Journal of Cellular Biochemistry 35:271–284 (1987)
Steroid Hormone Action 67–80

Genetic Evidence That the Steroid-Regulated Trafficking of Cell Surface Glycoproteins in Rat Hepatoma Cells Is Mediated by Glucocorticoid-Inducible Cellular Components

Gary L. Firestone, Nancy J. John, Omar K. Haffar, and Paul W. Cook

Department of Physiology-Anatomy and Cancer Research Laboratory, University of California at Berkeley, Berkeley, California 94720

The biological control of posttranslational maturation and compartmentalization reactions that operate upon proteins during transport to their final cellular destinations is crucial for normal cellular function. Using the expression of mouse mammary tumor virus (MMTV) glycoproteins as sensitive probes in the viral-infected rat hepatoma cell line M1.54, we have discovered and documented a novel glucocorticoid-regulated trafficking pathway that controls the cell surface localization of MMTV glycoproteins. One complement-selected derivative of M1.54 cells, CR4, failed to compartmentalize cell surface MMTV glycoproteins in the presence of dexamethasone. To test genetically if this glycoprotein trafficking pathway is mediated by cellular or viral gene products, CR4 cells were fused with uninfected Fu5 rat hepatoma cells. Indirect immunofluorescence of CR4 × Fu5 heterokaryons revealed that Fu5 complemented the defect in CR4 only after exposure to 1 μM dexamethasone. The glucocorticoid inhibition of Fu5 proliferation was exploited to recover the receptor-deficient uninfected derivative EDR3 that expressed a 100-fold lower level of [^{3}H]dexamethasone binding activity. Analysis of CR4 × EDR3 cell fusions by indirect immunofluorescence revealed

Nancy J. John's present address is Institut für Physiologische Chemie 1, Universität Düsseldorf, Moorenstr 5, D-4000 Düsseldorf 1, Federal Republic of Germany.

Omar K. Haffar's present address is Genentech, Inc., Department of Molecular Immunology, 460 Point San Bruno Blvd., South San Francisco, CA 94080.

Address all correspondence to G.L. Firestone, Department of Physiology-Anatomy and Cancer Research Laboratory, University of California at Berkeley, Berkeley, CA 94720.

Received February 23, 1987; revised and accepted June 28, 1987.

that EDR3 cells complemented CR4 in a dexamethasone-dependent manner, suggesting that EDR3 supplied a functional trafficking component while CR4 provided a functional glucocorticoid receptor to the heterokaryons. Taken together, our results demonstrate that cellular-encoded glucocorticoid-inducible components mediate the regulated trafficking of cell surface MMTV glycoproteins.

Key words: glycoprotein trafficking variant, mouse mammary tumor virus, heterokaryons, glucocorticoid receptor deficient variant

Glucocorticoid hormones act via specific intracellular receptor proteins to elicit a broad spectrum of characteristic responses in many types of target tissues and in in vitro cultured cell lines [1–4]. Similar to other steroids, glucocorticoids bind to and potentiate a functional change in its receptor which results in an increased DNA-binding activity and a selective recognition of transcriptional enhancer elements linked to promoters of regulated genes [4–10]. As a result of this apparent sequence specificity, glucocorticoid receptor complexes can directly modulate the rate of RNA synthesis which can account for certain glucocorticoid responses [1,4]. In most cases, however, the precise molecular processes under hormonal control have not been fully defined. Conceivably, these steroid responses may actually result from secondary or "regulatory" effects in which steroids modulate the transcription of genes encoding polypeptides whose function is to affect selectively the expression of other biologically active gene products at transcriptional, posttranscriptional, translational, or posttranslational levels.

A biological advantage for such regulatory responses is the capability to rapidly turn on or off many functionally related sets of gene products by altering the expression of only a few steroid-regulated genes. Because of the number of potentially regulatable reactions, a particularly intriguing question is whether steroid hormones can selectively modulate posttranslational pathways. For example, proteins can be processed by many types of modification reactions (such as glycosylation and phosphorylation) and can be transported through distinct routes into a variety of cellular and extracellular compartments [11–18]. Evidence to support the steroid-regulated control of posttranslational maturation and/or sorting of glycoproteins has been generated in only a few systems. For example, in oviduct membranes, estrogen regulates the level of specific glycosyl transferase activities which function to modify glycoproteins [19]. Also, in macrophages, glucocorticoid hormones have been shown to stimulate an increase in the level of mannose-6-phosphate receptors which control the proper localization of certain hydrolysases into the lysosome [20]. In general, most of the observed phenomena have not been amenable to detailed analysis owing to the lack of experimentally accessible glycoprotein targets for the steroid-regulated pathways.

We have been using the expression of mouse mammary tumor virus (MMTV) glycoproteins in viral-infected rat hepatoma tissue culture (HTC) cells as sensitive probes to explore a unique posttranslational glycoprotein trafficking circuit under the control of glucocorticoid hormones. M1.54 cells are a clonal isolate of MMTV-infected HTC cells containing ten integrated proviruses [21]. Treatment of these cells with dexamethasone, a synthetic glucocorticoid, selectively stimulates the compartmentalization of three new cell surface glycoproteins (gp78, gp70, and gp32) and an extracellular species (gp70) derived from a common glycosylated polyprotein (Pr^{env}). The major constitutive product is a 50,000-dalton (gp50) extracellular glycoprotein

which represents the aminoterminal two-thirds of the precursor [22–24]. Our recent evidence has shown that this glucocorticoid-regulated trafficking process requires receptor function and the de novo synthesis of components that appear to function in the medial to trans Golgi [25,43]. Dexamethasone also increased the rate of MMTV RNA synthesis and posttranslational processing of the phosphorylated precursor polyprotein but did not qualitatively alter the structure or function of expressed viral transcripts [22,26–29].

