Distinct Cytoplasmic Domains of the Growth Hormone Receptor Are Required for Glucocorticoid- and Phorbol Ester-induced Decreases in Growth Hormone (GH) Binding

THESE DOMAINS ARE DIFFERENT FROM THAT REPORTED FOR GH-INDUCED RECEPTOR INTERNALIZATION*

(Received for publication, December 29, 1995, and in revised form, April 16, 1996)

Anthony P. J. King†§, Min-J en Tseng†, Craig D. Logsdon†, Nils Billestrup§, and Christin Carter-Su§†

From the †Department of Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0622 and the §Hagedorn Research Laboratory, DK-2820 Gentofte, Denmark

Glucocorticoids inhibit growth in children and antagonize the growth-promoting action of GH in peripheral tissues. Recently, they have been shown to decrease GH binding. In this study we examine the molecular mechanisms by which the glucocorticoid dexamethasone (DEX) and the phorbol ester phorbol myristate acetate (PMA) decrease cellular GH binding. In 3T3-F442A fibroblasts, DEX and PMA decrease the number of GH receptors (GHRs) capable of binding GH by 50% (t1/2 = 6 h) and 70% (t1/2 = 15 min), respectively. Neither appear to decrease the total number of cellular GHR. Rather, they appear to redistribute GHRs away from the plasma membrane or inactivate GHRs on the membrane such that they cannot bind GH. DEX and PMA also decrease GH-induced tyrosyl phosphorylation of GHR and J AK2 with a magnitude and time course correlating with that of inhibition of GH binding. DEX- and PMA-induced reductions of GH binding are also observed in a Chinese hamster ovary (CHO) cell line stably transfected with a rat liver GHR cDNA, further arguing that DEX and PMA act post-translationally on GHR. Using mutant GHRs stably expressed in CHO cells, amino acids 455-506 and tyrosines 333 and/or 338 of GHR were shown to be required for maximal DEX-induced inhibition of GH binding. DEX decreased GH binding to a GHR mutant F346A, which is reported to be deficient in ligand-induced internalization, suggesting that DEX decreases GH binding by a mechanism distinct from that of ligand-induced GHR internalization. PMA reduced GH binding to CHO cells expressing all GHR mutants tested. However, deletion of the C-terminal 132 amino acids decreased this effect, suggesting that at least one component of PMA action on GHR requires amino acids 507-638. These data suggest that distinct pathways mediate the effects of GH, DEX, and PMA on GH number in the plasma membrane.

Inhibition of growth by glucocorticoids was suggested as early as 1944 to result from antagonism of GH1 action (1), and recently mechanisms for this antagonism have begun to be uncovered. There are multiple potential sites for glucocorticoid antagonism of GH action. Recent evidence indicates that glucocorticoids may antagonize GH action at several of these sites, consistent with their widespread and pleiotropic actions. Glucocorticoids decrease IGF-I expression in osteoblasts (2), apparently through decreasing the rate of transcription of the IGF-I gene (3, 4), and inhibit GH induction of IGF-I mRNA in hypophysectomized rats (5). Glucocorticoids alter the expression of IGF-binding proteins (6–8) and repress IGF-II receptor transcription in osteoblasts (9). They can also directly antagonize the cellular actions of GH. The synthetic glucocorticoid dexamethasone (DEX) recently has been shown in this laboratory to decrease in 3T3-F442A fibroblasts GH-stimulated tyrosyl phosphorylation of microtubule-associated protein kinases, signal transducers, and activators of transcription (Stats) 1 and 3, J AK2, and GH receptor (GHR) (10). This decrease in GH-stimulated tyrosyl phosphorylation closely correlates with a decrease in the number of GH binding sites in the plasma membrane, suggesting that glucocorticoids decrease tissue sensitivity to GH at least in part by decreasing the number of GHRs available to GH.

