Rat Liver Glucocorticoid Receptor Isolated by Affinity Chromatography Is Not a Mg^{2+}- or Ca^{2+}-Dependent Protein Kinase†

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ABSTRACT: The glucocorticoid hormone receptor (92 kDa), purified 9000-fold from rat liver cytosol by steroid affinity chromatography and DEAE-Sephacel chromatography, was assayed for the presence of protein kinase activity by incubations with [γ-^{32}P]ATP and the photoaffinity label 8-azido-[γ-^{32}P]ATP. Control preparations isolated by affinity chromatography in the presence of excess steroid to prevent the receptor from binding to the affinity matrix were assayed for kinase activity in parallel. The receptor was not labeled by the photoaffinity label under photoactivation conditions in the presence of Ca^{2+} or Mg^{2+}. A Mg^{2+}-dependent protein kinase (48 kDa) that could be photoaffinity labeled with 8-azido-ATP copurified with the receptor. This kinase was also present in control preparations. The kinase could phosphorylate several minor contaminants present in the receptor preparation, including a protein (or proteins) of similar molecular weight to the receptor. The phosphorylation of 90–92-kDa proteins was independent of the state of transformation or steroid-binding activity of the receptor. These experiments provide direct evidence that neither the glucocorticoid receptor nor the 90–92-kDa non-steroid-binding protein associated with the molybdate-stabilized glucocorticoid receptor possesses intrinsic Ca^{2+}- or Mg^{2+}-dependent protein kinase activity.

Protein phosphorylation/dephosphorylation reactions have been implicated in the mechanism of action of steroid hormone receptors. Dephosphorylation by exogenous phosphatases has been shown to decrease the steroid-binding activity of crude estrogen (Migliaccio et al., 1982) and glucocorticoid (Nielsen et al., 1977) receptors. Similarly, this loss can be decreased by the phosphatase inhibitors sodium molybdate and sodium fluoride (Leach et al., 1979; Grody et al., 1980). Dephosphorylation has also been linked to transformation since exogenous phosphatases can enhance the latter process (Barnett et al., 1980).

Whereas it is well established that various peptide hormone receptors residing in the plasma membrane possess intrinsic tyrosine-specific protein kinase activity [see Carter-Su & Pratt (1984) for a review], the evidence for protein kinase activity in steroid receptors is contradictory. Progesterone and glucocorticoid receptors have been isolated as phosphoproteins after "in vivo" labeling with radioactive inorganic phosphate (Dougherty et al., 1982; Grandics et al., 1984a). The 80- and 108-kDa progesterone receptor subunits can undergo phosphorylation by exogenous cAMP-dependent protein kinases in the presence of Mg^{2+} (Weigel et al., 1981). No evidence for intrinsic protein kinase activity in the presence of Mg^{2+} (Weigel et al., 1981) or Ca^{2+}, Mg^{2+}, or Mn^{2+} (Ghosh-Dastidar et al., 1984) was found by these authors, whereas others reported that the progesterone receptor has intrinsic protein kinase activity (Garcia et al., 1983). The androgen receptor can be selectively phosphorylated by a cAMP-independent kinase from the nuclei of rat prostate, but no intrinsic receptor protein kinase activity was detected (Goueli et al., 1984). The estrogen receptor can be phosphorylated by a calmodulin-dependent tyrosine-specific nuclear kinase. No intrinsic receptor protein kinase activity could be detected (Migliaccio et al., 1984). Some authors have found that highly purified glucocorticoid receptor preparations possess protein kinase activity (Kurl & Jacob, 1984; Singh & Moudgil, 1984; Miller-Diener et al., 1985). These reports are inconsistent with regard to the divalent cation dependency of the kinase activity and whether the receptor itself becomes phosphorylated by the kinase. Kurl and Jacob (1984) reported that the receptor was phosphorylated in a Mg^{2+}-dependent fashion by an endogenous kinase in highly purified transformed GHRC[1] preparations. Miller-Diener et al. (1985) found that Ca^{2+}-dependent protein kinase activity was associated with their purified transformed GHRC preparations. Their results strongly suggest an inherent receptor autokinase activity. Others have reported the absence of any autokinase activity of GHRC in the presence of Ca^{2+} or Mg^{2+}, but an ability of the purified GHRC preparation to phosphorylate exogenous substrates in the presence of these divalent cations (Singh & Moudgil, 1984). Immunoaffinity-enriched molybdate-stabilized L-cell GHRC did not exhibit intrinsic kinase activity (Sanchez & Pratt, 1986). A Mg^{2+}-dependent protein kinase contaminant was present in such complexes when polyclonal but not monoclonal anti-receptor antibodies were used.

