A Distinct Glucocorticoid Hormone Response Regulates Phosphoprotein Maturation in Rat Hepatoma Cells

KINLEY KARLSEN, ANNE K. VALLERGA, JENNIFER HONE, AND GARY L. FIRESTONE*

Department of Physiology-Anatomy, University of California, Berkeley, California 94720

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Glucocorticoid hormone-dependent maturation of the mouse mammary tumor virus (MMTV) phosphorylated polyprotein (Pr74) allows experimental access to certain posttranslational regulatory circuits under steroid control in M1.54 cells, an MMTV-infected rat hepatoma cell line. Pulse-chase experiments revealed that [³⁵S]methionine-labeled Pr74 synthesized in uninduced cells could be converted posttranslationally into p24, a stable phosphorylated maturation product, only after 4 h of exposure to 1 μ M dexamethasone, a synthetic glucocorticoid. This regulated processing could be prevented by prior exposure, during the chase period, to inhibitors of RNA (actinomycin D) or protein (cycloheximide or puromycin) synthesis. Moreover, half-maximal production of p24 occurred at 10 nM dexamethasone, a concentration that approximated half-maximal receptor binding and stimulation of MMTV transcript synthesis. Kinetic, hormonal, and genetic evidence suggest that p24 expression did not require or result from the overall glucocorticoid-dependent increase in polyprotein concentration. First, 20 h after dexamethasone withdrawal, Pr74 maturation was completely deinduced, whereas the absolute level of this MMTV precursor remained 10-fold over its basal level. Second, progesterone, which competes with dexamethasone for receptor binding, facilitated the regulated production of p24 but prevented the steroid-mediated accumulation of functional MMTV mRNA. Lastly, certain glucocorticoid-responsive variants, derived from M1.54 cells by resistance to complement cytolysis, expressed p24 in the presence or absence of glucocorticoid-induced levels of Pr74. Taken together, our results suggest that the glucocorticoid-regulated maturation of MMTV phosphopolyproteins resulted from an independent hormone response that required normal receptor function and de novo RNA and protein synthesis.

Glucocorticoid hormones elicit many types of responses in a variety of target tissues and in vitro-cultured cell lines (1, 4, 24; K. R. Yamamoto, *Annu. Rev. Genet.*, in press). These hormonal effects appear to be mediated by specific intracellular receptor proteins; the steroid-receptor interaction potentiates a functional change in the receptor that results in its increased DNA-binding activity and selective recognition of enhancer-like sites at or around genes under glucocorticoid control (1, 7, 10, 18, 26, 31-37, 44, 52; Yamamoto, in press). As a result of this apparent sequence specificity for glucocorticoid-receptor complexes, the rate of transcription of glucocorticoid-regulated genes is selectively modulated and accounts for gene expression changes of certain steroid responses (1; Yamamoto, in press). However, the mechanisms of most glucocorticoid responses are unknown, and many responses may result from secondary or regulatory steroid effects in which functional gene products under direct transcriptional control affect the expression of other biologically active genes at posttranscriptional or posttranslational levels. For example, the accumulation of α_1 -acid glycoprotein mRNA appears to result from selective message stabilization after glucocorticoid treatment (50), while steroid-mediated changes in the levels or activities of specific processing enzymes have also been reported (12, 27, 45); mechanisms of these regulated processes are largely unknown. We have been using the expression and subsequent maturation of mouse mammary tumor virus (MMTV) precursor polyproteins in virally infected cell lines as versatile and sensitive probes to explore posttranslational regulatory circuits under steroid control (16, 17).

The expression of MMTV gene products can be regulated by glucocorticoids at both transcriptional and posttransla-

tional levels. Hormonal stimulation of MMTV RNA synthesis in many virus-expressing cell lines is well documented (5, 38, 39, 48). For example, in M1.54 cells, a clonal MMTV-infected rat hepatoma tissue culture (HTC) cell line containing 10 integrated proviruses (17, 40), the basal rate of MMTV transcription is stimulated approximately 30-fold within 15 min of exposure to dexamethasone, a synthetic glucocorticoid (17, 47, 48). Hormone treatment does not functionally (16) or structurally (17, 48) alter the MMTV transcripts. In the presence or absence of glucocorticoids, M1.54 cells produce two distinct MMTV precursor proteins, a phosphorylated polyprotein encoded by the *gag* gene and an *env*-encoded glycosylated polyprotein, which are translated from the viral 7.8- and 3.8-kilobase RNA species, respectively. In addition to an increase in the absolute level of each precursor polyprotein, dexamethasone is required for their full posttranslational processing and compartmentalization (16). Several new MMTV polypeptides are expressed in glucocorticoid-induced M1.54 cells; two viral phosphoproteins (p35 and p24) are proteolytic cleavage products from a common phosphorylated precursor, while three cell surface glycoproteins (gp78, gp70, gp32) and a secreted species (gp70) are derived from their glycosylated precursor. The phosphorylated precursor remains essentially unprocessed in the absence of hormone; the major constitutive maturation product is a 51,000-dalton secreted glycoprotein (16). Moreover, selection and characterization of M1.54 variants that fail to produce cell surface MMTV glycoproteins have demonstrated that the glucocorticoid regulation of MMTV RNA synthesis and phosphoprotein maturation can be genetically uncoupled from the inducible glycoprotein maturation events (17).

To begin to assess the molecular details and cellular requirements of the steroid-regulated posttranslational path-

* Corresponding author.

ways in M1.54 cells, we have focused on the expression and processing of the phosphorylated precursor (Pr74), which is encoded by the MMTV *gag* gene. The viral core polypeptides are produced from this precursor by a sequential series of proteolytic reactions (5); in the heterologously infected rat hepatoma line M1.54, these processing events require glucocorticoid hormones. Of particular importance is whether the regulated maturation of Pr74 into p24 (a stable, phosphorylated proteolytic fragment) results from the glucocorticoid-induced de novo synthesis of another gene product. Moreover, is this hormone-regulated process independent of or dependent upon the stimulation of MMTV transcription? To test these notions, we have used an intact cell assay for analyzing regulated posttranslational changes affecting MMTV polyprotein maturation and have exploited the relative stability of Pr74 in the absence of hormone and its efficient processing of p24 in the presence of hormone. In this study, we report that the glucocorticoid-dependent maturation of MMTV phosphopolyproteins is a distinct, receptor-dependent process requiring de novo RNA and protein synthesis that can proceed independently of hormone-induced MMTV RNA synthesis.

