Phosphorylation of L-Cell Glucocorticoid Receptors in Immune Complexes: Evidence That the Receptor Is Not a Protein Kinase†

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ABSTRACT: Two phosphoproteins are adsorbed to protein A–Sepharose when cytosol from ^{32}P-labeled L-cells is incubated with a monoclonal antibody against the glucocorticoid receptor: one is a 98K phosphoprotein that contains the steroid binding site, and the other is a 90K non-steroid-binding phosphoprotein that is associated with the molybdate-stabilized receptor [Housley, P. R., Sanchez, E. R., Westphal, H. M., Beato, M., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 13810–13817]. In this paper we have incubated L-cell cytosol with rabbit antiserum against the mouse glucocorticoid receptor and show that incubation of protein A–Sepharose-bound immune complexes with [γ-^{32}P]ATP and Mg^{2+} results in phosphorylation of the 98K steroid-binding protein but not of the 90K receptor-associated protein. Phosphorylation occurs regardless of whether the receptor is unoccupied or is present as the untransformed or transformed steroid–receptor complex. No phosphorylation occurs in the presence of Ca^{2+} instead of Mg^{2+}. If protein A–Sepharose-bound immune complexes prepared with a monoclonal antibody against the receptor are incubated with [γ-^{32}P]ATP and Mg^{2+}, neither protein is phosphorylated. If the protein A–Sepharose pellet is obtained from molybdate-stabilized cytosol that has been incubated both with monoclonal antibody to provide the 98K receptor and its 90K associated protein and with preimmune rabbit serum, which causes the nonspecific adsorption of an L-cell protein kinase, then incubation with [γ-^{32}P]ATP and Mg^{2+} causes receptor phosphorylation. This study shows that L-cell cystol contains a protein kinase that phosphorylates the glucocorticoid receptor, but it fails to confirm recent reports that either the glucocorticoid receptor or the 90K receptor-associated protein has intrinsic protein kinase activity.

We have reported that molybdate-stabilized cytosol prepared from L-cells cultured in the presence of [^{32}P]orthophosphate contains two phosphoproteins (a 90–92K and a 98–100K protein)[1] that elute from an affinity resin of deoxycorticosterone-derivatized agarose in a manner consistent with the predicted behavior of the glucocorticoid receptor (Housley & Pratt, 1983; Housley et al., 1985). We have recently shown that both phosphoproteins are adsorbed to protein A–Sepharose after incubation of ^{32}P-labeled L-cell cytosol with a monoclonal antibody against the glucocorticoid receptor (Housley et al., 1985). The 98K phosphoprotein was shown by covalent affinity labeling with [^{3}H]dexamethasone 21-mesylate to contain the steroid binding site. The 90K phosphoprotein is a structurally different protein that does not bind steroid but copurifies with the uncleaved 98K form of the glucocorticoid receptor (Housley et al., 1985). The 90K non-steroid-binding phosphoprotein is the major Coomassie blue stained component in affinity column eluates and in protein A–Sepharose pellets obtained after incubation of L-cell cytosol with a monoclonal antibody that reacts specifically with the 98K steroid-binding phosphoprotein in Western blots (Housley et al., 1985).

A similar 90K non-steroid-binding phosphoprotein has been shown to copurify with the molybdate-stabilized avian progesterone receptor (Dougherty et al., 1982, 1984). Radanyi et al. (1983) have prepared a monoclonal antibody against the 90K non-steroid-binding protein of chicken oviduct. This antibody does not react with either the purified A or B progesterone-binding proteins (Renoir et al., 1984), but it does form an immune complex with the molybdate-stabilized 8S forms of progesterone, androgen, estrogen, and glucocorticoid receptors in chick oviduct cytosol (Joab et al., 1984). These observations support a general model in which the molybdate-stabilized, untransformed state of glucocorticoid, progesterone, and possibly other steroid receptors contains a 90K non-steroid-binding phosphoprotein.