Thus it is evident that steroid receptors can act as substrates for various kinases, resulting in phosphorylation at serine (Dougherty et al., 1982; Housley & Pratt, 1983), tyrosine (Ghosh-Dastidar et al., 1984; Migliaccio et al., 1984), or threonine residues (Miller-Diener et al., 1985). However, the biological significance of phosphorylation/dephosphorylation reactions remains to be elucidated. The presence of intrinsic

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[1] Abbreviations: dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; TA, triamcinolone acetonide, 9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone; [^{3}H]TA, [1,2,3(n)-^{3}H]triamcinolone acetonide; GHRC, glucocorticoid hormone receptor complex; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; DEAE, diethylaminoethyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ATP, adenosine 5′-triphosphate; UV, ultraviolet; ATPase, adenosinetriphosphatase; TCA, trichloroacetic acid; P_i, inorganic phosphate; TLC, thin-layer chromatography.

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protein kinase activity as a general feature of steroid receptors is highly controversial.

If the GHRC possesses intrinsic protein kinase activity, it should be possible to label the receptor with the photoaffinity label 8-azido-ATP on photoactivation. This ATP analogue labels the ATP binding sites of proteins exhibiting varying catalytic activity (Haley & Hoffman, 1974; Wagenvoord et al., 1977; Czarnecki et al., 1979; Hollemans et al., 1983; Roth & Cassell, 1983; Shia & Pilch, 1983; Clawson et al., 1984; Scheiner-Bobis & Schoner, 1985). We show that the purified GHRC does not bind 8-azido-[γ-^{32}P]ATP and does not exhibit intrinsic Mg^{2+}- or Ca^{2+}-dependent protein kinase activity in the transformed or untransformed state. We also show that a Mg^{2+}-dependent protein kinase is copurified with GHRC. This kinase (48 kDa) can be partially separated from GHRC on DEAE-Sephacel and phosphorylates contaminants present in the GHRC preparation.

Materials and Methods

Materials. [γ-^{32}P]ATP (3–6 Ci/μmol) was synthesized enzymatically by the method of Johnson and Walseth (1979), using [^{32}P]orthophosphate (carrier free) (Amersham) and adenosine diphosphate (Sigma). 8-Azido-[γ-^{32}P]ATP (3–6 Ci/μmol) was synthesized by the same enzymatic procedure by substituting 8-azidoadenosine diphosphate (Sigma) for adenosine diphosphate. [^{3}H]TA was from Amersham (25 Ci/mmol). Affi-Gel 102 was from Bio-Rad. Serum containing polyclonal anti-glucocorticoid receptor antibodies was a kind gift from Dr. M. V. Govindan. The preparation and characterization of the antibody are described by Govindan and Gronemeyer (1984).

Buffers. Buffer A was Tris (10 mM), EDTA (1 mM), β-mercaptoethanol (5 mM), and glycerol (10%), pH 7.4 (4 °C). Buffer B was potassium phosphate (50 mM), EDTA (1 mM), β-mercaptoethanol (5 mM), and glycerol (10%), pH 7.0. Buffer C was buffer A with the addition of 50 mM sodium chloride.

Purification of GHRC from Rat Liver Cytosol. The untransformed molybdate-stabilized GHRC was purified 9000-fold by affinity chromatography and DEAE-Sephacel chromatography. Molybdate (Na_2MoO_4) (10 mM) was present in all the isolation buffers unless otherwise stated. All procedures were performed at 4 °C. The biospecific adsorbent (2 μmol of steroid/mL of gel) was synthesized by coupling the 17β-carboxylic acid of dexamethasone to Affi-Gel 102 via the *N*-hydroxybenzotriazole ester of dexamethasone (Govindan & Gronemeyer, 1984; Idzioreck et al., 1985). Underivatized amino groups were acetylated to decrease nonspecific binding of receptor to the matrix. Cytosol (150 mL) was prepared from 15 rat livers and subjected to phosphocellulose chromatography as described by Govindan and Gronemeyer (1984). The phosphocellulose flow-through (200 mL) was incubated with 40 mL of affinity matrix for 16 h. After thorough washing of the affinity matrix, biospecific elution was achieved by incubation with 2 μM [^{3}H]TA (5.0 Ci/mmol) for 16 h (Idzioreck et al., 1985; Grandics et al., 1984a,b). The affinity eluate (80 mL) was then subjected to DEAE-Sephacel chromatography (Grandics et al., 1984b). The untransformed GHRC was eluted from DEAE-Sephacel in a total volume of 6 mL in the absence or presence of 10 mM molybdate, with either a 0–500 mM sodium chloride gradient in buffer A or a 50–500 mM potassium phosphate gradient in buffer B. For some experiments, the purified GHRC was precipitated with 50% $(NH_4)_2SO_4$ and resuspended in buffer C in the absence or presence of 10 mM molybdate. Control mock isolations were performed by incubation of cytosol with affinity matrix in the presence of 4 μM unlabeled dexamethasone. Experimental and control preparations were analyzed for the presence of the glucocorticoid receptor by the determination of steroid-binding activity, molecular weight on SDS–PAGE, and anti-receptor antibody binding.

