 100 μL of rabbit anti-MMTV antisera (diluted 1:100 in DMEM/1% FCS). After a 1-hr incubation on ice, the cells were washed twice with fresh DMEM/1% FCS followed by incubation in 100 μL of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (diluted 1:10 in DMEM/1% FCS). After a 1-hr incubation on ice, the cells were then washed twice in DMEM/1% FCS.

Abbreviations: MMTV, mouse mammary tumor virus; FCS, fetal calf serum.
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fixed with 1% paraformaldehyde, and analyzed by flow cytometry using a Becton Dickinson FACS IV (Flow Cytometry Facility of the Cancer Research Laboratory).

**Cell Fusions and Indirect Immunofluorescence.** Cells were fused in a monolayer with polyethylene glycol 1000 (Koch Chemicals Ltd., Hertford Herts, England) and dimethyl sulfoxide (Sigma) as previously described (16, 17). Heterokaryons were produced by fusing 3 × 10⁶ CR4 cells with an equivalent number of either uninfected HTC cells or isolated rat hepatocytes, whereas homokaryons were formed by fusing 6 × 10⁶ cells of a single type. Fused cells were allowed to recover in serum-supplemented medium for 48 hr and then were transferred to polylysine-coated (Sigma) coverslips; monolayers were cultured overnight in the absence or presence of 1 μM dexamethasone. Where indicated, actinomycin D at a final concentration of 5 μg/ml or cycloheximide at a final concentration of 300 μl/ml was added 30 min prior to hormone exposure. The cells were fixed with 3.7% formaldehyde, incubated with rabbit anti-MMTV antibody and rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochrannie, PA), and mounted on slides as described (17).

**Steady-State Radiolabeling and Immunoprecipitation of Cell Surface and Intracellular MMTV Proteins.** Fused cells were incubated in the absence or presence of 1 μM dexamethasone for 16 hr and next were radiolabeled with [³⁵S]methionine (100 μCi/ml; 1 Ci = 37 GBq) for 14 hr. Intact cells were then incubated with anti-MMTV antibodies, and cell surface-associated and intracellular MMTV proteins were immunoprecipitated and electrophoretically fractionated in NaDodSO₄/polyacrylamide gels as previously described (12, 13).

**RESULTS**

**Expression of Cell Surface MMTV Glycoproteins in Wild-Type M1.54 and Variant CR4 Cells.** To compare the relative levels of cell surface MMTV glycoproteins in wild-type M1.54, complement-selected CR4, and uninfected HTC cells, dexamethasone-treated cell populations were incubated with anti-MMTV glycoprotein antibodies and analyzed on a fluorescence-activated cell sorter. The mean fluorescence intensity of wild-type M1.54 cells was approximately 10-fold higher than that of variant CR4 cells, and there was virtually no overlap in the distribution of fluorescence (Fig. 1 Left). Importantly, the overall fluorescence distribution within populations of CR4 cells closely paralleled that of uninfected HTC cells. Anti-MMTV-antibody-directed immunofluorescence of individual dexamethasone-treated cells was examined after homologous cell fusions. Fused M1.54 cells displayed a bright immunofluorescent-capping pattern, which reflects the presence of plasma membrane-associated MMTV antigens (Fig. 1b). In contrast, CR4 and uninfected HTC homokaryons (Fig. 1d and f) lack cell surface-associated MMTV glycoproteins.