MATERIALS AND METHODS

Materials. Dulbecco modified Eagle medium and horse serum were supplied by the Cell Culture Facility, University of California, San Francisco. L-[³⁵S]methionine (1,000 Ci/mmol), [³²P]phosphate (131 Ci/mg), and [³H]dexamethasone (85 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill; dexamethasone, progesterone, actinomycin D, puromycin, and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo.; Pansorbin was obtained from Calbiochem-Behring, La Jolla, Calif.; rabbit complement was obtained from GIBCO Laboratories, Grand Island, N.Y.; En³Hance was obtained from New England Nuclear Corp., Boston, Mass.; and Kodak X-ray film was purchased from Merry X-Ray Chemical Corp., Burlingame, Calif. The antisera (6) used in this study were a generous gift from L. J. T. Young and R. D. Cardiff (Department of Pathology, University of California at Davis); the anti-MMTV antisera used for the immunoprecipitations and immunoselections were directed against total disrupted virions and recognized both the phosphorylated and glycosylated viral gene products. All other reagents were of the highest available purity.

Cells and methods of culture. M1.54 is a cloned line of HTC cells and contains 10 stably integrated MMTV proviruses (17, 40). Cultures were propagated as monolayers in Dulbecco modified Eagle medium supplemented with 10% horse serum at 37°C in a humid atmosphere of air-carbon dioxide (95:5). Unless otherwise indicated, dexamethasone was added to the culture medium to a final concentration of 1 μ M. Cell cultures were radiolabeled with [³⁵S]methionine in methionine-deficient medium supplemented with 0.5% dialyzed fetal calf serum or with [³²P]phosphate in phosphate-deficient medium supplemented with 0.5% dialyzed fetal calf serum.

Radiolabeling at steady state. Cells exposed to the indicated concentrations of steroids were radiolabeled with either 100 to 150 μ Ci of [³⁵S]methionine or 800 μ Ci of [³²P]phosphate for 16 h; in appropriate incubations, metabolic inhibitors were added 5 min before the addition of dexamethasone. Cellular fractions were harvested, washed three times in phosphate-buffered saline, and detergent solubilized as described below.

Pulse-chase posttranslational maturation assay. Monolayers of uninduced cells were preincubated with methionine-free medium for 30 min and radiolabeled with 50 μ Ci of [³⁵S]methionine for 2 h. After the cultures were rinsed several times in phosphate-buffered saline, the radiolabel was chased by incubating the cells in medium supplemented with 1% normal horse serum and unlabeled methionine (1 mg/ml). In certain experiments, dexamethasone was added 60 min after the start of the chase period (final concentration, 1 μ M). Actinomycin D (final concentration, 5 μ g/ml), cycloheximide (final concentration, 15 μ g/ml), and puromycin (final concentration, 300 μ g/ml) were added during the chase 15 min before hormone exposure (or 45 min after initiating the uninduced chase incubation). At the indicated times, monolayers were washed in phosphate-buffered saline, gently dislodged from the culture plates in phosphate-buffered saline containing 10 mM Tris–1 mM EDTA (pH 7.5), and centrifuged at 600 $\times$ g for 3 min, and cell pellets were stored at –20°C until they were detergent solubilized as described below.

Immunoabsorption of detergent-solubilized cells. MMTV proteins were solubilized from radiolabeled cells by homogenizing cell pellets (10⁷ cells) in 1 ml of 1% Triton X-100–0.5% deoxycholate–5 mM EDTA–250 mM NaCl–25 mM Tris hydrochloride (pH 7.5); cell homogenates were centrifuged at 20,000 $\times$ g for 10 min, and supernatant fractions were used as sources of solubilized polypeptides. The solubilized fractions were analyzed for total radiolabeled proteins by precipitating a small sample with 10% trichloroacetic acid.

Pansorbin (formaldehyde-fixed *Staphylococcus aureus* Cowen I containing cell surface-associated protein A [Staph A]), was used as an absorbant for antibodies complexed with radiolabeled antigens. Solubilized cell extracts (300 μ l) were first preadsorbed without added antibodies with 10 μ l of 10% (wt/vol) solution of fixed Staph A in solubilization buffer; simultaneously, the Staph A used for the immunoabsorption was preadsorbed with detergent-solubilized cell extracts that had not been radiolabeled. After each preabsorption, incubation was allowed to proceed for 15 min at room temperature; the preadsorbed radiolabeled extracts and the preadsorbed Staph A were each harvested by centrifugation for 3 min in an Eppendorf centrifuge. Subsequently, the radiolabeled extracts were added to 100 μ l of solubilization buffer containing 50 mg of bovine serum albumin per ml and 10 μ l of a 1:10 dilution (in phosphate-buffered saline) of either anti-MMTV or preimmune serum. After incubation for 10 min at room temperature, 10 μ l of 10% Staph A (preincubated with unlabeled cell extracts in solubilization buffer) was added, and the mixture was incubated for an additional 5 min. The entire reaction mixture was layered over 600 μ l of 1 M sucrose in solubilizing buffer and centrifuged for 2 min in an Eppendorf centrifuge, and the nonadsorbed material was removed by aspirating down to the sucrose interface. The tubes were subsequently washed by overlaying the sucrose cushion with 2 M urea in solubilization buffer containing 500 mM NaCl and aspirating down to the Staph A pellets. The pellets were washed first with solubilization buffer containing 250 mM NaCl and then with 5 mM EDTA–10 mM Tris hydrochloride (pH 7.5). The washed Staph A pellets were either stored at –20°C or immediately reimmunoprecipitated as described below.