It is now well-defined that several peptide hormone receptors are protein kinases that are capable of autophosphorylation [see Carter-Su & Pratt (1984) for a review], and five recent communications have suggested that components of progesterone or glucocorticoid receptors may possess protein kinase activity. Garcia et al. (1983) obtained extensive purification of the 90K non-steroid-binding and the 110K progesterone-

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[1] Abbreviations: dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; ATP, adenosine 5′-triphosphate; IgG, immunoglobulin G. 90K, 98K, etc. refer to bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with M_r of 90 000, 98 000, etc. The 90–92K non-steroid-binding phosphoprotein migrates faster than the phosphorylase *b* marker on SDS–polyacrylamide gel electrophoresis (Housley & Pratt, 1983; Housley et al., 1985) and will be referred to here as a 90K band, which is consistent with the molecular weight assigned by Dougherty et al. (1982, 1984) to a similar protein that is associated with the chicken oviduct progesterone receptor. The 98K steroid-binding protein migrates slightly slower than the 97K phosphorylase *b* marker, and for both mouse and rat receptors, it has been assigned an M_r of 98–100K in reports from various laboratories (Housley et al., 1985; Reichman et al., 1984; Gehring, 1983). The most accurate assignment of molecular weight for the glucocorticoid-binding protein is probably that of Reichman et al. (1984), who found that the [^{3}H]dexamethasone 21-mesylate labeled rat HTC cell receptor migrated slightly slower than the phosphorylase marker with an M_r of 98 600 ± 1500 (SD) as determined for 28 separate preparations.

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binding proteins of chick oviduct cytosol and demonstrated phosphorylation when the purified preparations were incubated with [γ-^{32}P]ATP and divalent cations. The 90K protein was phosphorylated in the presence of Ca^{2+}, but not of Mg^{2+}, whereas phosphorylation of the 110K progesterone-binding protein was observed only in the presence of Mg^{2+} and not in the presence of Ca^{2+}. Similarly, Kurl & Jacob (1984) have partially purified the glucocorticoid receptor from rat liver cytosol by steroid affinity and ion-exchange chromatography and shown that incubation with [γ-^{32}P]ATP and Mg^{2+} results in phosphorylation of a major Coomassie blue stained protein with an M_r of 90K.

These observations, combined with other recent reports that affinity-purified preparations of rat liver glucocorticoid receptor have protein kinase activity (Miller et al., 1984; Singh & Moudgil, 1984; Miller-Diener et al., 1985), have led to the speculation that steroid receptors have intrinsic protein kinase activity and that they may modulate gene transcription by phosphorylation of chromatin proteins (Garcia et al., 1983; Singh & Moudgil, 1984). In this report we use immunological methods to purify the L-cell glucocorticoid receptor and assess subsequent protein phosphorylation as it occurs in immune complexes adsorbed to protein A–Sepharose. We demonstrate that the 98K L-cell glucocorticoid receptor can be phosphorylated by an endogenous L-cell protein kinase, but we fail to confirm that either the 98K glucocorticoid binding protein or the 90K receptor-associated phosphoprotein has intrinsic protein kinase activity.

Materials and Methods

Materials. [6,7-^{3}H]Dexamethasone 21-mesylate[1] (48.9 Ci/mmol) and EN^{3}HANCE were obtained from New England Nuclear; [^{32}P]orthophosphate (carrier-free) and [γ-^{32}P]ATP (3 Ci/mmol) were from Amersham Corp. Normal nonimmune mouse IgG and molecular weight marker proteins were from Sigma Chemical Co.; protein A–Sepharose CL-4B was from Pharmacia.

Cell Culture and Cytosol Preparation. L929 mouse fibroblasts were grown in monolayer culture and harvested in log phase as previously described (Housley & Pratt, 1983). In the experiments shown in Figure 2 (lane 1) and Figure 3, cells in log phase of growth were washed with phosphate-free medium containing 10% dialyzed calf serum and incubated in this medium containing [^{32}P]orthophosphate (20 μCi/mL) at 37 °C for 18 h. After harvesting, cells were washed in iced Earle's saline, suspended in 1.5 volumes of 10 mM Hepes, 0.4 mM EDTA, pH 7.35, at 4 °C, and ruptured by Dounce homogenization. The homogenate was centrifuged first at 27000*g* for 30 min, and the mixture was centrifuged at 105000*g* for 1 h. The supernatant fluid is referred to as cytosol, and it was stored at −70 °C.