Transformation of Purified GHRC. Purified GHRC eluted off DEAE-Sephacel in the absence of molybdate could be heat-transformed to increase its DNA–cellulose- and nuclear-binding activity as found by some authors (Lan et al., 1984; Weisz et al., 1984; Idziorek et al., 1985). This transformation is prevented by molybdate. Ammonium sulfate precipitation (50%) of DEAE-Sephacel eluates in the presence of molybdate resulted in GHRC transformation. Precipitation in the absence of molybdate caused an 80% loss of steroid-binding activity (Hapgood and von Holt, unpublished results).

Labeling with [γ-^{32}P]ATP and 8-Azido-[γ-^{32}P]ATP. Purified GHRC preparations were incubated with 200 nM [γ-^{32}P]ATP (1.5 Ci/μmol) for ammonium sulfate treated samples and with 50 nM [γ^{32}P]ATP (0.75 Ci/μmol) for samples assayed directly after DEAE-Sephacel elution. In pilot experiments these two concentrations had been found to result in reproducible and comparable phosphorylation of GHRC and other proteins present in the respective GHRC preparations. Incubations were for 30 min at 20 °C (i.e., under heat transformation conditions) in the presence of 5 mM $MgCl_2$ and/or 5 mM $CaCl_2$, unless otherwise stated. Samples to be photoaffinity-labeled with 8-azido-[γ-^{32}P]ATP (40 nM, 0.75 Ci/μmol) were exposed to UV light for 15 min at 20 ± 5 °C. Samples were placed in quartz cuvettes on a UV transillumination box containing a mercury lamp giving maximum emission at 313 nm. For incubation volumes of 50 μL, the reaction was stopped by the addition of double-concentration SDS–PAGE sample application buffer. For incubation volumes of 200 μL, the reaction was stopped by the addition of deoxycholate (0.02%) and TCA precipitation (10%) at 4 °C (Idzioreck et al., 1985). The precipitated samples were redissolved in a minimal volume of 1 M sodium hydroxide followed by 50 μL of sample application buffer. All samples were heated in a boiling water bath for 1 min prior to SDS–PAGE.

SDS–PAGE. Samples were electrophoresed on 0.8-mm 10% SDS–polyacrylamide slab gels according to the method of O'Farrell (1975). Electrophoresis was for 15 h at 100-V constant voltage. Gels to be stained with Coomassie were soaked in 0.25% Coomassie Brilliant Blue in 50% (v/v) methanol and 10% (v/v) acetic acid for $1^1/_2$ hours. Destaining was in 25% (v/v) ethanol and 7% (v/v) acetic acid. Protein standards were from Sigma (MW SDS 200): carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

Autoradiography. Autoradiography of dried SDS–PAGE gels was by exposure in X-ray cassettes using Kodak X-omat film at −70 °C. The film has been preflashed twice on both sides, and intensifying screens were used.

Western Blotting and Detection with Anti-Glucocorticoid Receptor Antibodies. The procedure was as described in Govindan and Gronemeyer (1984). In all cases control incubations with preimmune serum gave negative results.

Results and Discussion

The purified untransformed glucocorticoid receptor eluted off DEAE-Sephacel at 0.25 M potassium phosphate in buffer B in the absence or presence of molybdate. It was identified as a 92-kDa steroid-binding protein that cross-reacted with anti-receptor antibodies (Figure 1). Mock isolations (see