To further reduce nonspecific adsorption of radiolabeled proteins, the antigen-antibody complexes were released from the Staph A in 30 μ l of 1% sodium dodecyl sulfate (SDS) by a 20-min incubation at 37°C and a 2-min Eppendorf

centrifugation; the supernatant fractions were added to 500 μ l of solubilization buffer containing 10 mg of bovine serum albumin per ml and 0.8 μ l of the appropriate immune or preimmune serum. After 15 min at room temperature, 10 μ l of 10% preadsorbed pansorbin was added, and the reaction was allowed to proceed for 5 min at room temperature. Staph A immunocomplexes were collected by centrifugation in an Eppendorf centrifuge and washed in solubilization buffer and then in 10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA. Final Staph A pellets were either stored at -20°C or prepared immediately for SDS-polyacrylamide gel electrophoresis. With the second immunoadsorption, backgrounds were reduced from 10^3 ppm to approximately 1 ppm, while lowering the levels of specifically bound material by only 30%; the pattern of immunoadsorbed MMTV proteins was not qualitatively altered.

SDS electrophoresis and autoradiography. Staph A pellets were solubilized in 25 μ l of SDS-gel sample buffer (28) and 5 μ l of 0.5 mM dithiothreitol at 100°C for 2 min; after a 2 min Eppendorf centrifugation, supernatant fractions were electrophoresed in SDS-polyacrylamide gels containing 9.5% acrylamide. Proteins were subsequently fixed and stained by incubation overnight with 0.4% Coomassie brilliant blue in 10% acetic acid–25% isopropyl alcohol. Gels were then destained in 10% acetic acid for 4 h, impregnated with En^3Hance (New England Nuclear Corp.) for 1 h, incubated in water for 1 h, dried at 60°C , and analyzed by fluorography on Kodak RP-Royal X-Omat film at -70°C .

Glucocorticoid receptor assays. Glucocorticoid-binding activity was monitored either by a whole-cell assay (54) or by a glass fiber filter method for cell extracts (9, 20). For the whole-cell assay, 2×10^6 cells were incubated for 40 min at 37°C in serum-free media containing various concentrations of [^3H]dexamethasone. Nonspecific binding of radiolabeled steroid was determined by incubating parallel cultures with a 100-fold excess of unlabeled dexamethasone in the presence of each concentration of radiolabeled hormone. Cells were then pelleted in an Eppendorf centrifuge for 15 s and washed four times in phosphate-buffered saline at room temperature, and cell-associated radioactivity was determined by liquid scintillation.

For the glass fiber filter assay, washed cell pellets (5×10^7 cells) were homogenized with a Teflon pestle in 2 ml of TEGBNO40 buffer (10 mM Tris hydrochloride [pH 8.1], 1 mM disodium EDTA, 10% [vol/vol] glycerol, 1 mM 2-mercaptoethanol, 100 μ g of crystalline bovine serum albumin per ml, 200 μ M phenylmethylsulfonyl fluoride, 40 mM NaCl) and centrifuged at $20,000 \times g$ for 15 min at 4°C . Aliquots (100 μ l) of the supernatant fractions were made 40 nM [^3H]dexamethasone in the presence of the indicated concentrations of either unlabeled dexamethasone or unlabeled progesterone. Binding reactions were continued for 30 min at 4°C , and the entire reaction mixture was spotted onto dry 2.1-cm GF/A glass microfiber filters (Whatman, Inc., Clifton, N.J.). After 5 min at room temperature, filters were washed three times (10 min per wash) in 20 ml of NET buffer (10 mM Tris hydrochloride [pH 8.1], 1 mM disodium EDTA, 40 mM NaCl) per filter at 4°C with continuous shaking. Radioactivity in the dried filters was monitored by liquid scintillation in toluene-based scintillation fluid. This filter assay takes advantage of the strong affinity of the glucocorticoid receptors to the glass filters. The background of free [^3H]dexamethasone binding to the filters was less than 0.02% of the added radioactivity.

Isolation of complement-resistant variants. Populations of M1.54 (5×10^6 cells) were mutagenized with UV radiation

FIG. 1. Dexamethasone-regulated expression of MMTV phosphoproteins. Monolayer cultures of M1.54 cells were incubated with the indicated combinations of 1 μ M dexamethasone and 5 μ g of actinomycin D per ml and simultaneously radiolabeled with either 100 μ Ci of [^{35}S]methionine (lanes A through E) or 500 μ Ci of [^{32}P]phosphate (lanes F through J) for 16 h. Cells were harvested by centrifugation at $600 \times g$ for 3 min, cell pellets were detergent solubilized, and solubilized extracts were immunoprecipitated with either anti-MMTV antibodies (lanes A through I) or preimmune serum (lanes E and J). Immunoprecipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. The molecular weight markers are β -galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (68,000), ovalbumin (43,000), and glyceraldehyde phosphate dehydrogenase (36,000).

by placing monolayer cultures 15 cm away from a tissue culture hood UV light source (Contamination Control, Inc.) for 60 s. This dose was lethal to approximately 50% of the cells, and the surviving cells proliferated at a normal rate. Cells were then allowed to recover for five generations, and dexamethasone-treated cultures were subjected to anti-MMTV antibody-mediated complement cytolysis as described previously (17). In the presence of anti-MMTV antibodies and rabbit complement, hormone-induced cells expressing cell surface viral antigens were lysed, while variants unable to express their surface MMTV glycoproteins survived this selection procedure. After four cycles of complement selections, 12 individual subclones were recovered and designated CRV1 through CRV12; the expression of [^{35}S]methionine-labeled MMTV proteins was analyzed electrophoretically in dexamethasone-induced CRV1, CRV2, CRV3, and CRV4 as described above.

RNA isolation and dot blot hybridization analysis. Total cellular RNA was isolated from 10^7 M1.54 cells exposed to 1 μ M dexamethasone for the indicated times by combining the guanidinium thiocyanate lysis technique of Chirgwin et al. (8) and the CsCl centrifugation procedure of Glisin et al. (19) as described previously (15). Small portions (3 μ l) of RNA (1

FIG. 2. Pulse-chase assay to detect glucocorticoid-regulated posttranslational maturation of MMTV polyproteins. Uninduced M1.54 cells were pulse-labeled with 150 μ Ci of [35 S]methionine for 2 h, followed by further incubation with an excess of unlabeled methionine for 60 min. The chase was then continued in either the absence (lanes A through D) or presence (lanes E through J) of 1 μ M dexamethasone, and at the indicated chase times (0, 4, 6, and 8 h), the cells were harvested. Detergent-solubilized extracts were then immunoprecipitated with either anti-MMTV (lanes A through H) or preimmune sera (lanes I and J) and fractionated by SDS-polyacrylamide gel electrophoresis, and radiolabeled proteins were analyzed by fluorography. The molecular weight standards are described in the legend to Fig. 1.