Incubation with Antibodies and Adsorption to Protein A–Sepharose. Two antibody preparations were used in this work. Monoclonal antibody (GR49) was prepared by Westphal et al. (1982) with the highly purified, transformed form of the rat liver glucocorticoid receptor as antigen (Wrange et al., 1979). GR49 monoclonal antibody cross-reacts extensively (96%) with the mouse glucocorticoid receptor (1982), and we have described in detail its reaction with the [^{3}H]dexamethasone 21-mesylate labeled and ^{32}P-labeled L-cell glucocorticoid receptor in a recent publication (Housley et al., 1985). The polyclonal antiserum was raised in rabbits by using glucocorticoid receptor partially purified from molybdate-stabilized L-cell cytosol by affinity chromatography on deoxycorticosterone-derivatized agarose. The preparation of the polyclonal antiserum will be described in detail in another report from this laboratory (Tienrungroj, Sanchez, Housley, and Pratt, unpublished results).

The following protocol was used in all of the experiments. Aliquots of 200–400 μL of unlabeled or, in the case of Figures 2 and 3, ^{32}P-labeled or [^{3}H]dexamethasone 21-mesylate labeled L-cell cytosol were mixed with an equal volume of TES buffer (10 mM TES, 50 mM NaCl, 10% glycerol, 4 mM EDTA, 20 mM DTT, with or without 20 mM sodium molybdate, pH 7.6 at 0–4 °C). Where noted in the figure legend, the mixture was incubated for 2–4 h at 0 °C with 50 nM nonradioactive triamcinolone acetonide or dexamethasone to form the steroid-bound receptor complex. Monoclonal antibody or nonimmune mouse IgG (at the same IgG concentration as that of the GR49 preparation), or rabbit antireceptor antiserum, or preimmune rabbit serum was added to the cytosol mixture at 2.5% or 5% of final volume, and the mixtures were incubated overnight on ice. Each mixture was added to a 150-μL bed volume of protein A–Sepharose preequilibrated in TES buffer and mixed by rotation at 4 °C for 2 h. Protein A–Sepharose was then pelleted by centrifugation, the supernatant was removed, and the pellet was washed 3 times by suspension in 1 mL of TES buffer. The pellet was washed sequentially with 3 × 1 mL volumes of TES buffer with 0.4 M NaCl, TES buffer with 0.4 M NaCl and 0.2% Triton X-100, and finally TES buffer without EDTA.

Labeling with [γ-^{32}P]ATP and Gel Electrophoresis. The washed protein A–Sepharose pellet was incubated for 30 min at 25 °C with 120 μM [γ-^{32}]ATP and 4 mM $MgCl_2$ or 4 mM $CaCl_2$ as indicated. At the end of the incubation, the mixture was centrifuged and washed 3 times with 1 mL of TES buffer and 2 times with 75 mM Tris buffer, pH 6.8, with 10% glycerol. The washed pellet was suspended at 4% SDS sample buffer and boiled for 4 min, and the eluted proteins were resolved by electrophoresis on 7% SDS–polyacrylamide gels with molecular weight standards as previously described (Housley et al., 1985).

Results and Discussion

If either the glucocorticoid receptor itself or the 90K receptor-associated phosphoprotein possesses intrinsic protein kinase activity or if a protein kinase copurifies with the molybdate-stabilized complex, the immunoabsorbed complex, like the complex purified by affinity chromatography (Kurl & Jacob, 1984; Miller et al., 1984; Miller-Diener, 1985), might be expected to exhibit protein kinase activity. The presence of protein kinase activity could be reflected by phosphorylation of the 90K or 98K protein or by phosphorylation of immunoglobulins and other proteins present in the material bound to the protein A–Sepharose pellet. Phosphorylation that is due to the presence of the glucocorticoid receptor or a receptor-associated kinase should occur in a manner that is immune-specific.