Phorbol esters which activate protein kinase C (PKC) have also been shown to decrease GH binding and GH-stimulated proliferation of IM-9 cells (11), although the effects of phorbol esters on GH binding are much more rapid than those of glucocorticoids. In the present study, we examine the respective mechanisms by which DEX and the phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA) decrease the number of GH binding sites on the plasma membrane. This study identifies distinct cytoplasmic domains of GHR reported to be required for DEX- and PMA-induced decreases in GH binding that are different from that required for ligand-induced internalization, suggesting that while GH, DEX, and PMA all appear to decrease GHR number in the plasma membrane by redistributing GHRs away from the plasma membrane, they may do so by different mechanisms.

EXPERIMENTAL PROCEDURES

Materials—Recombinant DNA-derived 22,000-dalton hGH was a gift of Eli Lilly Co. hGH was iodinated by the Reproductive Sciences Train-
Glucocorticoids and Phorbol Ester Decrease GH Binding

ing Grant Core Facility at the University of Michigan Medical School to a specific activity of ~2,000 ρCi/μmol. [125I-Tyr]-Bombesin (2,200 μCi/μmol) was obtained from DuPont NEN. Recombinant protein A-agarose was from Repligen, protein assay BCA was from Pierce Chemical Co., and Triton X-100, aprotinin, and leupeptin were purchased from Boehringer Mannheim. Ovalbumin, DEX, and PMA were purchased from Sigma. Pooled normal molecular weight standards were from Life Technologies, Inc., and nitrocellulose membranes were from Schleicher & Schuell. The enhanced chemiluminescence (ECL) detection system, anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase were from Amersham, and x-ray film was from DuPont.

Antisera—Antibody to GH (αGH) (NIDDK-anti-h-GH-IC3, lot C1208), which was from the National Institute of Diabetes and Digestive and Kidney Diseases/National Hormone and Pituitary Program. Anti-phosphotyrosine antibody (αPY) (4G10) was purchased from Upstate Biotechnology Inc. Antibody to JAK2 (αJAK2) was prepared in rabbits against a synthetic peptide corresponding to amino acids 758–776 as described previously (12) either in our laboratory in conjunction with Pel-Freeze Biologicals or by Dr. J. Ihle and colleagues (St. Jude Children's Research Hospital, Memphis, TN). Antibody to GHR (αGHR), kindly provided by Dr. W. R. Baumbach (American Cyanamid, Princeton, NJ), was produced in rabbits using recombinant rat GH-binding protein produced in Escherichia coli (13).

Immunoprecipitation and Western Blotting—Confluent 3T3-F442A cells were incubated 24 h in the absence of serum and with vehicle (0.02% ethanol) or DEX for the indicated times at 37°C in 95% air, 5% CO2, GH or bombesin was then added for 15 min at 37°C. Cells were rinsed with three changes of ice-cold 10 μM sodium phosphate, pH 7.4, 137 mM NaCl, 1 mM Na2VO4, and scraped on ice in lysis buffer (50 mM Tris, pH 7.5, 0.1% Triton-X100, 137 mM NaCl, 2 mM EGTA, 1 mM Na2VO4, 1 μM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cell lysates were centrifuged at 12,000 × g for 10 min and the resulting supernatants were incubated on ice for 120 min with antibodies to GHR and JAK2, which appear much later (14). Thereafter, the immunoprecipitates and lysates were subjected to SDS-PAGE followed by Western blot analysis with the indicated antibody using the ECL detection system. Bands on autoradiographs were quantified by scanning laser densitometry, using a BioMed Instruments scanning laser densitometer and Videoephor II program.

Construction of Mutant GHRs and Stable Expression in CHO Cell Lines—The expression plasmid, pLM108, containing the full-length rat GH receptor cDNA under the transcriptional control of the human metallothionein IIa promoter and the simian virus 40 enhancer was constructed as described previously (14). The cDNAs encoding GH receptor constructs were generated from a Bamos/EcoRI fragment of the pLM108 plasmid, which was subcloned into M13 mp19 for in vitro mutagenesis as described previously (15). The resulting mutant cDNAs were constructed using the polymerase chain reaction to splice out or alter regions as described (16). Oligonucleotides carrying the different mutations were synthesized and used as primers in the polymerase chain reaction to introduce stop codons (GHR1–380, GHR1–454, deletions (GHR297–311), and point mutations (GHR1–294, GHR1–454 Y333F, Y338F), and 0.5 and 5.7 (CHO GHR1–454 Y333F, Y338F). Therefore, binding data have been normalized within individual experiments to the level of binding observed in the same cell line treated with vehicle (i.e. percent of control). Effects of DEX and PMA treatment on binding are expressed as the mean of normalized values from independent experiments ± S.E. Values are considered significantly different from control when a 95% confidence interval (calculated by multiplying the S.E. by the t value for the relevant degree of freedom) excludes 100%. Experimental values are considered different from each other when their respective confidence intervals do not overlap.