μ g/ml in water) from each sample were spotted onto nitrocellulose filters that had been preincubated with $20\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the filters were baked in vacuo, preincubated with hybridization buffer, and hybridized with 32 P-labeled cloned MMTV DNA as described previously (17). The relative level of MMTV RNA expressed in each sample was quantitated by scanning the autoradiographs with a soft laser densitometer.

RESULTS

Dexamethasone alters the steady-state expression of MMTV phosphoproteins. To examine the full spectrum of MMTV phosphoproteins produced in dexamethasone-induced and uninduced M1.54 cells, [35 S]methionine- or [32 P]phosphate-labeled cells were detergent solubilized and immunoprecipitated with anti-MMTV antibodies, and radiolabeled polypeptides were electrophoretically analyzed on SDS-polyacrylamide gels. Hormone exposure resulted in the striking expression of several new viral proteins (Fig. 1, cf. lanes A and B); of particular interest is the proteolytic fragment designated p24 (M_r , 24,000). We have previously shown that p24 is produced by the proteolytic cleavage of a larger precursor polypeptide (16). Immunoprecipitation of [32 P]phosphate-labeled extracts revealed that this maturation

product was phosphorylated (Fig. 1, cf. lanes B and G), as was the precursor polypeptide designated Pr74 (M_r , 74,000), from which it was derived. Phosphorylated Pr74 appeared relatively stable and unprocessed in the absence of hormone (Fig. 1, lanes A and F) while p24 was one of the two predominantly phosphorylated maturation products detected after 24 h of exposure to dexamethasone (lane G); the other stable MMTV phosphoprotein that was derived from Pr74 only in the presence of dexamethasone was p35. The polyclonal antisera used were directed against total disrupted virions (6) and specifically bound both MMTV phosphoproteins and MMTV glycoproteins (Fig. 1, lanes E and J). Both p24 and p35, as well as Pr74, were selectively recognized by anti-MMTV phosphoprotein (gag) antibodies (data not shown), while [35 S]methionine-labeled MMTV polypeptides which migrated at M_r s of approximately 50,000, 60,000, 70,000, and 78,000 and failed to be radiolabeled with [32 P]phosphate were glycosylated maturation products derived from the *env*-encoded precursor in a hormone-dependent manner (16). The apparent heterogeneity of [35 S]methionine-labeled Pr74 (Fig. 1, lane B) most likely represented multiple forms of closely migrating MMTV glycoproteins.

Effect of metabolic inhibitors on the regulated posttranslational expression of p24. Preincubation of cells with a con-

centration of actinomycin D which inhibits *de novo* RNA synthesis but not protein synthesis (see below) prevented the dexamethasone-mediated expression of p24 (Fig. 1, cf. lanes B and D and lanes G and I); basal synthesis of either [³²P]phosphate-labeled or [³⁵S]methionine-labeled Pr74 was not affected by this inhibitor (lanes C and H). This result suggests that the maturation of Pr74 to p24 requires the product of a newly synthesized mRNA; however, by this steady-state protocol, posttranslational events *per se* were not delineated. For example, can the Pr74 produced in the absence of hormone undergo regulated posttranslational maturation in the presence of hormone, or is a precursor unique to hormone-treated cells actually the precursor to p24? To test these possibilities, a whole-cell posttranslational assay was developed which exploited the stability of Pr74 in the absence of dexamethasone and its efficient processing to p24 in the presence of hormone. This assay was a modified pulse-chase protocol in which uninduced M1.54 was pulsed with [³⁵S]methionine for 2 h and the cells were subsequently incubated with an excess of unlabeled methionine in either the presence or absence of 1 μ M dexamethasone. Any chase-specific differences detected in MMTV protein expression must result from hormone-regulated posttranslational reactions. Pr74 remained unprocessed throughout an 8-h chase period in uninduced cells (Fig. 2, lanes A through D), while exposure to 1 μ M dexamethasone in the chase period resulted in the processing of Pr74 into p24 after several hours (lanes E through H). These results demonstrate that the same phosphorylated polyprotein synthesized in the absence of hormone can be efficiently processed in the presence of dexamethasone. If a different precursor had been produced in hormone-treated cells, the production of radiolabeled p24 would not have been detected. Thus, Pr74 synthesized in the absence of hormone fails to be proteolytically matured unless appropriate glucocorticoid-regulated posttranslational events are functioning.

The above pulse-chase protocol represents an intact-cell assay to determine cellular requirements of hormone-regulated phosphoprotein maturation. As mentioned, if dexamethasone stimulates *de novo* synthesis of a cellular factor required for the maturation of Pr74, the posttranslational expression of p24 should be dependent upon *de novo* RNA and protein synthesis. Importantly, the posttranslational assay described above allowed the use of inhibitors of both RNA (actinomycin D) and protein (puromycin or cycloheximide) synthesis. Inhibitors were added during the chase period 15 min before the addition of dexamethasone; after 8 h of chase, the production of p24 was examined electrophoretically on SDS-polyacrylamide gels. Prior exposure to actinomycin D prevented the dexamethasone-dependent maturation of Pr74 to p24 (Fig. 3, cf. lanes B and D), suggesting the need for *de novo* RNA synthesis. Under these conditions, actinomycin D inhibited [³H]uridine incorporation into total RNA by greater than 98% with only a minor effect on total protein synthesis and did not affect the synthesis of Pr74 from basal levels of MMTV RNA (Fig. 3, cf. lanes A and C).