Using complement-mediated cytolysis, we have selected and characterized a M1.54-derived variant (designated CR4) that is defective in the glucocorticoid-regulated compartmentalization of cell surface MMTV glycoproteins [24,26]. Relatively little is known about the cellular mechanism and function of this posttranslational regulatory circuit. Of particular importance is whether cellular or viral sequences encode the glucocorticoid regulated trafficking activity. Moreover, are variant CR4 cells defective in the expression of this pathway or is there a subtle lesion in its glucocorticoid receptors? In a genetic approach to understand the molecular details of this phenomenon, we have utilized CR4 cells and selected receptor-defective variants from uninfected hepatoma cells to demonstrate that cellular encoded gene products act in trans to mediate the glucocorticoid-dependent trafficking of MMTV glycoproteins to the cell surface.

MATERIALS AND METHODS

Materials

All media and sera used for tissue culture were purchased from the UCSF Tissue Culture facility. L-[^{35}S]Methionine (1,000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL); dexamethasone, dimethyl sulfoxide, and polylysine (type B-1, average molecular weight of 150,000) were purchased from Sigma Chemical Company (St. Louis, MO); polyethylene glycol 1000 was obtained from Koch Chemical Ltd. (Hartford Harts, England); Pansorbin was acquired from Calbiochem (La Jolla, CA); and Kodak X-ray film was purchased from Merry X-Ray Chemical Corp. (Burlingame, CA). Rhodamine-conjugated goat antirabbit IgG was obtained from Capell (Malvern, PA) and the MMTV and preimmune antisera [30] used in this study were generous gifts of L.J.T. Young and R.D. Cardiff (Department of Pathology, University of California, Davis). The Fu5 rat hepatoma cell line was a gift of J.A. Peterson (John Muir Research Institute, Walnut Creek, CA). All other reagents were of highest available purity.

Cells and Method of Culture

M1.54 is a cloned cell line isolated from MMTV-infected rat populations of cultured hepatoma (HTC) cells and contains ten stably integrated MMTV proviruses [21]. Variant CR4 cells are a clonal derivative of M1.54 selected for its resistance to anti-MMTV-antibody directed complement cytolysis [24,26]. Fu5 cells are derived from the Reuber H35 hepatoma, and its origin and characteristics have been described previously [31]. EDR3 cells are a single-cell-derived growth variant of Fu5 (see below) that expressed less than 1% of its dexamethasone binding activity. FM7 is a clonal cell line derived from an MMTV-infected population of Fu5 cells. The HTC-derived cell lines were propagated as monolayers in Dulbecco's modified Eagle's medium (DME) supplemented with 10% horse serum, while the Fu5-derived cell lines were grown in monolayers in a 1:1 mixture of DME:F12 medium supplemented with 10% fetal calf serum (Hyclone) on Falcon tissue culture plates. All cells were cultured at 37°C in a humid atmosphere of air/CO_2 (95%:5%). Cells were radiolabeled with [^{35}S]methionine in methionine-free medium supplemented with 0.5%

dialyzed fetal calf serum. Cells were allowed to incubate 30 min in methionine-free medium prior to addition of the radiolabel. Glucocorticoid-treated cells received 1 μM dexamethasone.

Selection of Fu5 Variants Defective in Glucocorticoid Responsive Growth Inhibition

The Fu5 cell population was mutagenized with 900 μg/ml EMS for 48 hr. Surviving cells (approximately 5–10%) were washed extensively with phosphate buffered saline (PBS) and grown to confluency. This mutagenized population was then repeatedly passaged from low to high density in the presence of 1 μM dexamethasone for 11 mo. Random dilution subclones were recovered, examined for their ability to proliferate in the presence of dexamethasone, and screened for their inability to bind [^{3}H]dexamethasone as described previously [26]. One subclone with a particularly low level of hormone binding activity (denoted EDR3) was used for further studies.

Scatchard Analysis of Glucocorticoid Binding Activity

Monolayer cultures were grown to 80% confluency, washed three times in PBS at 4°C, and harvested by centrifugation at 600g for 5 min. Pelleted cells (0.5 ml) were then disrupted by polytron homogenization in 3.5 ml of TEGBN040 [10 mM Tris-HCl (pH 8.1), 1 mM EDTA (pH 8.1), 1 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin, and 40 mM NaCl] at 4°C. Cell extracts were centrifuged at 10,000g for 15 min at 4°C. Aliquots (100 μl) of the supernatant fraction were then incubated with several concentrations of [^{3}H]dexamethasone (0, 1.25, 2.5, 5, 10, 20, 40, 80, 100, and 150 nM) in the presence or absence of 100 μM unlabeled dexamethasone and assayed for [^{3}H]dexamethasone binding activity by a glass fiber filter assay as described previously [32]. From the calculated free and experimentally determined specifically bound levels of radiolabeled dexamethasone, a Scatchard plot of hormone binding activity was constructed. Protein concentrations were determined by the method of Lowry et al [33] using bovine serum albumin as a standard.

Recovery of MMTV-Infected Subclones of Fu5

Fu5 cell populations were infected with mouse mammary tumor virus (MMTV) by incubating subconfluent monolayers of Fu5 cells with medium from GR mouse mammary carcinoma cells for 48 hr. Since the GR cells were cultured in the presence of 100 nM dexamethasone to stimulate the production of MMTV, RU 38486 (1 μM), a potent glucocorticoid antagonist, was added during the infection period in order to allow the Fu5 cell population to proliferate normally. This procedure was repeated four times, and the infected cell population dilution subcloned. Individual cell colonies expressing secreted MMTV glycoproteins were identified by a filter immunoassay using anti-MMTV antibodies as the primary antibodies as described previously [34].

Steady-State Incorporation of Radiolabeled Material

Cells were incubated in the presence or absence of 1 μM dexamethasone for 16 hr and subsequently radiolabeled with 30 μCi/ml [^{35}S]methionine for 10 hr. Appropriate cultures contained dexamethasone throughout the radiolabeling period. The cell monolayers were washed three times with PBS and prepared for immunoprecipitations as described below.