In the experiment shown in Figure 1, unlabeled L-cell cytosol containing sodium molybdate was incubated with rabbit antiserum raised against the mouse glucocorticoid receptor or with preimmune serum adsorbed to protein A–Sepharose, and the adsorbed material was incubated with [γ-^{32}P]ATP and magnesium. It can be seen from lanes 1 and 2 that material adsorbed in the presence of polyclonal antibody contains major ^{32}P-labeled bands at 116K, 98K, and about 37K that are not phosphorylated in the protein A–Sepharose pellet of cytosol incubated with preimmune serum. It is clear from lanes 4, 5, and 6 that one or more cytosol proteins that bind to protein A–Sepharose in the absence of immunoglobulin have detectable protein kinase activity but protein A–Sepharose incubated with immune or preimmune serum alone does not.

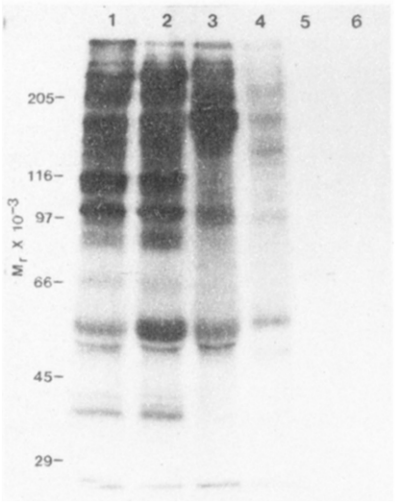

FIGURE 1: Phosphorylation of proteins after L-cell cytosol has been incubated with rabbit antiserum to the murine glucocorticoid receptor and absorbed to protein A–Sepharose. Aliquots (300 μL) of L-cell cytosol were diluted with an equal volume of TES buffer containing 20 mM sodium molybdate and incubated with 60 μL of rabbit antiserum (lanes 1 and 2), preimmune serum (lane 3), or no serum (lane 4) and adsorbed to protein A–Sepharose. Lanes 5 and 6 represent immune and preimmune sera, respectively, adsorbed to protein A–Sepharose in the absence of L-cell cytosol. The protein A–Sepharose pellets were washed, incubated with [γ-^{32}P]ATP in TES buffer with 4 mM $MgCl_2$ and 20 mM sodium molybdate, and analyzed by electrophoresis on 7% SDS–polyacrylamide gels and autoradiography as described under Materials and Methods. Lanes 1 and 2 represent two different preparations of L-cell cytosol incubated with immune serum.

In the experiment shown in Figure 2, the L-cell glucocorticoid receptor was labeled with [^{3}H]dexamethasone 21-mesylate and immunoadsorbed to protein A–Sepharose with the rabbit antiserum (lanes 2 and 3). The same preparation of L-cell cytosol was also immunoadsorbed and incubated with [γ-^{32}P]ATP and Mg^{2+} (lane 4). A sample of cytosol prepared from L-cells that had been cultured for 18 h in the presence of [^{32}P]orthophosphate was immunoadsorbed in the same manner (lane 1) to permit comparison of the proteins phosphorylated in the intact cell with proteins phosphorylated after addition of [γ-^{32}P]ATP to the immunoadsorbed material in the protein A–Sepharose pellet. It is clear that two proteins (one just above the phosphorylase *b* marker at 98K and one below the marker at 90K) that are phosphorylated in the intact cell are immunoadsorbed by the antiserum. It is also clear that there is a line of continuity between the [^{3}H]dexamethasone 21-mesylate labeled band in lane 3, the upper band in the doublet recovered from ^{32}P-labeled cytosol in lane 1 and the 98K band phosphorylated in the immunoprecipitate (lane 4). This provides strong evidence that incubation of the immunoadsorbed material with [γ-^{32}P]ATP is leading to phosphorylation of the glucocorticoid receptor. It is clear from the experiment shown in Figure 3 that the rabbit antiserum to the L-cell receptor behaves just like the monoclonal antibody in that it causes immune-specific adsorption to protein A–Sepharose of both the 90K and 98K proteins labeled with ^{32}P in intact cells.