Exposure to cycloheximide or puromycin quantitatively inhibited the hormone-dependent processing of Pr74 (Fig. 3, cf. lanes B and F and lanes B and H), suggesting that *de novo* protein synthesis is also required for this regulated event. Interestingly, Pr74 produced in either hormone-treated or untreated cells (Fig. 3, lanes G and H) appeared to be stabilized in the presence of cycloheximide, a phenomenon that has been observed previously in rat hepatoma cells (13,

FIG. 3. Effect of RNA synthesis and protein synthesis inhibitors on dexamethasone-regulated phosphoprotein maturation. Uninduced M1.54 cells were radiolabeled with 150 μ Ci of [³⁵S]methionine for 2 h, followed by further incubation with an excess of unlabeled methionine; 45 min into the chase incubation, cells were exposed to 5 μ g of actinomycin D per ml (lanes C and D), 300 μ g of puromycin per ml (lanes E and F), or 15 μ g of cycloheximide per ml (lanes G and H) or were not treated with any metabolic inhibitors (lanes A, B, and I). After an additional 15 min, appropriate cultures were treated with 1 μ M dexamethasone (lanes B, D, F, H, and I), and the chase incubation was allowed to continue for a total of 8 h. Cell cultures were then harvested, detergent solubilized, and immunoprecipitated with either anti-MMTV (lanes A through H) or preimmune serum (lane I), and immunoprecipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by fluorography. The molecular weight markers are described in the legend to Fig. 1.

22, 23). The concentrations of protein synthesis inhibitors used prevented the incorporation of [³H]leucine into macromolecular material (trichloroacetic acid-precipitable radioactivity) by greater than 95% without significantly affecting either [³H]uridine incorporation into RNA or the dexamethasone induction of MMTV transcripts as examined by dot blot analysis (data not shown). Taken together, these results are consistent with the notion that dexamethasone induces the synthesis of a cellular component which functions to alter gene expression at a posttranslational level.

Receptor dependency of Pr74 maturation. To assess the role of glucocorticoid receptors in induction of Pr74 maturation, p24 production and hormone binding were monitored over a range of dexamethasone concentrations. The saturation of glucocorticoid receptors with [³H]dexamethasone was determined by a whole-cell binding assay, and, consis-

FIG. 4. Glucocorticoid receptor-dependent phosphoprotein maturation. To assess glucocorticoid receptor-binding activity, 2×10^6 cells were incubated with the indicated concentrations of [3 H]dexamethasone for 30 min at 37°C in the absence or presence of a 100-fold excess of unlabeled hormone and specific cell-associated radioactivity, determined as described in the text. To monitor the overall level and maturation state of mouse mammary tumor virus (MTV, MMTV) proteins, M1.54 cells were treated with the indicated concentrations of dexamethasone in the presence of $100 \mu\text{Ci}$ of [^{35}S]methionine for 16 h, detergent solubilized, and immunoprecipitated with either anti-MMTV or preimmune serum. The specific immunoprecipitated radioactivity in each sample was quantitated as described in the text. Equivalent immunoprecipitations with anti-MMTV (gel insert, lanes A through G) or preimmune (gel insert, lanes H and I) serum were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. The molecular weight markers are described in the legend to Fig. 1. The p24/Pr74 ratio was determined by scanning the fluorographs with a soft laser densitometer, and the percent induction of maturation was plotted versus the concentration of dexamethasone.

tent with previous published work (3), half-maximal binding occurred at approximately 10 nM dexamethasone (Fig. 4). At the indicated steroid concentrations, the production of p24 was analyzed by immunoprecipitation of [^{35}S]methionine-labeled cells and subsequent electrophoresis in SDS-polyacrylamide gels. The gel insert to Fig. 4 shows the expressed MMTV polypeptides normalized to the absolute level of MMTV proteins; the ratio of p24 to Pr74 was determined by densitometry to assess the efficiency of polyprotein processing. The induction of phosphoprotein maturation closely correlated with receptor occupancy; half-maximal maturation occurred at approximately 10 nM dexamethasone (Fig. 4). This concentration of hormone is necessary for half-maximal induction of many other HTC cell glucocorticoid-responsive gene products (42, 44, 46, 49), including the accumulation of MMTV RNA (3, 41) and total MMTV protein levels (also shown in Fig. 4). In support of a glucocorticoid receptor-dependent process, we have recently shown that the synthetic glucocorticoid antagonist, RU 38486, which fully antagonizes both *in vitro* and *in vivo* effects of dexamethasone (30), prevents the dexamethasone-

mediated Pr74 maturation (S. Winguth and G. L. Firestone, manuscript in preparation).

Induction and deinduction kinetics of phosphoprotein maturation. Several lines of evidence suggest that the glucocorticoid regulation of MMTV transcription and posttranslational maturation of Pr74 are independent responses. To examine the time course of induction of Pr74 maturation, p24 was monitored at various 2-h time windows after exposure to dexamethasone by radiolabeling with [^{35}S]methionine. The level of MMTV RNA throughout the time course was measured either by dot blot hybridization of isolated RNA with ^{32}P -labeled cloned MMTV RNA or by determining the absolute levels of synthesized MMTV proteins. A four hour time lag was needed before the onset of polyprotein processing (Fig. 5, bottom), while the induction of synthesis of total MMTV RNA and proteins occurred with no detectable time lag (top panel). In fact, the maximal rate of MMTV transcription can be obtained within 15 min of hormone exposure (40, 47, 48, 53). The time lag before observable phosphoprotein maturation is intriguing and may reflect the time required for a putative dexamethasone-regulated matu-

ration factor to be synthesized, to be properly compartmentalized, to recognize Pr74 substrate, and, finally, to produce p24.

Glucocorticoid-regulated Pr74 processing and accumulation of MMTV proteins were also differentially deinduced after withdrawal of dexamethasone. The gel insert to Fig. 6 displays the viral polypeptides produced at various 4-h time windows during hormonal withdrawal; the loss in p24 production indicates that Pr74 maturation returned to its basal

FIG. 5. Time course of induction of phosphoprotein maturation and MMTV transcript levels. M1.54 cells were incubated with 1 μ M dexamethasone, and at the indicated times, cells were harvested, and total RNA was isolated and spotted onto nitrocellulose filters, baked in vacuo, and then hybridized with 32 P-labeled cloned MMTV DNA as described in the text. The level of MMTV transcripts was quantitated by densitometry of the autoradiographs and then the percent maximal induction was calculated at each time. Parallel cultures were exposed to 1 μ M dexamethasone, and 2 h before harvesting, the cells were incubated with 150 μ Ci of [35 S]methionine. At the indicated times, including the 2-h radiolabeling period, the cells were harvested, detergent solubilized, and immunoprecipitated with either anti-MMTV or preimmune serum, and either the radioactivity in the final Staph A pellets was determined directly by liquid scintillation or the immunoprecipitated material was fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by fluorography. The p24/Pr74 ratio was determined by densitometry. Symbols: Δ , MMTV RNA induction; $\circ$, MMTV protein induction; $\bullet$, p24/Pr74 ratio.

state within 20 h. In contrast, the absolute amount of MMTV protein returned to its basal level at a significantly slower rate (Fig. 6, graph) and, in fact, was still 10-fold above uninduced concentrations when Pr74 maturation was completely shut down. Taken together, our kinetic information suggests that Pr74 maturation is not a simple consequence of its increased accumulation due to the hormone-dependent increase in MMTV RNA but is, instead, a distinct hormone response.