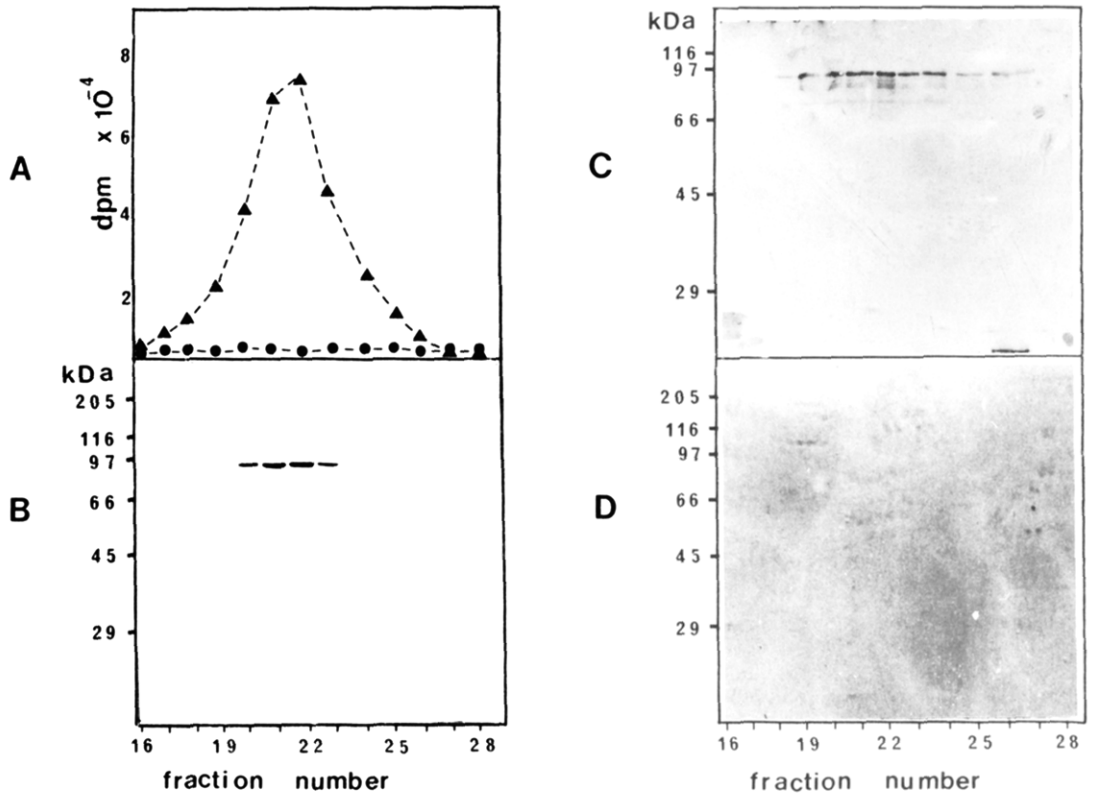

FIGURE 1: Characterization of purified GHRC eluted from DEAE-Sephacel after affinity chromatography and comparison with control mock isolations. Experimental and control preparations were eluted with a 40-mL 50–500 mM potassium phosphate gradient from DEAE-Sephacel in buffer B. Fractions of 1 mL were collected. (A) The radioactive elution profile of [^{3}H]TA was plotted for 30 μL of individual eluate fractions for experimental (▲) and control (●) isolations. (B) Coomassie staining pattern for individual fractions of experimental isolation after SDS–PAGE. (C and D) Anti-glucocorticoid receptor polyclonal antibody binding pattern of individual fractions after SDS–PAGE and Western blotting for experimental (C) and control (D) isolations. For experimental details see Materials and Methods.

Materials and Methods) did not yield steroid-binding proteins on elution from the affinity matrix and subsequent DEAE-Sephacel chromatography (Figure 1A). However, after SDS–PAGE a Coomassie-stained protein of 90–92-kDa molecular mass could be detected in the eluate from DEAE-Sephacel in the absence or presence of molybdate (Figure 6A). As judged by the intensity of staining, the mock isolation decreased the yield of protein migrating at 90–92 kDa by 30–50% as found by Idziorek et al. (1985). We have shown that this protein obtained in the mock isolation is immunochemically distinct from the glucocorticoid receptor (Figure 1C,D). A similar non-steroid-binding protein of approximately 90 kDa was found by several authors to be associated with molybdate-stabilized untransformed steroid receptors (Jaob et al., 1984; Housley et al., 1985; Sanchez et al., 1985). Further details on the characterization of our purified untransformed receptor will be the subject of a forthcoming publication (Hapgood and von Holt, unpublished results).

Affinity-purified GHRC eluted off DEAE-Sephacel in buffer A or B in the absence or presence of 10 mM molybdate, and in some cases subjected to an additional purification step of 50% $(NH_4)_2SO_4$ precipitation, was assayed for ^{32}P incorporation after incubation with [γ-^{32}P]ATP under various conditions. These samples were also assayed for the presence of ATP-binding proteins by photoaffinity labeling with 8-azido-[γ-^{32}P]ATP. For further details on transformation conditions see Materials and Methods.