Miller-Diener et al. (1985) have reported that phosphorylation of affinity-purified rat liver glucocorticoid receptor is steroid-dependent and is inhibited by molybdate. Accordingly, in the experiment shown in Figure 4, we asked whether the presence of either molybdate or steroid in the cytosol affects the profile of proteins that are phosphorylated after addition of [γ-^{32}P]ATP to the immunoadsorbed material. The 98K band is phosphorylated regardless of whether or not receptors

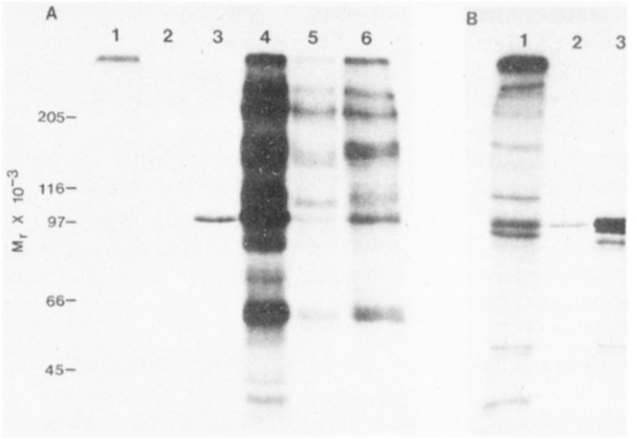

FIGURE 2: Comparison of [^{3}H]dexamethasone 21-mesylate labeled receptor with immunoadsorbed cytosol proteins from ^{32}P-labeled L-cells and proteins that are phosphorylated by incubating immunoadsorbed L-cell proteins with [γ-^{32}P]ATP. Aliquots (300 μL) of cytosol incubated with [^{3}H]dexamethasone 21-mesylate or cytosol prepared from ^{32}P-labeled L-cells or unlabeled L-cell cytosol (150 μL) were mixed with an equal volume of TES buffer containing 10 mM sodium molybdate and 10 mM dithiothreitol, incubated for 16 h in ice with rabbit antireceptor antiserum at 5% of final volume, and adsorbed to protein A–Sepharose. The protein A–Sepharose pellets were washed sequentially with TES buffer and TES buffer containing 400 mM NaCl and 0.2% Triton X-100. The immunoadsorbed pellets prepared from unlabeled cytosol were washed additionally with TES buffer without EDTA prior to incubation with [γ-^{32}P]ATP and $MgCl_2$ as described under Materials and Methods. All buffers contained 10 mM molybdate and dithiothreitol. Proteins were extracted from all pellets by boiling in SDS sample buffer, electrophoresed in a 7% polyacrylamide gel, and visualized by autoradiography. Panel A: lane 1, immunoadsorbed material from cytosol prepared from L-cells that were incubated with [^{32}P]orthophosphate; lane 2, cytosol incubated with [^{3}H]dexamethasone 21-mesylate in the presence of a 100-fold excess of competing nonradioactive dexamethasone; lane 3, cytosol incubated with [^{3}H]dexamethasone 21-mesylate alone; lane 4, immunoadsorbed pellet incubated for 30 min at 25 °C with [γ-^{32}P]ATP and Mg^{2+}; lane 5, same as lane 4 except incubated on ice; and lane 6, same as lane 4 except that a later, lower titer bleed of rabbit antiserum was used to adsorb receptor to protein A–Sepharose. Panel B: longer exposure of lanes 1, 2, and 3 from the same gel in panel A.

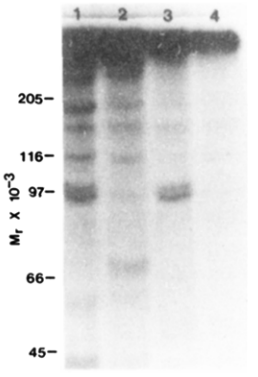

FIGURE 3: Comparison of phosphoproteins immunoadsorbed by rabbit antireceptor serum and GR49 monoclonal antibody. Aliquots (150 μL) of cytosol prepared from L-cells incubated with [^{32}P]orthophosphate were diluted with an equal volume of TES buffer containing 10 mM molybdate and dithiothreitol and incubated with 5% rabbit antiserum (lane 1), preimmune serum (lane 2), 2.5% GR49 monoclonal antibody against the receptor (lane 3), and 2.5% nonimmune mouse IgG (lane 4). All samples were adsorbed to protein A–Sepharose and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

are bound with steroid and whether or not molybdate is present. The 116K band is phosphorylated only when molybdate is present during the immunoadsorption procedure, and we have found that its phosphorylation is rather specific for