Effect of progesterone on glucocorticoid-regulated phosphoprotein maturation. We have been able to uncouple dexamethasone-regulated Pr74 processing and MMTV RNA synthesis in the presence of progesterone, which is a known antagonist of certain glucocorticoid responses (41, 42, 49). An excess of progesterone effectively competed with 40 nM [3 H]dexamethasone for binding to glucocorticoid receptors (Fig. 7, left panel). The competition curve for unlabeled progesterone was shifted to higher concentrations than those for unlabeled dexamethasone, indicating that progesterone binds to glucocorticoid receptors with an affinity approximately 10-fold lower than that of dexamethasone.

In parallel, intact cells were treated with 40 nM dexamethasone in the presence of various progesterone concentrations, incubated with [35 S]methionine, and the induction of both Pr74 maturation and MMTV protein synthesis monitored electrophoretically. The dexamethasone induction of functional MMTV transcripts (as monitored by total MMTV protein level) was inhibited at concentrations of progesterone that competed with [3 H]dexamethasone binding to its receptors (Fig. 7, middle panel). In striking contrast, progesterone did not significantly affect the glucocorticoid-regulated maturation of Pr74 to p24 since at the higher progesterone levels, basal Pr74 was clearly processed into p24 (Fig. 7, middle panel, lanes G and H). Under these radiolabeling conditions, no maturation was detected in the absence of steroids (lane A). The ratio of p24 to Pr74 at each concentration of progesterone was quantitated by densitometry and compared with the total synthesis of MMTV polypeptides (Fig. 7, right panel). As indicated, the maturation of Pr74 proceeded with 60% efficiency in the absence of glucocorticoid-induced levels of precursor, suggesting that progesterone might be acting selectively as a glucocorticoid agonist. Consistent with this notion, we have recently demonstrated that exposure to progesterone alone induces phosphoprotein maturation in manner similar to dexamethasone (S. Winguth and G. L. Firestone, unpublished data).

It is important to point out that one mechanism consistent with the observed glucocorticoid-dependent production of p24 is that the hormone-mediated increase in the absolute level of precursor polypeptide may bring the concentration of Pr74 above a critical K_m range of preexisting maturation factor. However, at the highest concentrations of progesterone, a basal level of Pr74 was clearly matured into p24. Thus, the simple increase in Pr74 accumulation cannot account for its hormone-dependent maturation.

Regulated polyprotein maturation in immunoselected M1.54 variants. To test genetically whether glucocorticoid-regulated phosphoprotein maturation and MMTV RNA synthesis are independent responses, UV-mutagenized populations of M1.54 (MV cells), were subjected to anti-MTV antibody mediated complement cytolysis. We have demonstrated previously that this immunoselection procedure can be used to recover cells that fail to produce cell surface MMTV glycoproteins in a glucocorticoid-dependent manner (17). This selection procedure is targeted at one of the last stages of gene expression, that is, the incorporation of

FIG. 6. Deinduction of dexamethasone-regulated phosphoprotein maturation and mouse mammary tumor virus (MTV, MMTV) total protein levels. M1.54 cells were exposed to $1 \mu\text{M}$ dexamethasone for 12 h, and then hormone-containing medium was withdrawn and replaced with steroid-free medium. During 4-h time windows, just before and throughout the deinduction, cells were radiolabeled with $150 \mu\text{Ci}$ of [^{35}S]methionine; uninduced cultures were also radiolabeled (gel insert, lane A). Cells were harvested at the indicated times, detergent solubilized, and immunoprecipitated with either anti-MMTV (lanes A through I) or preimmune (lanes J and K) serum, and the immunoprecipitated material was fractionated by SDS-polyacrylamide gel electrophoresis and finally analyzed by fluorography. The molecular weight markers are described in the legend to Fig. 1. The fluorogram was scanned by a soft laser densitometer, and the percent induction of total MMTV polypeptides and Pr74 maturation (p24/Pr74 ratio) was calculated at each time of dexamethasone withdrawal.

accessible cell surface-associated glycoproteins, and, therefore, recovered variants may be defective at any one of many prior reactions. Importantly, variants defective in glucocorticoid-regulated MMTV transcription have been isolated previously (17), although glucocorticoid-regulated phosphoprotein maturation should proceed normally since the viral phosphoproteins remain intracellular and are not a direct target of the selection.

Dexamethasone-treated MV cells were exposed to anti-MMTV antibodies in the presence of rabbit complement, and surviving cells were subjected to four cycles of immunoselection. Four individual subclones of recovered variants (CRV1 through CRV4), as well as starting MV populations, were radiolabeled with [^{35}S]methionine in the presence or absence of $1 \mu\text{M}$ dexamethasone, the absolute levels of MMTV protein were quantitated by determining the radioactivity in the final immunopellets, and the induction of MMTV polypeptides was indicated in Table 1. Parental MV populations displayed a 9.8-fold induction of total MMTV proteins, whereas two of the immunoselected variants (CRV1 and CRV3) expressed basal levels of MMTV protein in the presence of dexamethasone (0.9- and 1.4-fold induction, respectively) which is presumably due to an inability to increase the rate of MMTV transcription after hormonal exposure (17). In contrast, another of the variants (CRV2)

showed nearly normal levels of hormone-inducible MMTV gene products, while CRV4 cells were partially inducible; most likely CRV2 and CRV4 cells survived the complement-mediated selection procedure as a result of their failure to produce accessible cell surface-associated MMTV glycoproteins. We have characterized other complement-selected variants with similar phenotypes (16, 17).