To determine if the fusion process itself had an effect on the expression of MMTV glycoproteins, the production of intracellular and cell surface viral glycoproteins was monitored in hormone-induced homokaryons of either wild-type M1.54, variant CR4, or uninfected HTC cells. Dexamethasone-treated, [³⁵S]methionine-labeled cells were incubated with anti-MMTV glycoprotein antibodies, washed extensively, and detergent solubilized. Fixed *Staphylococcus aureus* strain A was added to cell extracts containing antibody-bound antigens to immunoadsorb cell surface-associated MMTV glycoproteins, whereas additional antibodies and S. aureus strain A were added to the resulting supernates to immunoadsorb intracellular viral glycoproteins. NaDodSO₄/polyacrylamide gel electrophoresis revealed that hormone-induced homokaryons of M1.54 competently expressed a wild-type pattern of intracellular and cell surface MMTV glycoproteins (Fig. 2, lanes A and D, respectively). Homokaryons of variant CR4 synthesized nearly the same overall level of viral glycoproteins as fused M1.54 cells (compare lanes B and A in Fig. 2). However, this variant produced an altered array of intracellular viral glycoproteins (Fig. 2, lane B) and failed to localize any viral species to the cell surface (Fig. 2, lane E). Importantly, similar to results previously reported for unfused hormone-induced CR4 cells, the viral glycoprotein pattern detected in CR4 homokaryons

![Fig. 1. Fluorescence analysis of expressed cell surface MMTV glycoproteins in glucocorticoid-inducible fused and unfused cell populations. (Left) Unfused cell populations were treated with 1 μM dexamethasone for 16 hr and were analyzed for expression of cell surface MMTV glycoproteins by flow cytometry. The number of fluorescent cells is plotted as a function of the relative logarithm (log) of the fluorescence intensity. (Right) Dexamethasone-treated homokaryons of M1.54, CR4, and uninfected HTC cells were analyzed for the expression of cell surface MMTV glycoproteins by indirect immunofluorescence. Cells were visualized by phase (a, c, and e) and fluorescence (b, d, and f) microscopy. (Bar = 30 μm.)](image-url)
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dexamethasone
glycoproteins
(lanes Mr intracellular IC; and anhydrase serum NaDodSO4/polyacrylamide uninfected HTC MMTV transcripts viral ple, does protein synthesis, localization To examine when protein synthesis, localization of cell surface MMTV glycoproteins should be dependent upon de novo RNA and protein synthesis. Heterokaryons formed from pairwise fusions of uninfected HTC cells and other variant CR4 or wild-type M1.54 cells were treated with potent inhibitors of RNA (actinomycin D) or protein (cycloheximide) synthesis prior to the addition of dexamethasone. Both metabolic inhibitors prevented the dexamethasone-dependent localization of cell surface MMTV glycoproteins in CR4–HTC and M1.54–HTC heterokaryons (Fig. 3 j, l, n, and p). Under these conditions, actinomycin D inhibited [3H]uridine incorporation into total RNA by >98% and had no effect on total and MMTV protein synthesis, whereas cycloheximide prevented the incorporation of [3H]leucine by >95% without significantly affecting total transcription or MMTV RNA synthesis (13, 19).

The results shown in Fig. 3 (as well as the homologous fusions shown in Fig. 1) were quantitated by determining the percentage of fused cells (500 cells counted) that expressed cell surface MMTV glycoproteins. The complementation efficiency of dexamethasone-induced CR4–HTC fusions was 15.3%, whereas the complementation efficiency of uninduced heterokaryons and fusions carried out in the presence of cycloheximide was less than 1% (Fig. 4). Approximately 2.3% of hormone-induced heterokaryons treated with actinomycin D displayed a fluorescent pattern at their cell surface; this value closely corresponded to the level of RNA synthesis in the presence of this inhibitor. A similar low level of immunochemical positive heterokaryons (2.7%) was observed in M1.54–HTC heterokaryons exposed to hormone and actinomycin D.

Normal Rat Hepatocytes: Complement the Defect in CR4 Cells. Heterokaryon formation using CR4 cells as a recipient line provides an assay to demonstrate the existence of the glucocorticoid-regulated glycoprotein-trafficking pathway in any donor cell type. Of particular importance is if normal, fully-differentiated rat liver cells express the steroid-regulated sorting pathway. To test this notion, isolated rat hepatocytes were fused with CR4, and the localization of cell surface-associated MMTV glycoproteins in the absence or presence of dexamethasone was determined by indirect immunofluorescence. Normal rat hepatocytes complemented CR4 only after hormone exposure, suggesting that glucocorticoid-inducible normal adult liver components can mediate the compartmentalization of MMTV glycoproteins to the cell surface (compare c and d with g and h in Fig. 5). The complementation efficiency for normal hepatocytes was 18% for hormone-induced heterokaryons and less than 1% for uninduced heterokaryons (Fig. 5). As expected for uninfected cells, rat hepatocytes do not express cell surface MMTV glycoproteins (Fig. 5 b and f). Since this complementation is dependent upon dexamethasone treatment, the glucocorticoid-regulated trafficking activity that mediates the localization of cell surface glycoproteins appeared to be a normal component of the transcriptionally responsive glucocorticoid domain in adult rat liver.