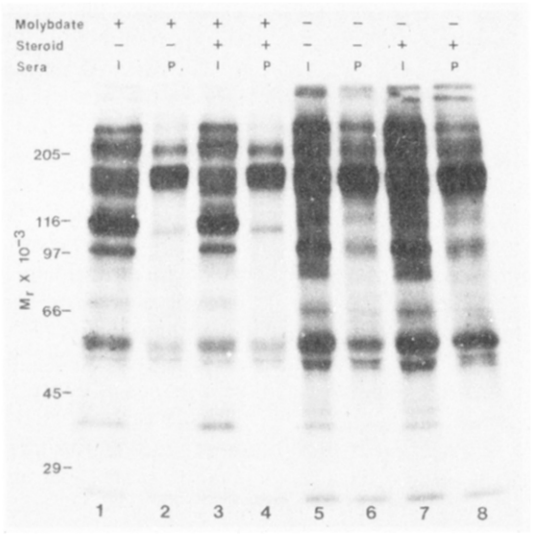

FIGURE 4: Effect of the presence of molybdate or steroid during reaction with rabbit antiserum on the pattern of proteins phosphorylated when the subsequent protein A–Sepharose pellet is incubated with [γ-^{32}P]ATP and magnesium. Aliquots (300 μL) of L-cell cytosol containing molybdate (20 mM) and/or dexamethasone (50 nM) were incubated with immune or preimmune rabbit serum, bound to protein A–Sepharose, and incubated with [γ-^{32}P]ATP and magnesium chloride as described under Materials and Methods. The presence (+) or absence (−) of molybdate or steroid is indicated above each gel lane, as is the use of immune (I) or preimmune (P) antiserum.

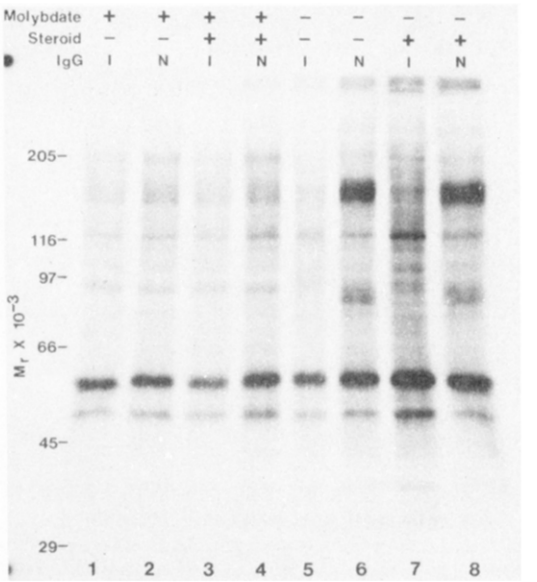

FIGURE 5: Phosphorylation pattern obtained in protein A–Sepharose pellets prepared after incubating L-cell cytosol with the monoclonal antibody against the glucocorticoid receptor. Aliquots (300 μL) of L-cell cytosol were incubated with GR49 monoclonal antibody or nonimmune mouse IgG, adsorbed to protein A–Sepharose, incubated with [γ-^{32}P]ATP and Mg^{2+}, and analyzed by SDS–polyacrylamide gel electrophoresis. The presence (+) or absence (−) of molybdate or steroid is indicated above each gel lane, as is the use of monoclonal antibody (I) or nonimmune mouse IgG (N).

immunoadsorbed pellets prepared with the earliest and highest titer bleed of antiserum. Later batches of antiserum show little phosphorylation of the 116K band (e.g., Figure 2, lane 6, and Figure 7, lanes 7–9). At this time, we do not think that the 116K protein is associated specifically with the molybdate-stabilized receptor.