Cell Surface Immunoprecipitations

The expression of metabolically radiolabeled MMTV polypeptides at the cell surface was selectively examined by modifications of the method of Krangel et al [35]. Briefly, washed monolayers of intact cells were incubated with 4 μl polyclonal MMTV antibodies or preimmune sera per 1 ml PBS at 4°C for 15 min. The cells were washed once with medium containing 10% horse serum, three times with PBS, and then harvested. Cells were detergent solubilized as described above and incubated with fixed *Staphylococcus aureus* A (Staph A) for 5 min to immunoadsorb the surface-associated viral proteins. After centrifugation, the immunoprecipitation was continued on the Staph A-pelletable MMTV proteins to monitor the cell surface-associated species, whereas the direct addition of anti-MMTV antibodies to supernates (non-Staph A-pelletable material) monitors the intracellular MMTV proteins. Under these conditions, only surface-associated MMTV polypeptides are immunoadsorbed in the Staph A-pelletable material, since p24, a known intracellular and highly immunogenic MMTV phosphoprotein, fails to be detected. The immunoprecipitated MMTV proteins from cell surface and intracellular fractions were electrophoretically fractionated in SDS polyacrylamide gels as described below.

Immunoprecipitation and Electrophoretic Analysis of MMTV Proteins

Immunoprecipitations were carried out with either polyclonal anti-MMTV glycoprotein antibodies or total anti-MMTV antibodies using a highly sensitive Staph A-immunoadsorption procedure that detects low-level proteins in the virtual absence of nonspecific background [36,37]. MMTV proteins bound to the final Staph A pellets were electrophoretically fractionated in SDS polyacrylamide gels and analyzed by fluorography as described previously [23,36,38].

Cell Fusions

Heterokaryons were produced by cell fusion in the presence of polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) according to the method of Murname and Painter [39]. For optimal heterokaryon formation, a 1:2 ratio of CR4:Fu5 or EDR3 (2.5×10^6 cells:5×10^6 cells) was mixed together and added to 100-mm tissue culture dishes (Corning). After a 6-hr incubation for cell attachment, cells were fused by a 1-min treatment with 40% (wt/vol) solution of PEG dissolved in DME containing 10% DMSO. Cells were washed four times with DME containing 10% DMSO followed by three washes in DME alone. The cells were allowed to recover in DME supplemented with 10% horse serum for 48 hr before further analysis.

Indirect Immunofluorescence

Cells were transferred to polylysine-coated coverslips and cultured overnight in the presence or absence of 1 μM dexamethasone, washed several times with PBS, and fixed by incubating with 3.7% formaldehyde in PBS for 20 min at room temperature. After washing extensively in PBS containing 10 mM glycine, the cells were incubated with 1 ml PBS containing 80 μl of a 1:10 dilution of anti-MMTV antibody and 100 μl of bovine serum albumin solution (50 mg/ml) for 30 min on ice. The antibody solution was then aspirated off, the cultures washed several times in PBS-glycine, and then incubated with 500 μl PBS containing 120 μl of a 1:100 dilution of rhodamine-conjugated goat antirabbit IgG. After 30 min on ice, the coverslips were washed four times in PBS-glycine and then mounted on slides in 90%

glycerol, 10% Tris-HCl (100 mM), pH 7.5 [40]. Mounted slides were either examined immediately by fluorescence microscopy or stored at −20°C before visualization.

RESULTS

Glucocorticoid-Regulated Compartmentalization of Cell Surface MMTV Glycoproteins in Wild-Type M1.54 and Variant CR4 Cells

The glucocorticoid-regulated localization of cell surface MMTV glycoproteins was compared in wild-type M1.54 and variant CR4 cells by direct plasma membrane immunoprecipitation to distinguish metabolically labeled cell surface viral glycoproteins from immunoadsorbed intracellular species. [^{35}S]Methionine-labeled dexamethasone-induced and uninduced cells were incubated with anti-MMTV glycoprotein antibodies, washed extensively, and detergent solubilized. To immunoprecipitate cell surface-associated viral glycoproteins (denoted CS), fixed Staph A was added directly to the cellular extracts and pelleted after a short incubation. A second aliquot of anti-MMTV glycoprotein antibodies was added to the supernates, and the intracellular MMTV glycoproteins (denoted IC) immunoprecipitated after a subsequent incubation with Staph A. SDS-polyacrylamide gel electrophoresis of immunoadsorbed material revealed that both M1.54 and CR4 cells competently synthesized intracellular MMTV glycoproteins in the presence or absence of hormone (Fig. 1, lanes A and B vs. E and F). However, MMTV glycoproteins (in particular the polyprotein gp70) were transported to the cell surface in M1.54 cells only after exposure to dexamethasone (lane C vs. D), while in CR4 cells gp70 fails to be compartmentalized to the cell surface in a dexamethasone-regulated manner (lane G vs. H). For this particular experiment, the cells were treated with the ionophore monensin to accumulate high levels of gp70 at its appropriate cellular location depending on the presence of hormone [25].

Indirect immunofluorescence of intact hormone-induced M1.54 and CR4 cells further confirmed that M1.54 localized MMTV glycoproteins to the cell surface while CR4 cells were defective in this process. Dexamethasone-treated M1.54 displayed a clearly visible fluorescent pattern indicative of cell surface viral antigens (Fig. 2a,b). In contrast, a similar fluorescent pattern was not visible in hormone-induced CR4 (Fig. 2c,d). We have shown previously that CR4 cells synthesize normal MMTV transcripts and glycosylated precursor polyproteins, express wild-type levels of functional glucocorticoid receptors, proliferate normally, and are not globally defective in the expression of intracellular and cell surface glycoproteins, as well as total functional mRNA [24,26].