Detection of Kinase Activity by Incubation with [γ-^{32}*P*]*-ATP.* Figure 2 demonstrates that, prior to $(NH_4)_2SO_4$ precipitation, incubation of GHRC preparations in the presence of $MgCl_2$ results in a considerable number of phosphorylated proteins, including a 90–92-kDa protein (or proteins). GHRC

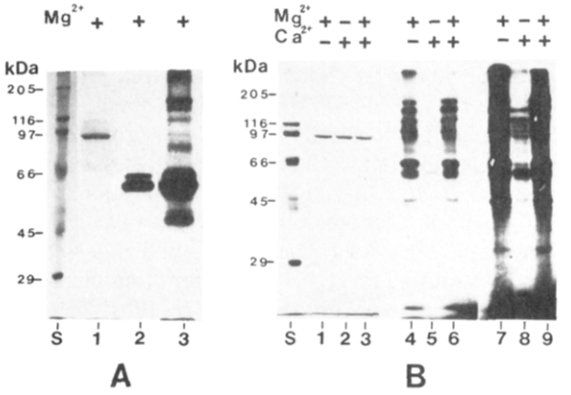

FIGURE 2: Kinase activity in purified GHRC preparations assayed directly after DEAE-Sephacel elution. Affinity-purified GHRC eluted from DEAE-Sephacel in buffer B minus molybdate (A) or in buffer A plus molybdate (B) was diluted to a final GHRC concentration of 1 μg/mL, a final potassium phosphate concentration of 50 mM (in buffer B), or a final sodium chloride concentration of 50 mM (in buffer A). One milliliter of these solutions was incubated with [γ-^{32}P]ATP in the presence of $MgCl_2$ and/or $CaCl_2$ as shown in the figure. The reactions were stopped by TCA precipitation. The samples were subjected to gel electrophoresis and Coomassie staining (A, lane 1; B, lanes 1–3) and exposed to X-ray film after drying (further experimental details under Materials and Methods). The autoradiograms were exposed for 2 (A, lane 2; B, lanes 4–6) or 10 days (A, lane 3; B, lanes 7–9).

preparations eluted off DEAE-Sephacel with a potassium phosphate gradient in buffer B (minus molybdate) (Figure 2A) contain two major phosphorylated bands of 50–60 kDa. On elution with a sodium chloride gradient in buffer A (plus molybdate), the larger proteins appear to undergo more phosphorylation (Figure 2B). This difference in phosphory-

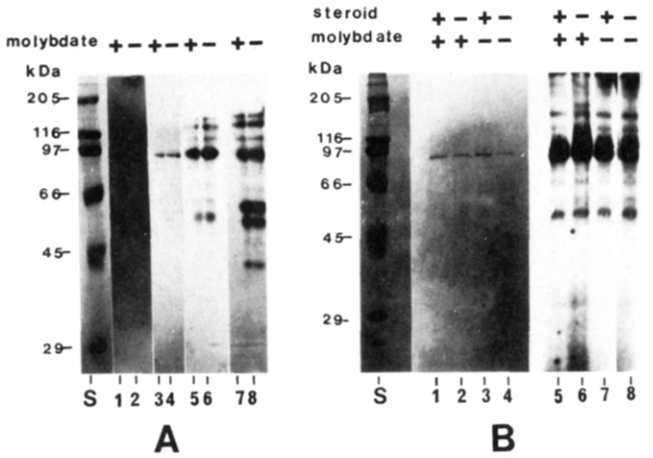

FIGURE 3: Kinase activity in purified GHRC preparations after $(NH_4)_2SO_4$ precipitation. Affinity-purified GHRC eluted from DEAE-Sephacel in buffer A minus molybdate (A, lanes 1 and 2), buffer B minus molybdate (A, lanes 3 and 4), or buffer A plus molybdate (B, lanes 1–4) was precipitated with $(NH_4)_2SO_4$ and resuspended in buffer C. Molybdate (10 mM) and/or unlabeled TA (1 μM) was added to samples as shown in the figure. Fifty microliters of each sample (0.5 μg of GHRC) was incubated with [γ-^{32}P]ATP in the presence of $MgCl_2$. The reactions were stopped by the addition of SDS–PAGE sample application buffer, and the samples were analyzed as described for Figure 2. The Coomassie-stained gels (A, lanes 1–4; B, lanes 1–4) and the autoradiograms of these gels exposed for 2 days (A, lanes 5–8; B, lanes 5–8) are shown.

lation pattern appeared to depend on the nature of the salt gradient rather than on the absence or presence of molybdate, since GHRC preparations eluted from DEAE-Sephacel in buffer A (minus molybdate) resulted in the same phosphorylation pattern as in Figure 2B (data not shown). Incubations in the presence of Ca^{2+} resulted in considerably less ^{32}P incorporation than in the presence of Mg^{2+} under all conditions tested.