RESULTS

DEX Decreases GH Binding and GH-induced Tyrosyl Phosphorylation of GHR and JAK2 in 3T3-F442A Fibroblasts—Phorbol esters have been shown to decrease GH binding in IM-9 cells (11). Therefore, we investigated whether, like DEX, the phorbol ester PMA causes a concomitant decrease in GH signaling. In 3T3-F442A cells, PMA rapidly decreases tyrosyl phosphorylation, as assessed by αPY Western blots, of both GHR (Fig. 1A) and JAK2 (Fig. 1B). In Fig. 1C, we compare the effects of TPA and DEX on GH binding and GH-induced tyrosyl phosphorylation of GHR and JAK2. PMA (100 nM) very rapidly decreases GH binding in 3T3-F442A cells (t1/2 = 15 min), to an even greater extent than reported for IM-9 cells (11). The time course of PMA-induced inhibition of GH-induced tyrosyl phosphorylation closely parallels that of PMA-induced decrease in GH binding. These rapid effects of PMA on GH binding and signaling are transient, however, GH binding and GH-induced tyrosyl phosphorylation of GHR and JAK2 return to basal levels by 18 h of PMA treatment. This contrasts with the effects of DEX on GH binding and tyrosyl phosphorylation of GHR and JAK2, which appear much later (t1/2 = 6 h) (Fig. 1C), and continue for at least 48 h (10).

DEX and PMA Decrease the Level of GH-bound GHR Protein, but Not the Level of Total Cellular GHR Protein—Previous studies indicate that DEX and phorbol esters decrease the number of GH receptors in the plasma membrane of cells without affecting their affinity, as determined by Scatchard analysis using 125I-hGH (10, 11). To determine whether these decreases in GHR in the plasma membrane represent a decrease in the expression of GHR or some other event, such as a change in the subcellular distribution (e.g. internalization) of GHRs or inhibition of GHRs, we examined whether DEX or PMA alters the total number of cellular GHRs expressed in 3T3-F442A cells. Levels of GH-bound and total cellular GHR protein were measured in control, DEX, and PMA-treated cells by Western blotting with an antibody directed against the extracellular domain of GHR (αGHR). GH-bound GHRs were immunoprecipitated as a complex with αGH. These GHRs thus represent GH-bound GHRs on the plasma membrane and those internalized during the 10-min GH treatment but still bound to GH. Total cellular GHRs (e.g. GH bound and unbound, plasma membrane, and intracellular) were immunoprecipitated with αGHR. Consistent with its effects on GH binding, DEX (24 h) decreases the number of GH-bound, plasma membrane GHRs by approximately 60% (Fig. 2, A, compare lanes B and C; and
cells were solubilized and GHR was immunoprecipitated with αGH and Western blotted with αPY. αGHR bands in autoradiographs of Western blots were quantified by scanning laser densitometry and normalized to levels of GHR in control cells. Data are from five (DEX) or two (PMA) separate experiments, and are expressed as the mean ± S.E. (DEX), range (PMA), or the coefficient of variability (control measurements).