Using this immunofluorescence assay, CR4 should provide a particularly useful recipient cell line for cell fusions to test biological features of the glucocorticoid-regulated pathway that mediate the trafficking of cell surface glycoproteins. Of particular importance is whether the crucial glucocorticoid-inducible component is viral or cellular encoded or if CR4 has a subtle defect in its receptor per se. To test this notion, an appropriate CR4 fusion partner would be an uninfected, glucocorticoid receptor-defective cell line derived from a glucocorticoid-responsive parent line that has the potential to express cell surface viral antigens. However, the currently available receptor-defective HTC cell lines contain stably integrated MMTV proviruses and therefore another hepatoma line was used for these studies.

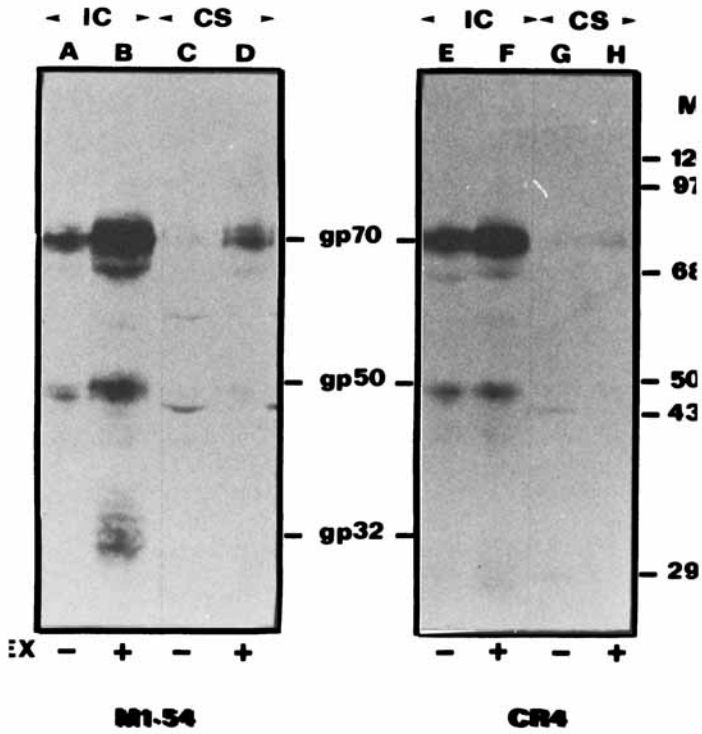

Fig. 1. Glucocorticoid-regulated expression of cell surface and intracellular MMTV glycoproteins. Wild-type M1.54 and variant CR4 cells were treated in the presence or absence of 1 μM dexamethasone for 16 hr and then radiolabeled with [^{35}S]methionine for 10 hr. Cell monolayers were extensively washed and cell surface (**lanes C,D,G,H**; denoted CS) and intracellular (**lanes A,B,E,F**; denoted IC) MMTV glycoproteins immunoprecipitated as described in the text. Immunoprecipitated proteins were then electrophoretically fractionated in SDS-polyacrylamide gels and radioactive proteins visualized by fluorography. Molecular weight standards are as follows: β-galactosidase (120,000 M_r), phosphorylase b (97,000 M_r), bovine serum albumin (68,000 M_r), ovalbumin (43,000 M_r), and carbonic anhydrase (29,000 M_r).

Isolation and Characterization of Fu5 Hepatoma-Derived Glucocorticoid Receptor-Defective Cells

The Fu5 rat hepatoma cell line is relatively differentiated and highly glucocorticoid responsive [31]. Immunoprecipitation of radiolabeled Fu5 revealed that this cell line does not express MMTV proteins and as expected failed to display a cell surface immunofluorescent pattern using anti-MMTV antibodies (Fig. 2e,f). To test the potential to express cell surface MMTV glycoproteins, populations of Fu5 cells were infected with MMTV. One of the stable subclones recovered from this infection (FM7) was characterized for the production of MMTV gene products. Dexamethasone-induced and uninduced FM7 as well as HTC-derived M1.54 cells (see above) were radiolabeled with [^{35}S]methionine, and cellular and secreted MMTV proteins were examined by immunoprecipitation. Electrophoretic analysis in SDS-polyacrylamide gels (Fig. 3) revealed that FM7 and M1.54 expressed a similar molecular weight spectrum of cellular viral proteins in the presence of hormone including the viral glycosylated polyproteins (gp78 and gp70) and maturation products (gp50 and gp32). One notable difference was the lack of secreted gp70 from FM7. Indirect