In an intact-cell assay, the level of [^3H]dexamethasone binding activity per cell was monitored by determining the amount of cell-associated radioactivity. Each of the recovered variants displayed normal levels of hormone-binding activity (Table 1), suggesting that the concentration of glucocorticoid receptors in the variants is not significantly altered and that the regulated phosphoprotein maturation reactions might be functional. To examine MMTV phosphoprotein maturation (production of p24) in starting MV populations and recovered variants, cells were incubated with [^{35}S]methionine in the presence of dexamethasone, and immunoprecipitated MMTV proteins were analyzed electrophoretically. As expected, dexamethasone regulated phosphoprotein maturation in parental MV populations since p24 was only produced in hormone-treated cells (Fig. 8, cf. lanes H and F; Table 1); all detected polypeptide species were encoded by MMTV since they were not recognized by preimmune serum (lanes E and G). Densitometric analysis of

FIG. 7. Effect of progesterone on dexamethasone-regulated phosphoprotein maturation, induction of mouse mammary tumor virus (MTV, MMTV) total proteins, and binding to glucocorticoid receptors. (Left panel) M1.54 cell extracts were incubated with 40 nM [^3H]dexamethasone in the presence of the indicated concentrations of either unlabeled dexamethasone or unlabeled progesterone mixtures and spotted onto glass fiber filters, and the filter-bound radioactivity was determined by liquid scintillation. (Middle panel) M1.54 cells were exposed to 40 nM dexamethasone in the presence of the indicated concentrations of progesterone and 100 μCi of [^{35}S]methionine for 16 h. Uninduced cells were also radiolabeled in a similar manner (lane A). After harvesting, detergent-solubilized cell extracts were immunoprecipitated with either anti-MMTV (lanes A through H) or preimmune (lanes I and J) serum, and the immunoprecipitated material was fractionated by SDS-polyacrylamide gel electrophoresis and then analyzed by fluorography. (Right panel) The fluorogram displayed in the middle panel was scanned by a soft laser densitometer, and the relative total level of immunoprecipitated MMTV polypeptides and the efficiency of phosphoprotein maturation (p24/Pr74 ratio) were determined at each concentration of progesterone. The relative level of MMTV proteins was also determined in uninduced cells (*).

the fluorograph displayed in Fig. 8 revealed that in uninduced MV cells (lane H) the p24/Pr74 ratio was 0.00 since no p24 was detected; whereas, in dexamethasone-treated MV cells (lane F), this same ratio increased to 0.53 under the radiolabeling conditions (Table 1).

The two complement-selected variants (CRV1 and CRV3) that failed to induce total MMTV protein levels in the presence of dexamethasone expressed p24 in the presence of hormone, although at somewhat different efficiencies (Fig. 8, lanes D and B; Table 1 shows p24/Pr74 ratios), demonstrating that regulated phosphoprotein maturation can competently proceed from near basal levels of Pr74 substrate. In fact, p24 was expressed in each of the variants regardless of the overall glucocorticoid-inducible level of MMTV polypeptides (Table 1). In the presence of 1 μM dexamethasone, the variants (in particular CRV1, CRV3, and CRV4) failed to express normal quantities of the enveloped-encoded MMTV glycoproteins (lack of radiolabeled MMTV polypeptide species at M_r s of 32,000, 50,000, 70,000, and 78,000), suggesting, along with other characterized variants (17), that the regulated phosphoprotein maturation events are not dependent upon the expression of viral glycoproteins (Fig. 8). Thus, these genetic data, in combination with our kinetic and hormonal evidence, demonstrate that dexamethasone-regulated phosphoprotein maturation in rat hepatoma cells is a distinct hormone response that can function independently of the glucocorticoid stimulation in MMTV transcription and other subsequent events affecting the expression of MMTV gene products.

DISCUSSION

The processing and compartmentalization of MMTV precursor polyproteins are dependent upon host cell compo-

nents and therefore provide sensitive and perhaps general probes to assess posttranslational mechanisms that regulate the expression of protein products within the cell. MMTV phosphoproteins are differentially matured in the presence or absence of glucocorticoid hormones (16), and their unambiguous detection in infected rat hepatoma cells has allowed experimental access to a novel steroid-dependent posttranslational regulatory circuit. Given current concepts on glucocorticoid hormone action, which suggest that binding of steroid-receptor complexes to high-affinity enhancer-like sites at and around a regulated gene results in transcriptional changes of that gene (1; Yamamoto, in press), a likely mechanism by which dexamethasone can posttranslationally affect protein expression is the stimulation in de novo synthesis of a crucial cellular factor with a maturation function. Our results suggest that the viral phosphorylated precursor polyprotein is a substrate target for such a hormone-regulated processing activity.

In this study, we focused on the proteolytic maturation of the phosphorylated precursor (Pr74) since it remains virtually unprocessed in uninduced cells and is efficiently matured into p24 (and several other proteolytic fragments) only in the presence of dexamethasone. The absolute dependence upon hormone for maturation was exploited in a pulse-chase assay that monitored selectively hormone-regulated post-translational changes in Pr74 expression and allowed an evaluation of cellular requirements of these events. Consistent with the induced synthesis of a maturation factor, dexamethasone-regulated phosphoprotein processing required both de novo RNA and protein synthesis and was initiated approximately 4 h after hormone exposure. This lag may reflect the intrinsic time required for dexamethasone-receptor complexes to induce transcription, for the cell to

synthesize and compartmentalize the regulated maturation factor, and, finally, for the factor to recognize and mature Pr74. The pulse-chase assay also demonstrated that the same Pr74 molecules synthesized in the absence of hormone could be converted posttranslationally to p24 only in the presence of hormone. Thus, a distinct phosphoprotein precursor is not produced selectively after exposure to dexamethasone.