DEX and PMA Do Not Decrease Bombesin Binding in CHO Cells Expressing Murine Bombesin Receptors—The inhibitory effects of DEX on signaling events such as stimulation of tyrosyl phosphorylation of ERKs, Stats, and JAK2 appear reasonable specific, since DEX does not block stimulation of tyrosyl phosphorylation of these substrates by EGF, PMA, and/or leukemic inhibitory factor in 3T3-F442A cells (10). In contrast, phorbol esters internalize a number of membrane receptors in addition to GHR (36). To examine further the specificity of DEX and PMA for GHR, we examined the effects of DEX and PMA on 125I-bombesin binding in CHO cells stably expressing bombesin receptor as a positive control for bombesin receptor internalization, pretreatment of these cells with bombesin for 15 min was shown to decrease subsequent 125I-Tyr6 bombesin binding by >75%, presumably by sequestration of bombesin receptors (17). In contrast to their inhibitory effects on GH binding in CHO cells expressing GHR, neither DEX nor PMA decreases bombesin binding in CHO cells which express bombesin receptors (Fig. 4).

Specific Cytoplasmic Domains of GHR Are Required for Maximal DEX- and PMA-induced Decreases in GH Binding—To gain insight into whether specific region(s) of GHR are required for the observed abilities of DEX and PMA to decrease GH binding, CHO cells expressing mutated GHRs were treated...
suberate. Complexes composed of 125I-GH cross-linked to CHO cells expressing the mutated GHRs were incubated with mutated GHRs expressed in CHO cells to the correct size, 125I-GH followed by the cross-linking reagent disuccinimidyl A

trol), and represent mean ± S.E. (or ± coefficient of variability in the case of the control bars) of three separate experiments performed in duplicate.

with DEX or PMA and GH binding was examined. The GHR tested (Fig. 5A) include a series of truncated GHRs (GHR1–506, GHR1–454, GHR1–380, and GHR1–294); GHR lacking the proline-rich "Box 1" region required for association and activation of JAK2 (GHR1–311) (17), full-length GHR in which Phe-346 (18), GHR1–638 and GHR1–506 have previously been shown by 125I-bombesin binding was assayed. Bars show 125I-bombesin binding to cells treated with DEX or PMA expressed as a percentage of binding to cells treated with vehicle (control), and represent mean ± S.E. (or ± coefficient of variability in the case of the control bars) of three separate experiments performed in duplicate.

with DEX or PMA and GH binding was examined. The GHR tested (Fig. 5A) include a series of truncated GHRs (GHR1–506, GHR1–454, GHR1–380, and GHR1–294); GHR lacking the proline-rich "Box 1" region required for association and activation of JAK2 (GHR1–297–311) (18), full-length GHR in which Phe-346 has been replaced with Ala and which is deficient in GH-induced internalization (GHRF346A) (19), and GHR1–454 in which tyrosines 333 and 338 have been substituted by phenylalanines (GHR1–454 Y333F, Y338F) (Fig. 5A). To verify that the mutated GHRs expressed in CHO cells were of the correct size, CHO cells expressing the mutated GHRs were incubated with 125I-GH followed by the cross-linking reagent disuccinimidyl suberate. Complexes composed of 125I-GH cross-linked to GHR1–638, GHR1–454, GHR1–454 Y333F, Y338F, GHR1–380, GHR1–294, and GHR1–297–311 have previously been shown by this laboratory to migrate in SDS-PAGE as proteins of the appropriate predicted molecular weight (18, 20). Similarly, GHRF346A and GHR1–506 produce complexes with mobilities appropriate for their predicted weight (Fig. 5B, lanes B and C).

Fig. 5C shows the effect of DEX on GH binding to CHO cells expressing these GHR mutants, expressed as a percentage of GH binding to the same cell line treated with vehicle (control). As shown in Fig. 3, when cells expressing GHR1–638 are treated with DEX for 24 h, GH binding is reduced to an average of 55% of that of control cells. DEX decreases GH binding in cells expressing GHR1–506 to a similar extent (e.g. 63% of control). However, DEX decreases binding to cells expressing GHR1–454 to only 85% of control, and this small inhibitory effect of DEX is lost upon truncation of GHR to amino acid 380. GHR1–454 contains four tyrosines, of which only tyrosines 333 and 338 appear to be tyrosyl phosphorylated in response to GH (20). These tyrosines appear not to be phosphorylated in GHR1–380 in response to GH.2 We therefore investigated if tyrosines 333 and 338 are required for the inhibitory effect of DEX on GHR1–454. Substitution of tyrosines 333 and 338 with phenylalanines abolishes the relatively small effect of DEX upon GHR1–454, suggesting that tyrosines 333 and 338 may be involved in mediating the actions of DEX on GHR. DEX decreases GH binding in the cells expressing GHR1–454, GHR1–380, and GHR1–294; GHR lacking the proline-rich "Box 1" region and hence JAK2 association and activation, is not necessary for this action of DEX on GHR. GH binding to the GHRF346A mutant is also decreased by DEX to a similar extent as binding to wild-type GHR, suggesting that DEX may decrease GH binding in a manner that is independent of ligand-induced internalization pathways.