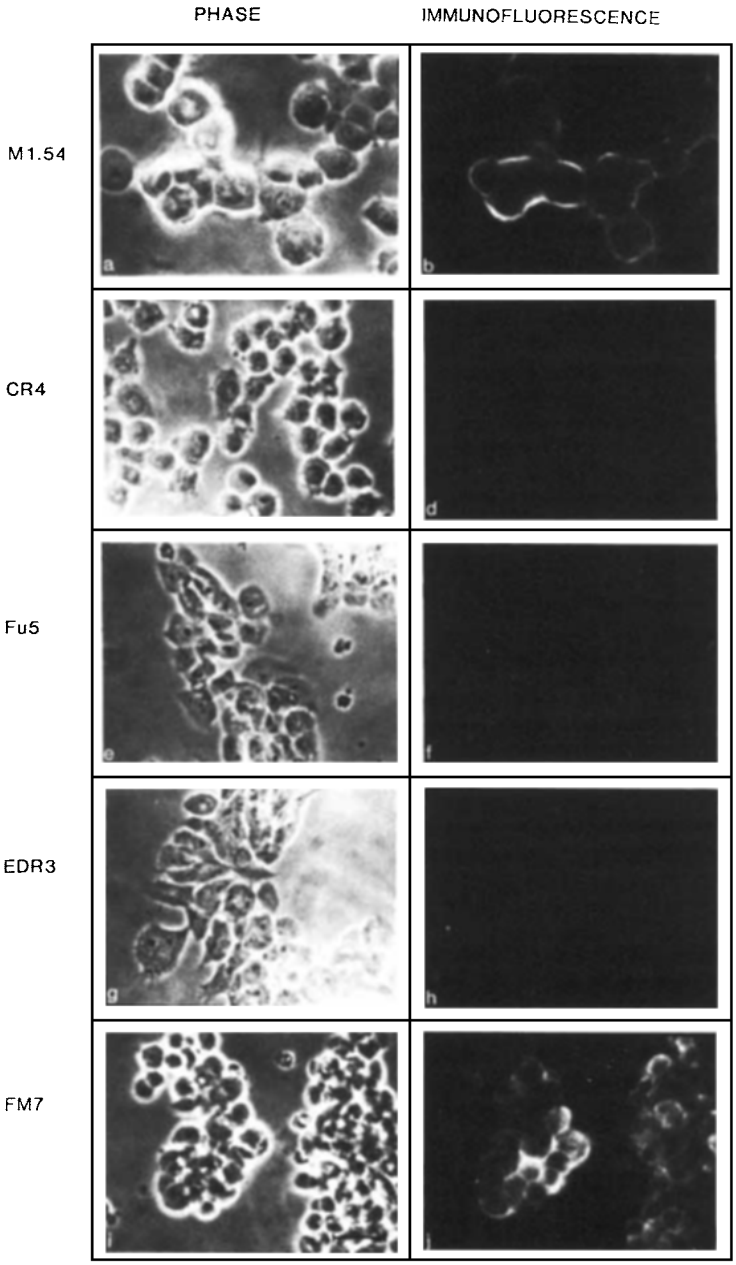

Fig. 2. Immunofluorescence analysis of cell surface MMTV glycoproteins. Cells were plated onto polylysine-coated coverslips, incubated with 1 μM dexamethasone for 16 hr, fixed in formaldehyde, and incubated sequentially with anti-MMTV antibodies and rhodamine-conjugated goat antirabbit IgG as described in the text. Populations of cells were examined by both phase (**a,c,e,g,i**) and fluorescence (**b,d,f,h,j**) microscopy. The bar in panel i represents 30 μm.

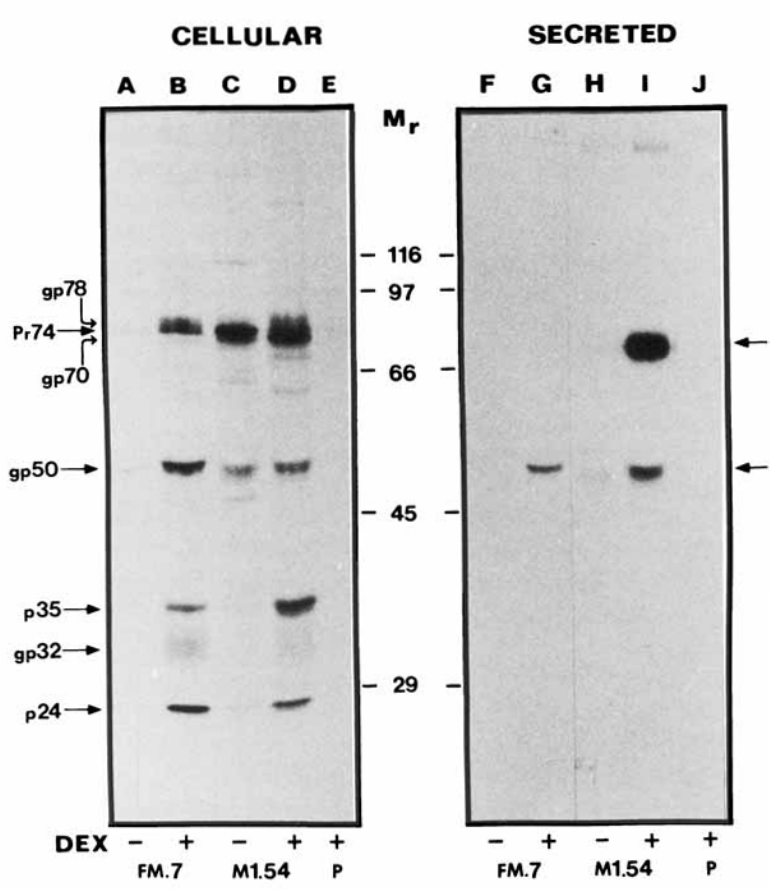

Fig. 3. Glucocorticoid-regulated expression of cellular and secreted MMTV proteins in viral-infected cell lines. Dexamethasone-induced and uninduced M1.54 and FM7 cells were radiolabeled with [^{35}S]methionine for 10 hr and cellular (**lanes A–E**) and secreted (**lanes F–J**) fractions harvested. Detergent-solubilized fractions were immunoprecipitated with either anti-MMTV antibodies (lanes A–D and F–I) or preimmune serum (lanes E and J) and immunoprecipitated material electrophoretically fractionated in SDS-polyacrylamide gels. The radiolabeled proteins were visualized by fluorography. The molecular weight standards are described in Figure 1.

immunofluorescence of hormone-induced FM7 (Fig. 2i,j) further revealed that Fu5-derived cell lines localized MMTV glycoproteins to the cell surface in the presence of dexamethasone.

We have shown that treatment with glucocorticoid hormones caused an inhibition of Fu5 proliferation rate that resulted in a lower saturation density [41]. This hormone-responsive growth property provided a selective strategy, unrelated to the expression of MMTV gene products, which allowed the recovery of receptor-defective Fu5 derivatives. Briefly, ethyl methanesulfonate (EMS) mutagenized, uninfected Fu5 cells were cultured continuously in 1 μM dexamethasone for 11 mo; cells were always passaged from low to high density to enrich for cell populations that proliferated in the presence of dexamethasone at high cell densities. Using this procedure, several variant subclones resistant to the growth-inhibitory effects of dexamethasone were recovered. Scatchard plot analysis of one such subclone, EDR3, revealed that

this cell line had a 100-fold reduction in the level of [^{3}H]dexamethasone binding activity (Fig. 4). This cell line also proved to be functionally defective since several glucocorticoid-mediated responses, such as tyrosine aminotransferase, alpha$_1$-acid glycoprotein, and glutamine synthetase were not induced in EDR3 (data not shown) while dot-blot analysis using a cloned rat glucocorticoid receptor cDNA probe revealed that EDR3 expressed approximately 1% wild-type levels of receptor transcripts [41]. Furthermore, as expected, indirect immunofluorescence revealed that hormone-induced EDR3 did not express cell surface MMTV glycoproteins (Fig. 2g,h) since this variant was derived from uninfected Fu5 cell populations. RNA dot blots and immunoprecipitations also confirmed the lack of expressed viral gene products in this cell line (data not shown).