Figure 3 demonstrates that after $(NH_4)_2SO_4$ precipitation, incubation in the presence of $MgCl_2$ yields a major 90–92-kDa phosphorylated fraction, with several other proteins also undergoing phosphorylation. The absence or presence of molybdate during incubation or during precipitation with $(NH_4)_2SO_4$ does not affect the phosphorylation pattern. Similarly, the absence or presence of excess stabilizing steroid has no effect. However, $(NH_4)_2SO_4$ precipitation changed the phosphorylation pattern, probably as a result of selective removal or enrichment of catalytic and noncatalytic proteins.

The phosphorylation pattern for GHRC preparations will depend on the relative amounts of contaminating kinase and substrates. This differs for GHRC eluted from DEAE-Sephacel by using either a 50–500 mM potassium phosphate gradient in buffer B or a 0–500 mM sodium chloride gradient in buffer A (Figure 2). The ratio of kinase to substrate also differs for $(NH_4)_2SO_4$ precipitated vs. unprecipitated samples (Figures 2 and 3). The reason for the relative increase in phosphorylation of 90–92-kDa polypeptides in $(NH_4)_2SO_4$-precipitated samples could also reflect some change in the structure of the GHRC, making it a better substrate for a kinase.

We have monitored the formation of ^{32}P-labeled inorganic phosphate by TLC and autoradiography for the experiment in Figure 3B and compared this to control incubations with [γ-^{32}P]ATP in buffer alone. We found no difference in the very small amounts of [^{32}P]P_i for experiment and control incubations (data not shown). In Figure 2, the absence or presence of molybdate (a known phosphatase inhibitor) (Barnett et al., 1980) gave identical ^{32}P incorporation. Taken

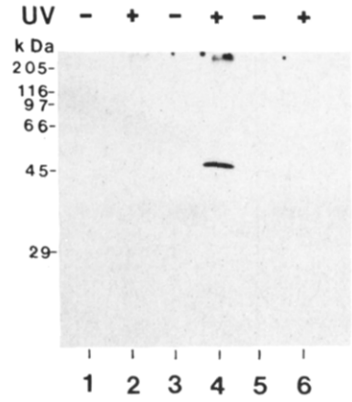

FIGURE 4: 8-Azido-[γ-^{32}P]ATP photoaffinity labeling of a contaminant kinase in purified $(NH_4)_2SO_4$-precipitated GHRC preparations. GHRC purified and $(NH_4)_2SO_4$ precipitated as for Figure 3B was resuspended in buffer C in the presence of 1 μM unlabeled TA. Two hundred microliters (0.5 μg) of GHRC was incubated with 8-azido-[γ-^{32}P]ATP in the presence of $MgCl_2$, in the absence and presence of UV. Unlabeled ATP (1 mM) (lanes 1 and 2) or unlabeled 8-azido-ATP (1 μM) (lanes 5 and 6) was added. Samples were analyzed as described for Figure 3. The autoradiogram was exposed for 2 days.

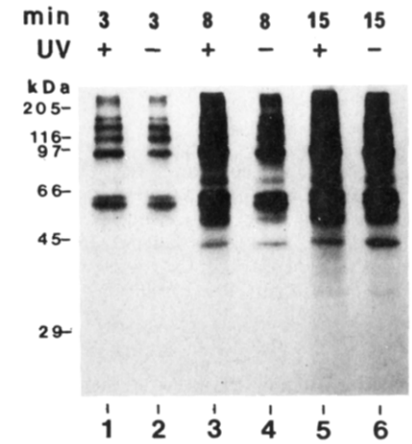

FIGURE 5: Kinase activity under photoactivation conditions. Purified, $(NH_4)_2SO_4$-precipitated GHRC (200 μL; 0.5 μg) in buffer C (as in Figure 3A, lanes 4 and 8) was exposed to UV photoactivation conditions (in the absence of 8-azido-ATP) at 20 °C for the times indicated, in the presence of [γ-^{32}P]ATP, $MgCl_2$, and 1 μM TA. Control incubations in the dark were also performed. The reactions were stopped by TCA precipitation, and samples were processed as in Figure 2. The autoradiogram was exposed for 2 days.

together, these results indicate that the GHRC preparation is not contaminated with a phosphatase or an ATPase.