Fig. 5D shows the effects of PMA (1 h) on GH binding in CHO cells expressing the various mutant GHRs. PMA decreases binding of 125I-GH to all CHO cell lines expressing mutant GHRs. However, whereas PMA treatment reduces GH binding to cells expressing GHR1–638 and GHR1–297–311 to approximately 20% of control, the effect of PMA on GH binding to all of the truncation mutants is blunted, i.e. GH binding is decreased to only 45–60% of control. This suggests that C-terminal amino acids of GHR may be required for maximal effects of PMA. Thus, there appear to be two components of the PMA-induced decrease in GH binding; one of which requires residues within the region of 507–638, and one which does not depend upon the cytoplasmic portion of GHR.

DISCUSSION

DEX and PMA Appear to Internalize GHR—Both DEX and PMA decrease GH binding and subsequent signaling through the GHR tyrosine kinase activity in 3T3-F442A fibroblasts and other cell types. The observed decreases in GH binding induced by DEX and PMA do not appear to be due to decreases in the level of expression of the GHR. The decrease in GH binding caused by PMA seems too rapid (t½ = 15 min). Furthermore, neither DEX nor PMA alter the total amount of cellular GHR protein measured by Western blotting. That DEX and PMA can decrease GH binding to CHO-GHR cells, in which the GHR gene is under the transcriptional control of a heterologous and actually glucocorticoid-inducible (21) promoter argues against DEX and PMA having their effects by decreasing transcription of the endogenous GHR gene. Even stronger evidence for these effects being post-translational is that DEX decreases GH binding in CHO cells stably transfected with cDNAs for wild-type GHR and some mutant GHRs, but not other mutants. All of these GHR cDNAs are driven by the same promoter. The small increase in GH binding consistently observed in CHO cells expressing GHR1–380 and GHR1–297–311 treated with DEX may reflect increased expression of these cDNA.

A decrease in the number of GH-bound plasma membrane GHRs without a decrease in the total number of cellular GHRs is consistent with DEX and PMA altering the distribution of

2 L. S. Smit and C. Carter-Su, unpublished observation.
GHRs away from the plasma membrane, either by internalizing (sequestering) GHRs previously on the membrane, or by directing the subcellular trafficking of newly synthesized GHRs to an intracellular site. Alternately, it is conceivable that glucocorticoids and/or phorbol esters could inactivate GHRs still on the plasma membrane such that they no longer bind GH, and thus decrease the number of GH binding sites without decreasing the total number of GHR proteins. However, inactivation of this sort would have to be an all-or-nothing event, since both DEX and PMA decrease the number of GH-binding sites but appear not to alter the affinity of GHR (10, 11). All of these possibilities are consistent with the finding that specific domains of GHR appear to be involved in these effects, as these domains could represent recognition sites for targetted endocytosis or trafficking, or a binding site for a regulatory protein.