It is important to point out that EDR3 is a stable mutant. This cell line has retained its glucocorticoid-unresponsive phenotype after 6 mo in continous culture, and therefore has a particularly low spontaneous reversion frequency. In fact, we have recently demonstrated that only by transfection of cloned glucocorticoid receptor DNA back into EDR3 do we detect wild-type [^{3}H]dexamethasone binding properties, receptor transcripts, and glucocorticoid-regulated growth inhibition [41]. Thus, results described in the following cell fusion experiments are not due to spontaneous reversion of the mutant cell lines.

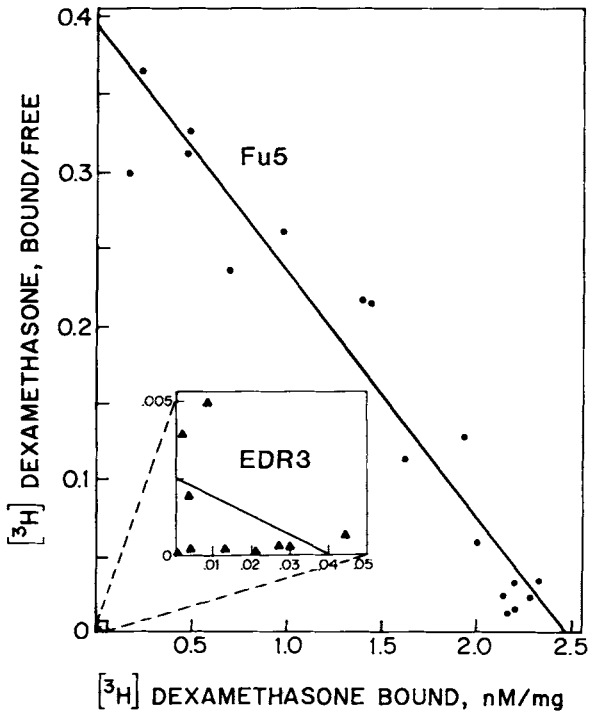

Fig. 4. Scatchard plot analysis of dexamethasone binding activity in Fu5 and EDR3 cells. Cell extracts were incubated with 0, 1.25, 5, 10, 20, 40, 80, 100, and 150 nM [^{3}H]dexamethasone in the presence or absence of 100 μM dexamethasone and assayed for [^{3}H]dexamethasone binding activity as described in the text. From the calculated free and experimentally determined specific bound levels of [^{3}H]dexamethasone, a Scatchard plot of hormone binding activity was constructed. The insert shows the EDR3 Scatchard plot on a magnified scale.

Cell Fusions Using the MMTV Glycoprotein Trafficking Variant CR4

Transient heterokaryons were formed by PEG-mediated fusions of CR4 and either receptor-containing uninfected Fu5 or receptor-defective uninfected EDR3 cells. Fused cells were cultured in the presence or absence of 1 μM dexamethasone for 12 hr, and fixed cells incubated sequentially with anti-MMTV antibodies and rhodamine-conjugated goat antirabbit IgG. Both Fu5 and EDR3 complemented the glycoprotein trafficking variant (CR4) in a glucocorticoid-dependent fashion (Fig. 5). As shown, dexamethasone-induced heterokaryons (CR4 × Fu5; CR4 × EDR3) displayed an immunofluorescent pattern indicative of the presence of cell surface MMTV glycoproteins (Fig. 5d,h), whereas uninduced heterokaryons failed to show any cell surface immunofluorescence (Fig. 5b,f). It is important to point out that MMTV-directed immunofluorescence was not observed in homologous fusions of either CR4, Fu5, or EDR3 (data not shown).

Quantitation of 300 heterokaryons revealed that 29% of CR4 × Fu5 fusions and 18% of CR4 × EDR3 fusions were positive for cell surface immunofluorescence in the presence of hormone. These complementation efficiencies represented a significant fraction of fused cells. However, the 1:2 (CR4:Fu5; CR4:EDR3) fusion ratio used for complementation may not have been optimal, and it was difficult to control the number of nuclei per heterokaryon and the level of homologous fusions. In any case, the presence of significant numbers of fluorescence-positive heterokaryons demonstrated that the glycoprotein-sorting defect in CR4 cells can be complemented by uninfected hepatoma cells only in the presence of dexamethasone. Thus, cellular-encoded gene products under control of glucocorticoid hormones mediate the regulated trafficking of cell surface glycoproteins in hepatoma cells. Moreover, the CR4 × EDR3 further demonstrated that CR4 does not contain a subtle lesion in its