The results of Figures 2 and 3 demonstrate the presence of a considerable amount of Mg^{2+}-dependent protein kinase activity in GHRC preparations. This activity did not depend on the absence or presence of molybdate, the state of transformation, or the steroid-binding activity of the GHRC. However, these results cannot be unequivocally interpreted to show intrinsic protein kinase activity of the receptor. 8-Azido-[γ-^{32}P]ATP was thus used to probe for the presence of ATP-binding proteins by photoaffinity labeling.

Detection of ATP-Binding Proteins. On incubation of purified $(NH_4)_2SO_4$-precipitated GHRC preparations with 8-azido-[γ-^{32}P]ATP, only one polypeptide (48 kDa) is labeled in a UV-dependent fashion (Figure 4). The photospecific labeling is suppressed by competition with unlabeled ATP or unlabeled 8-azido-ATP. The photoaffinity labeling conditions did not affect the kinase activity, as exposure of GHRC preparations to UV light for increasing times in the presence of [γ-^{32}P]ATP does not cause a decrease in kinase activity, nor does it lead to a change in the pattern of ^{32}P incorporation

(Figure 5). These results clearly exclude an ATP-binding property of the GHRC, a prerequisite for kinase activity.

A comparison of chromatographic fractions eluting near the GHRC off DEAE-Sephacel for experimental and control mock affinity isolations should reveal additional information. Figure 6 shows the results of these experiments. It is obvious that the ^{32}P incorporation pattern from [γ-^{32}P]ATP is identical for experimental and control isolations, i.e., it is independent of the presence or absence of the GHRC (Figure 6B). The ^{32}P incorporation pattern for ATP-binding proteins photoaffinity labeled with 8-azido-[γ-^{32}P]ATP is also identical for both preparations (Figure 6C). Those fractions with the most photoaffinity labeling show the most kinase activity. The Coomassie-stained pattern for the two preparations (Figure 6A) only differs in the absence or presence of GHRC. The contaminant kinase of 48 kDa elutes just before the GHRC on DEAE-Sephacel (Figure 6). A Coomassie-stained fraction of 48 kDa superimposes with the maximum of 8-azido-[γ-^{32}P]ATP binding (Figure 6A,C).

Only a 48-kDa protein becomes photoaffinity labeled regardless of whether 90–92-kDa proteins become phosphorylated or not in GHRC preparations (Figures 3B and 6B compared to Figures 4 and 6C). In the presence of Ca^{2+}, the same 48-kDa band was again photoaffinity-labeled, but with a much reduced intensity (data not shown).

Taken together, these results show the following: (1) A Mg^{2+}-dependent contaminant protein kinase of 48 kDa copurified with the GHRC on steroid affinity chromatography. This kinase can be partially separated from the GHRC on DEAE-Sephacel chromatography. (2) The kinase can phosphorylate several contaminating proteins present in the GHRC preparation, possibly including the GHRC. Some of the contaminants are undetectable by Coomassie staining, while others are major contaminants of the GHRC preparation. (3) The ability of the 90–92-kDa protein(s) to serve as substrate(s) for the kinase appears to be unrelated to steroid binding of the GHRC or to the absence or presence of molybdate. (4) Neither the purified GHRC nor the 90–92-kDa non-steroid-binding protein associated with the molybdate-stabilized GHRC is a Ca^{2+}- or Mg^{2+}-dependent protein kinase.

In light of the results reported here, the contradictory findings in the literature (see the introduction) as to the presence or absence in the GHRC of intrinsic protein kinase activity can in most cases be explained by contamination of GHRC preparations with copurified protein kinase activated by various cofactors. Such protein kinases, according to their respective properties, may be present in different proportions in GHRC preparations isolated by different authors. However, Miller-Diener et al. (1985) found that their observed kinase activity did not appear to be due to a protein kinase contaminant in their GHRC preparations. These authors were able to link the kinase activity to the steroid-binding activity and state of transformation of the GHRC. The untransformed GHRC eluted from DEAE-Sephacel at 200–250 mM KCl had no kinase activity. The same preparation, after heat transformation and subsequent DEAE chromatography, eluted at 50–100 mM KCl and had Ca^{2+}-dependent protein kinase activity. They concluded that the GHRC is most likely a Ca^{2+}-dependent autokinase when it is in the transformed state only. On analysis of their results, we conclude that it is still possible that a contaminant kinase is present in both their transformed and untransformed GHRC fractions eluted from DEAE-Sephacel, possibly in different amounts due to its elution position not coinciding with either GHRC fraction. The GHRC would have to be a substrate for the contaminant kinase only when in the transformed state to explain the results. Our results on the absence of Mg^{2+}- or Ca^{2+}-dependent intrinsic receptor protein kinase activity of the GHRC are in agreement with the findings of Sanchez and Pratt (1986).