GHR appear to internalize by both constitutive and GH-dependent pathways (22, 23). GHR has a very high rate of turnover in the membrane even in the absence of GH. GH binding is reduced by half after treatment with cycloheximide for 45 min in freshly isolated adipocytes (24) and 75 min in 3T3 fibroblasts (25). Upon binding GH, GH-GHR complexes are rapidly internalized and both GH (26–28) and GHR are degraded (22). Unlike many other receptor systems, most GHRs appear not to be recycled. A region of the rat GHR between amino acids 319 and 380 has been reported to be required for GH-induced internalization, with a single phenylalanine (Phe-342) being necessary (19). This region does not share consensus with motifs necessary for internalization identified in other receptors (for review, see Ref. 29). Interestingly, the Box 1 proline-rich region of GHR (amino acids 297–311), which is required for association and activation of JAK2 tyrosine kinase (18), is not required for GH-induced internalization, nor are tyrosines within amino acids 319–380 (Tyr-333, Tyr-338). Thus, it would appear that GH-induced internalization of GHR may not require GH-induced tyrosine kinase activity. In this study, DEX and PMA were added in the absence of GH and serum, and GH binding subsequently assessed at 4 °C so that movement of GHR within the cell would occur at greatly reduced levels. The observed effects of DEX and PMA therefore appear to be on the unbound GHR, rather than on the rate of GH-induced internalization.

Consistent with DEX leading to the internalization of GHRs, DEX has previously been reported to cause redistribution of glucose transporter proteins from the cell membrane to intracellular membranes (30–32). Glucocorticoid inhibition of glucose uptake in rat adipocytes is blocked by inhibitors of RNA and protein synthesis (35), suggesting that a glucocorticoid-induced protein may be involved in internalizing glucose transporter proteins. It was not possible to use inhibitors of protein synthesis such as cycloheximide to determine if the DEX-in-
Glucocorticoids and Phorbol Esters Decrease GH Binding

**Fig. 6. Domains of GHR involved in effects of GH, DEX, and PMA on GH binding.** Schematic diagram of GHR depicting cytoplasmic domains required for GH-induced receptor internalization (19), and maximal decreases in GH binding induced by DEX and PMA.

Reduced decrease in GH binding is dependent upon newly synthesized protein due to the high turnover rate of GHRs. Cycloheximide causes a 90% decrease in GH binding within 4 h in 3T3-F442A fibroblasts (25) and CHO-GHR cells (25), i.e. well before the maximal inhibitory effects of DEX are obtained.

The very rapid time course of the effect of PMA on GHR is consistent with PMA-induced internalization of GHRs being mediated through PKC phosphorylation events rather than being due to effects of newly synthesized proteins. PMA has been shown to lead to the rapid (within min) internalization of a number of membrane receptors (reviewed in Ref. 36), including receptors for transferrin (37), EGF (38, 39), tumor necrosis factor α (38), T-cell antigen (40), and muscarinic acetylcholine receptors (41). Most of these internalization events are attributed to a PMA-induced increase in the rate of endocytosis (37, 39, 41), although other effects of PMA on receptors, such as decreasing the rate of exocytosis (42), decreasing endocytosis (43), and decreasing receptor affinity (44, 45) have also been observed. Mutational analysis of EGF receptor (46, 47) and receptors for T-cell antigen (48) and Class I MHC antigen (49) indicate that the PKC phosphorylation sites are necessary for PMA-induced internalization, suggesting direct phosphorylation of receptors may be involved in their internalization.

In addition to multiple specific effects on the intracellular distribution and trafficking of membrane receptors, phorbol esters have general effects on membrane dynamics (50) and alter cellular morphology of 3T3-F442A, CHO, and H35 cells, observable by microscopy (data not shown), suggesting that PMA may cause a significant alteration of cellular membranes. However, PMA internalization of GHR does not appear to be a nonspecific consequence of a general increase in endocytosis or membrane rearrangement. In contrast to its effects on GH binding, PMA does not decrease binding of bombesin in CHO cells stably expressing bombesin receptors, and previously has been shown to increase, rather than decrease, the number of plasma membrane transferrin receptors in CHO cells (51).