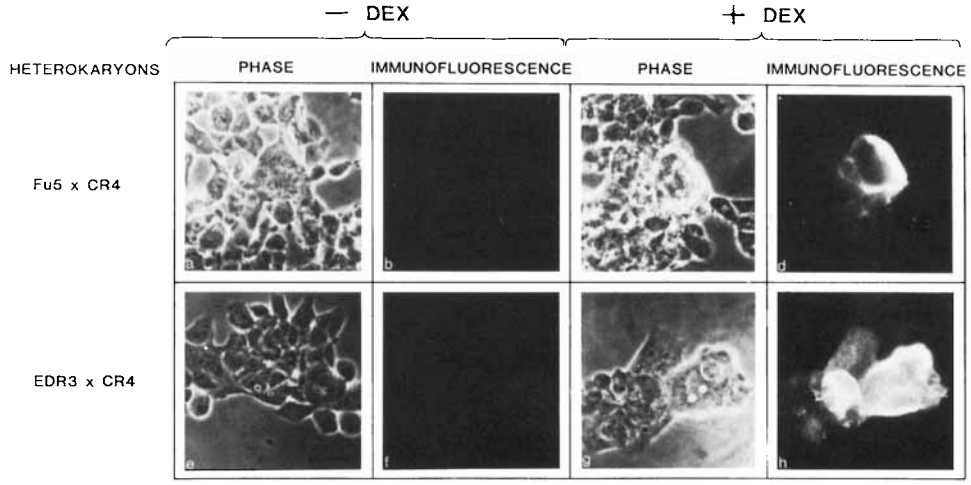

Fig. 5. Glucocorticoid-regulated expression of cell surface MMTV glycoproteins in heterokaryons. Variant CR4 cells were fused with either uninfected Fu5 (**a–d**) or receptor-defective uninfected EDR3 (**e–h**) cells and treated in the presence (c,d,g,h) or absence (a,b,e,f) of 1 μM dexamethasone for 16 hr. The cells were then fixed in formaldehyde and analyzed for the expression of cell surface MMTV glycoproteins by indirect immunofluorescence as described in the text. Cells were visualized by phase (a,c,e,g) and fluorescence (b,d,f,h) microscopy. The bar in panel e represents 30 μm.

receptor, since, in this case, EDR3 supplied a functional glycoprotein trafficking gene, while CR4 supplied a functional glucocorticoid receptor to the heterokaryon.

DISCUSSION

The posttranslational sorting and maturation of cell surface-associated MMTV glycoproteins are dependent upon an array of host cell components. Viral substrates therefore provide sensitive and perhaps general probes to assess mechanisms directing the trafficking of cellular glycoproteins. In MMTV-infected rat hepatoma cells, the localization of MMTV glycoproteins from an intracellular compartment to the cell surface is observed only after exposure to glucocorticoid hormones such as dexamethasone [22,24–26]. Given current concepts of glucocorticoid hormone action [1,4], one likely mechanism by which dexamethasone can alter glycoprotein compartmentalization is by stimulating the de novo synthesis of cellular components which function at a specific site within a regulated posttranslational trafficking pathway. To test this notion genetically, a series of heterokaryons were formed using a previously characterized trafficking variant (CR4) defective in the glucocorticoid-regulated localization of cell surface MMTV glycoproteins. Importantly, indirect cell surface immunofluorescence revealed that both uninfected Fu5 hepatoma cells as well as an uninfected Fu5-derived glucocorticoid receptor defective variant (EDR3) complemented the defect in CR4 only in the presence of dexamethasone. These results demonstrated that the glucocorticoid-regulated compartmentalization of cell surface MMTV glucoproteins is mediated by cellular encoded genetic elements whose gene products act in trans on MMTV glycoprotein substrates. Furthermore, CR4 cells do not have a subtle defect in their glucocorticoid receptor but rather are unable to express a functional glycoprotein trafficking gene (or genes) in a steroid-regulated manner.

The uninfected Fu5 rat hepatoma cell line was used for the heterokaryon studies because of the capability to select receptor defective variants by selection procedures unrelated to MMTV gene expression. In this case, variants were readily recovered by their ability to proliferate rapidly in the presence of 1 μM dexamethasone; wild-type Fu5 populations reach quiescence at a lower saturation density in the presence of hormone. Although Fu5 are a more differentiated hepatoma cell line than HTC cells (from which M1.54 and CR4 were derived), analysis of expressed viral glycoproteins in MMTV-infected subclones demonstrated that Fu5-derived cells produce cell surface MMTV glycoproteins only in the presence of glucocorticoids. Complementation of CR4 was therefore accomplished by the functional expression of the steroid-regulated compartmentalization pathway in both Fu5 and EDR3 cells. In this latter fusion, EDR3 supplied a functional trafficking gene, while CR4 supplied a functional glucocorticoid receptor, thus establishing a minimal requirement for two genetically defined complementation groups or genetic elements whose function are needed for the regulated expression of cell surface MMTV glycoproteins.

Uninfected HTC cells can also complement CR4 in a glucocorticoid dependent manner, while exposure to inhibitors of RNA and protein synthesis prevented the glucocorticoid-regulated expression of cell surface MMTV glycoproteins in the heterokaryons [42]. The need for a glucocorticoid-inducible de novo synthesized component is consistent with our previous biochemical studies [25] and suggests that the trafficking gene(s) may be a primary-steroid-regulatable genetic element. Thus, two

developmentally distinct hepatoma cell types express gene products that mediate a glucocorticoid-regulated glycoprotein trafficking pathway. Our recent studies have also shown that isolated normal rat hepatocytes complemented CR4 in a glucocorticoid-dependent manner, demonstrating that the regulated compartmentalization of cell surface glycoproteins is also expressed as part of the glucocorticoid-responsive domain of fully differentiated nontumorigenic tissues.

The role of a regulated trafficking pathway in normal liver function and/or development remains obscure, although regulation of entry into and through cellular sorting routes is crucial for localizing biologically active glycoproteins to their proper site of function in a precisely matured form. Vesicle- or organelle-associated "sorting receptors" have been postulated to bind, and help segregate selectively appropriate glycoprotein substrates during their intracellular transit [14–18,20]. Conceivably, glucocorticoids might induce cellular components which either functionally modify glycoprotein substrates such that they acquire a specific sorting signal or induce unique sorting receptors. In this regard, our recent cell fractionation experiments have shown that CR4 fails to express several nonviral membrane-associated proteins (most notably a 120,000 species) that cofractionate with intracellular Golgi membranes (D.A. Bravo and G.L. Firestone, unpublished). Moreover, the MMTV glycoproteins are inefficiently galactosylated in either uninduced wild-type M1.54 or hormone-induced variant CR4 cells which together indicates that a glucocorticoid-regulated branchpoint in glycoprotein trafficking exists in the Golgi [43]. Through further analysis of CR4, and by isolation and characterization the glucocorticoid-regulated gene products that mediate the compartmentalization of cell surface glycoproteins, it should be possible to delineate the biological significance of this unique steroid-modulated regulatory phenomenon.