DISCUSSION

By somatic complementation of the glycoprotein-trafficking variant CR4 with uninfected HTC cells and rat hepatocytes,
we have established that glucocorticoid-inducible cellular-encoded components act intrans to regulate the compartmentalization of cell surface MMTV glycoproteins. The precise mechanism by which this glucocorticoid regulated trafficking activity functions remains to be elucidated. Conceivably, rat hepatoma and liver cells may encode a positive sorting factor, analogous to the actions of \( \beta_2 \)-microglobulin (20, 21), egasyn (22), heavy-chain binding protein (23), or the mannos-6-phosphate receptor (4, 24), which directs the transport of specific glycoproteins into and through distinct intracellular routes or may help retain glycoproteins in a given vesicle population. Alternatively, glucocorticoid hormones may induce a processing activity that modifies specific classes of glycoproteins in a manner that allows them to be recognized by preexisting sorting receptors. Based on a comparison of oligosaccharide processing and expression of proteins in fractionated intracellular vesicle populations, our recent evidence suggests that the hormone-inducible components defective in variant CR4 most likely control MMTV glycoprotein sorting by acting in the Golgi.

Glucocorticoids induce biological responses by binding to and potentiating a functional change in a cytoplasmic receptor protein, which results in the selective recognition of transcriptional enhancer elements linked to promoters of hormone-regulated genes (18). As a result of this apparent sequence specificity, glucocorticoid–receptor complexes can directly modulate the rate of RNA synthesis (18, 25). Consistent with this mechanism of action, treatment with inhibitors of de novo RNA and protein synthesis (actinomycin D and cycloheximide, respectively) prevented the glucocorticoid-induced complementation of CR4 by uninfected HTC cells. The low level of complementation (2.3%) detected in fused cells exposed to actinomycin D corresponded closely to the level of de novo transcription observed (5%) under the conditions of this experiment.

The biological function of hormone-regulated glycoprotein trafficking in liver-derived tissues is unknown. Conceivably, this pathway may control the expression and activity of functionally related sets of genes at precise developmental stages or in response to specific environmental stimuli. For example, during inflammation \textit{in vivo}, glucocorticoids regulate the transcription of a variety of secreted liver glycoproteins (19, 26). Perhaps the rapid externalization of these glycoproteins may also reflect concomitant alterations in intracellular protein trafficking. With this in mind, the immunofluorescence assay was used to examine fully differentiated normal rat hepatocytes as well as hepatoma cells with different apparent developmental properties. Our results demonstrate that the relatively dedifferentiated HTC hepatoma cells (Fig. 3), the more differentiated Fu5 hepatoma cells (17), and normal liver cells (Fig. 5) complemented CR4...
cells in a hormone-dependent manner and therefore express the glucocorticoid-regulated trafficking activity. These studies also established that the hormone-dependent defect in CR4 is encoded by a complementation group distinct from the glucocorticoid receptor (17).

We have recently observed that transfection of genomic DNA isolated from uninfected HTC cells can complement the defect in variant CR4 (D.A.B. and G.L.F., unpublished work). Thus, the use of a well-characterized immunoselected variant defective in the localization of MMTV glycoprotein substrates has uncovered a glucocorticoid-inducible regulatory circuit that functions to direct the localization of exogenous viral glycoproteins to the cell surface. This regulated compartmentalization process may have general importance in mediating the trafficking of perhaps many other cellular glycoproteins, and variant CR4 cells will be particularly important for genetic approaches to define the biological significance of this phenomenon.

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