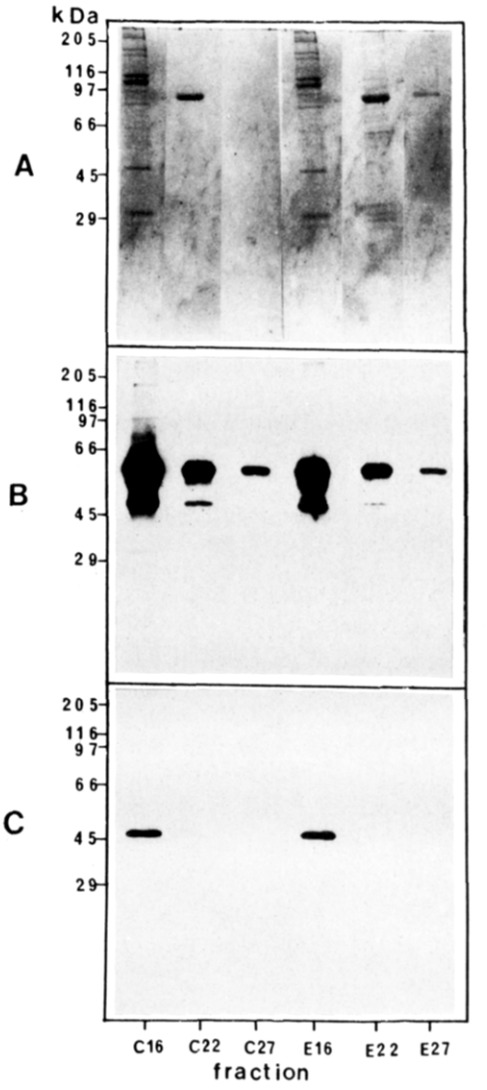

FIGURE 6: Protein kinase contaminant in purified GHRC. GHRC isolated by affinity chromatography was chromatographed on DEAE-Sephacel in buffer B minus molybdate. A control mock isolation was done in parallel. Kinase activity and 8-azido-[γ-^{32}P]ATP binding were determined in fractions 16, 22, and 27 as identified in Figure 1. Three equal volumes of each of fractions 16, 22, and 27 for experimental (E) and control (C) isolations were diluted to 600 μL to give a final potassium phosphate concentration of 100 mM in buffer B and a concentration of 1 μg/600 μL of GHRC for fraction 22 of experimental isolation. Unlabeled TA (1 μM) and $MgCl_2$ (5 mM) were added to each. (A) Coomassie-stained pattern of fractions for E and C isolations after treatment as in panel B. (B) Aliquots (600 μL) of each fraction were incubated with [γ-^{32}P]ATP for 30 min at 20 °C. The reactions were stopped by TCA precipitation, and the samples were processed as in Figure 2. The autoradiogram was exposed for 2 days. (C) Aliquots (2 × 600 μL) of each fraction were heat transformed by incubation for 30 min at 20 °C. Six hundred microliters of each was then incubated for 15 min at 20 °C in the presence of 8-azido-[γ-^{32}P]ATP either in the presence (lanes 1–6) or in the absence of UV. The reactions were stopped by TCA precipitation, and samples were processed as in Figure 2. The autoradiogram was exposed for 24 h. Exposure for 5 days did not reveal labeling of proteins in the 90–92-kDa region. In the absence of UV, samples analyzed as for panel C showed no detectable labeled bands (data not shown). Upon longer exposure for 5 days, only a 55-kDa band became visible, coinciding with the major phosphorylated band in panel B. This may indicate that the kinase can utilize 8-azido-ATP as a substrate in the absence of UV to phosphorylate the 55-kDa protein.

These authors could not detect intrinsic protein kinase activity in their GHRC preparations purified by immunoadsorption.

The experiments presented here show unequivocally that the Mg^{2+}-dependent protein kinase activity detected in our purified GHRC preparations is due to a contaminant protein kinase. The glucocorticoid receptor does not bind 8-azido-ATP in the presence of Mg^{2+} or Ca^{2+} under the conditions tested. By these criteria the GHRC does not possess intrinsic Mg^{2+}- or Ca^{2+}-dependent protein kinase activity.

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Registry No. Protein kinase, 9026-43-1.

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