Different Domains of GHR Are Required for the Effects of DEX and PMA—The effects of both DEX and PMA require specific domains of GHR. Amino acids located between residues 455 and 506 and tyrosines 333 and 338 of GHR are required for a maximal effect of glucocorticoids on GHR (Fig. 6). DEX also has a small but consistent effect on GHR1–454, which is not present with GHR1–380 or GHR1–294. This could reflect contributions to DEX actions by amino acids between 381 and 454. However, the small effects of DEX on GHR1–454 are abolished by substitution of tyrosines 333 and 338 by phenylalanines. As these tyrosines appear not to be phosphorylated in GHR1–380, although they appear to be phosphorylated in response to GH in GHR1–454 and wild-type GHR (20), this favors the conclusion that the small effect of DEX observed in GHR1–454 probably involves tyrosines 333 and 338 rather than amino acids between 381 and 454. The regions of GHR which appear to be required for the actions of DEX on GHR are not required for ligand-induced internalization (19); and Phe-346 of the GHR, required for internalization, does not appear to be necessary for the inhibitory effects of DEX. Thus, if DEX leads to increased internalization of GHRs from the plasma membrane, it would appear to do so by a mechanism different from that used by ligand-induced GHR internalization. The domains of GHR required for the effects of DEX may contain phosphorylation site(s) for a glucocorticoid-induced kinase or be involved in binding sites for glucocorticoid-inducible binding protein(s) which may direct GHRs on the plasma membrane toward endocytic pathways, target newly synthesized GHRs to an internal site, or inhibit GHRs on the plasma membrane. The proline-rich Box 1 region of GHR is not necessary for glucocorticoid-induced inhibition of GH binding, making it unlikely that JAK2 association with GHR or tyrosyl phosphorylation of GHR plays a direct role in glucocorticoid-induced effects on GHR. Furthermore, in these studies, cells were treated with glucocorticoids or phorbol esters in the absence of GH. In this state, tyrosine kinase activity associated with GHR is virtually undetectable.

The effects of PMA on GHR appear to be mediated by a mechanism distinct from both that of DEX and ligand-induced internalization. There appear to be two components of phorbol ester internalization of GHRs: one which seems to require no or only minimal cytoplasmic amino acids of GHR, and one which appears to require C-terminal amino acids (Fig. 6). Although the 507–638 region of GHR does not contain exact consensus sequences for protein kinase C phosphorylation sites, it does contain serines and threonines closely associated with lysine and arginine residues, making it attractive to speculate that this component of phorbol ester internalization of GHR may be mediated by direct phosphorylation of GHR by protein kinase C. However, a previous study in IM-9 cells failed to detect phorbol ester-induced incorporation of radiolabeled phosphate into immunoprecipitated GHR (11), suggesting that phorbol ester activation of PKC may not lead to serine/threonine phosphorylation of GHR in this cell type. This previous study did detect a 55-kDa phosphoprotein that was precipitated with GH antibodies, presumably in a complex with GHR, from cells treated with phorbol esters. This suggests that rather than causing a PKC-mediated phosphorylation of GHR itself, as is apparently the case for EGF receptor (46), phorbol esters may lead to the phosphorylation of a protein which binds GHR and may mediate its rapid internalization. It will be interesting to see whether GHR is phosphorylated on serine or threonine by PKC and/or other cellular kinases in 3T3-F442A fibroblasts and/or CHO cells, and whether a similar 55-kDa GHR-binding phosphoprotein is present in these cell types. As suggested for GH, phosphorylation by PKC of receptors for EGF (46), trans-

---

3 A. P. J. King and C. Carter-Su, unpublished observation.

4 E. Adkins, G. S. Campbell, and C. Carter-Su, manuscript in preparation.
Conclusions—Glucocorticoids and phorbol esters both appear to decrease GH binding by internalizing GHRs, however, the novel possibility that DEX and/or PMA may inactivate GHRs on the plasma membrane and thus be a potential site of cross-talk...Glucocorticoids...that glucocorticoids may act within the stress response...they provide a molecular mechanism by which glucocorticoids might decrease tissue sensitivity to GH...and act at the level of peripheral tissues, and thus these findings might provide a molecular mechanism by which glucocorticoids can down-regulate cellular physiological activities. Internalization of GHR and glucose transporters by glucocorticoids is generally consistent with the theory that glucocorticoids may act within the stress response to prepare the individual for future stressors. Increased levels of glucocorticoids generated during stress could curtail metabolically expensive activities such as growth and high peripheral metabolism by internalizing GHR and glucose transporter proteins in targeted peripheral tissues within a...Glucocorticoids and phorbol esters decrease GH binding...Glucocorticoids and phorbol esters decrease GH binding.