Glucocorticoid-receptor complexes act in *trans* to mediate selective alterations in gene expression; in total, the set of genes under hormone control can be considered the glucocorticoid-responsive domain of the cell (4, 17, 24; Yamamoto, in press). The crucial role of glucocorticoid receptors in regulating phosphoprotein maturation was inferred by the close dose-dependent correlation between receptor binding and dexamethasone induction of p24 production. We are attempting to confirm this notion genetically by selecting receptor-defective variants of M1.54 cells. Furthermore, several distinct classes of complement-resistant variants have been derived from M1.54 which display a partial pleiotropy with respect to their hormone responsive domain (17). For example, the phenotypes of CRV1 and CRV3 cells are generally similar to certain other complement-selected variants (17) since they process MMTV phosphoproteins in a hormone-regulated manner but do not increase the absolute level of MMTV proteins, most likely due to a failure to stimulate the synthesis of MMTV transcripts in the presence of dexamethasone. Furthermore, the variant phenotypes recovered from this study, as well as the results of other investigations (16, 17), suggest that the glucocorticoid-regulated phosphoprotein maturation events are independent of the cellular processes involved with the expression of the envelope-encoded viral glycoproteins.

Other recently recovered variants failed to express the hormone responsive maturation activity among several defective responses (such as tyrosine amino transferase) while remaining competent to express the other glucocorticoid inducible gene products (A. K. Vallerga, D. A. Bravo, and G. L. Firestone, unpublished data). Thus, selection against expression of hormone-inducible surface MMTV glycoproteins results in a coordinate loss of specific combinations of unselected hormone responses; including regulatable Pr74 processing. All of the complement-selected variants which

TABLE 1. Analysis of dexamethasone-binding activity, glucocorticoid induction of total MMTV proteins, and regulated phosphoprotein maturation

Cell line	Glucocorticoid-binding sites per cell ^a	Fold induction of MMTV proteins ^b	Phosphoprotein maturation in hormone-treated cells (p24/Pr74 ratio) ^c
MV population	85,400	9.8	0.53
CRV1	94,700	0.9	0.18
CRV2	77,100	7.1	0.40
CRV3	69,100	1.4	0.34
CRV4	95,300	2.7	0.56

^a Determined from [³H]dexamethasone-binding activity in an intact cell assay described in the text.

^b Calculated as the ratio of [³⁵S]methionine-labeled, immunoprecipitable MMTV proteins in hormone-treated cells to the amount detected in untreated cells. In uninduced cells, the basal level of immunoprecipitable MMTV proteins was approximately 150 cpm per 10⁶ trichloroacetic acid-precipitable cpm in each cell line.

^c Gel lanes in Fig. 8 were scanned with a soft laser densitometer, and phosphoprotein maturation was calculated as the ratio of p24 to Pr74. In uninduced cells, this ratio was 0.00 for each cell line since no p24 was produced.

FIG. 8. Analysis of hormone-regulated phosphoprotein maturation in complement-selected variants. Uninduced (lanes G and H) and dexamethasone-induced (lanes E and F) populations of MV cells as well as dexamethasone-treated CRV1 (lane D), CRV2 (lane C), CRV3 (lane B), and CRV4 (lane A) cells were incubated with 100 μ Ci of [³⁵S]methionine for 16 h. After harvesting, detergent-solubilized extracts were immunoprecipitated with either anti-MMTV (lanes A through D, F, and H) or preimmune (lanes E and G) serum, the immunoprecipitated material was fractionated by SDS-polyacrylamide gel electrophoresis, and the radioactive proteins were visualized by fluorography.

have been characterized contained normal levels of glucocorticoid receptors, suggesting that nonreceptor components may selectively influence the ability of a cell to express the processing function in a glucocorticoid-dependent manner in parallel with other hormone-responsive genes. Taken together, our results demonstrate that the regulatable phosphoprotein maturation is a normal part of the glucocorticoid-responsive domain of the hepatoma cell.

Depending on the variant phenotype, Pr74 processing could occur either in the presence or in the absence of glucocorticoid-induced levels of MMTV transcripts (Fig. 8), demonstrating that the maturation effect is not simply a consequence of increased levels of polyprotein substrate. Moreover, these two responses could also be kinetically uncoupled since phosphoprotein maturation was deinduced at a significantly faster rate than were total MMTV protein levels after withdrawal of dexamethasone. Other studies have shown that retroviruses can encode protease activities (55-57); however, our genetic evidence strongly suggests

that Pr74 maturation results from an independent response activated by glucocorticoid-regulated cellular components. Consistent with this notion, MMTV transcripts produced in hormone-induced and uninduced cells are structurally and functionally identical (16, 47), while the precursor polypeptides migrate indistinguishably in two-dimensional electrophoretic gels (data not shown).

Progesterone appears to act as a weak inducer or agonist of Pr74 maturation while simultaneously functioning as an antagonist of MMTV RNA synthesis. Assuming that there is one class of glucocorticoid receptors in HTC cells (29), it is interesting to speculate that unique chromosomal structural features at the putative maturation gene (14, 53) may allow transcriptional activation either by glucocorticoid receptors bound by progesterone or by progesterone-receptor complexes. In this regard, Von der Ahe et al. have reported that both progesterone receptors and glucocorticoid receptors can bind to the same DNA sites on two different regulated promoters (51). Whatever the mechanism, the detection of progesterone-regulated Pr74 processing demonstrates that nearly maximal phosphoprotein maturation can proceed with basal levels of precursor substrate, and the simplest explanation for our results is the existence of a distinct hormone-inducible processing gene.

Cellular targets and functional significance of the regulated phosphoprotein maturation are unknown although constitutive polypeptide maturation comparable to Pr74 processing is clearly an integral aspect of maintaining cell function and the activity of a wide variety of biologically important gene products. For example, certain hepatoma cell lines and liver cells proteolytically process γ -glutamyl transpeptidase during transport to the cell surface (2) and process haptoglobin during secretion (21). Moreover, specific mammalian protein hormones (11) and eucaryotic hormone-like molecules, such as yeast alpha factor (25), must be properly proteolytically matured to elicit their biological activities. Virtually nothing is known about the steroid-regulated gene products that function to selectively alter the posttranslational expression of cellular polypeptides. The detection and characterization of glucocorticoid-regulated MMTV polypeptide maturation is the first step in identifying the precise gene products and posttranslational pathways under hormonal regulation and may eventually help elucidate its normal function in the glucocorticoid domain of HTC cells.

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