ACKNOWLEDGMENTS

We thank Nicholas Black, Deborah A. Bravo, Karen S. Brown, Kinley Karlsen, Emily J. Platt, Anne K. Vallerga, and Sandra D. Winguth for their helpful comments on this manuscript and suggestions during the course of this study. We also thank Aileen K. Kim for her typing and preparation of this manuscript and John Underhill for his skillful photography.

This work was supported by a grant from the National Science Foundation (DCB 8616446). G.L. Firestone is recipient of a National Science Foundation Presidential Young Investigator Award (PCM-8351884). N.J. John and O.K. Haffar were postdoctoral trainees and P.W. Cook was a predoctoral trainee supported by National Research Service grant CA 09041 awarded by the National Institutes of Health, DHHS. In the later stages of the work, N.J. John was supported by a postdoctoral fellowship from the Americal Cancer Society, Inc. (PF-2892).

REFERENCES

1. Anderson JH: In Goldberger RF, Yamamoto KR (eds): "Biological Regulation and Development, Vol. 3B: Hormone Action." New York: Plenum Press, 1984, pp 169–212.
2. Baxter JD, Forsham PH: Am J Med 53:573–579, 1972.
3. Ivarie RD, O'Farrell PH: Cell 13:41–55, 1978.
4. Yamamoto KR: Annu Rev Genet 19:209–252, 1985.
5. Chandler VL, Maler BA, Yamamoto KR: Cell 33:489–499, 1983.

6. Karin M, Haslinger A, Holtgrave A, Richards RC, Krauter P, Westphal HM, Beato M: Nature 308:513–519, 1984.
7. Payvar F, DeFranco D, Firestone GL, Edgar B, Wrange O, Okret S, Gustafsson JA, Yamamoto KR: Cell 35:381–392, 1983.
8. Payvar F, Firestone GL, Ross SR, Chandler VL, Wrange O, Carlstedt-Duke J, Gustafsson JA, Yamamoto KR: J Cell Biochem 19:241–247, 1982.
9. Payvar F, Wrange O, Carlstedt-Duke J, Okret S, Gustafsson JA, Yamamoto KR: Proc Natl Acad Sci USA 78:6628–6632, 1981.
10. Ponta H, Kennedy N, Skroch P, Hynes NE, Groner B: Proc Natl Acad Sci USA 82:1020–1024, 1985.
11. Wold F: Annu Rev Genet 50:783–814, 1981.
12. Kornfeld R, Kornfeld S: Annu Rev Biochem 45:217–237, 1976.
13. Kornfeld R, Kornfeld S: Annu Rev Biochem 54:631–664, 1985.
14. Farquhar MG: Annu Rev Cell Biol 1:447–488, 1985.
15. Kelly R: Science 230:25–32, 1985.
16. Sabatini DD, Kreibich G, Morimoto T, Adesnik M: J Cell Biol 92:1–22, 1982.
17. Dunphy WG, Rothman JE: Cell 42:13–21, 1985.
18. Griffiths G, Simons K: Science 234:438–443, 1986.
19. Starr CM, Lucas JJ: Arch Biochem Biophys 237:261–270, 1985.
20. Sheperd VL, Konish MG, Stahl P: J Biol Chem 260:160–164, 1985.
21. Ringold GM, Shank PR, Varmus HE, Ring J, Yamamoto KR: Proc Natl Acad Sci USA 76:665–669, 1979.
22. Firestone GL, Payvar F, Yamamoto KR: Nature 300:221–225, 1982.
23. Firestone GL: J Biol Chem 258:6155–6161, 1983.
24. Firestone GL, John NJ, Yamamoto KR: J Cell Biol 103:2323–2331, 1986.
25. Haffar OK, Vallerga AK, Marenda SA, Witchel HJ, Firestone GL: Mol Cell Biol 7:1508–1517, 1987.
26. Firestone GL, Yamamoto KR: Mol Cell Biol 3:149–160, 1983.
27. Ucker DS, Ross SR, Yamamoto KR: Cell 27:257–266, 1981.
28. Ucker DS, Firestone GL, Yamamoto KR: Mol Cell Biol 3:551–561, 1983.
29. Karlsen D, Vallerga AK, Honè J, Firestone GL: Mol Cell Biol 6:574–585, 1986.
30. Cardiff RD, Puentes MJ, Young LJT, Smith GH, Teramoto YA, Altrack BW, Pratt TS: Virology 85:157–167, 1978.
31. Peterson JA, Weiss MC: Proc Natl Acad Sci USA 69:571–575, 1972.
32. Goldfeld AE, Firestone GL, Shaw PA, Gluecksohn-Waelsch S: Proc Natl Acad Sci USA 80:1431–1434, 1983.
33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
34. McCraken AA, Brown JL: Biotechniques 2:82–87, 1984.
35. Krangel MS, Orr HT, Strominger JL: Cell 18:979–991, 1979.
36. Platt EJ, Karlsen K, Lopez-Valdivieso A, Cook PW, Firestone GL: Anal Biochem 156:126–135, 1986.
37. John NJ, Firestone GL: Biotechniques 4:404–406, 1986.
38. Laemmli UK: Nature 227:680–685, 1970.
39. Murname JP, Painter RP: Proc Natl Acad Sci USA 79:1960–1963, 1982.
40. Satake M, Lutwig RB: Virology 124:259–273, 1983.
41. Cook PC, Swenson KT, Edwards CE, Firestone GL: Submitted.
42. John NJ, Bravo DA, Haffar OK, Firestone GL: Submitted.
43. Haffar OK, Aponte GW, John NJ, Bravo DA, Hess RT, Firestone GL: Submitted.