If phosphorylation of the 98K protein in the immunoadsorbed protein A–Sepharose pellet is due to a kinase activity that is intrinsic to the receptor itself or to a receptor-associated protein kinase, then the 98K protein should be phosphorylated when [γ-^{32}P]ATP is added to protein A–Sepharose pellets prepared with GR49 monoclonal antibody. As shown in Figure 5, there is no phosphorylation of a 98K protein when the receptor has been immunoadsorbed with the monoclonal antibody. Indeed, there is no immune-specific phosphorylation of IgG or other proteins.

The observations presented to this point could be explained if rabbit antiserum caused the nonspecific adsorption of an L-cell protein kinase that phosphorylates the 98K glucocorticoid receptor. That this is the case is demonstrated by the mixed antibody experiment shown in Figure 6. As can be seen from lane 2, the protein A–Sepharose pellet obtained from molybdate-stabilized cytosol incubated with preimmune rabbit serum has protein kinase activity, but no 98K protein is phosphorylated. The protein A–Sepharose pellet derived from molybdate-stabilized cytosol incubated with both preimmune rabbit serum as a source of kinase activity and monoclonal antibody to provide the receptor and its associated proteins (lane 5) contains a prominent phosphorylated band at 98K. There is no phosphorylation of a 116K band in lane 5, which supports our impression that this protein is not associated with the receptor.

It is interesting that none of the various immunoadsorbed samples has yielded significant phosphorylation of the 90K receptor-associated protein, although it is clearly present by Coomassie blue stain in samples that have been incubated with either the rabbit antiserum or the monoclonal antibody. Garcia et al. (1983) observed phosphorylation of the 90K receptor-

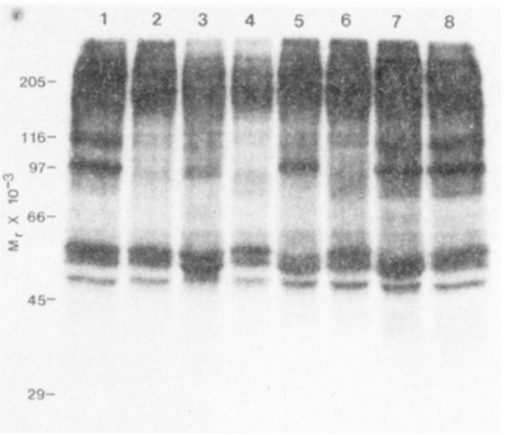

FIGURE 6: Phosphorylation of 98K protein after adsorption to protein A–Sepharose with the monoclonal antireceptor antibody. Aliquots (200 μL) of L-cell cytosol were incubated with 4 μL of rabbit immune or preimmune serum in the presence or absence of 20 μL of monoclonal antibody or nonimmune IgG. The rabbit immune serum was from a later, lower titer bleed. All samples were bound to protein A–Sepharose and incubated with [γ-^{32}P]ATP and magnesium chloride as described under Materials and Methods. Cytosol was incubated with the following: lane 1, rabbit immune serum alone; lane 2, preimmune serum alone; lane 3, monoclonal antibody alone; lane 4, nonimmune mouse IgG alone; lane 5, monoclonal antibody plus preimmune rabbit serum; lane 6, nonimmune mouse IgG plus preimmune rabbit serum; lane 7, monoclonal antibody and immune rabbit serum; lane 8, nonimmune mouse IgG and immune rabbit serum.

associated protein from chicken only when Ca^{2+} (without calmodulin) was present instead of Mg^{2+}. Miller-Diener et al. (1985) have reported that phosphorylation of the affinity-purified rat liver glucocorticoid receptor is stimulated by calcium and that the receptor must be both bound by hormone and transformed to the DNA-binding state in order for phosphorylation to occur. In the experiment shown in Figure 7, unbound receptor, steroid-bound but untransformed receptor, and steroid-bound transformed receptors were immunoadsorbed in the presence or absence of molybdate and in-

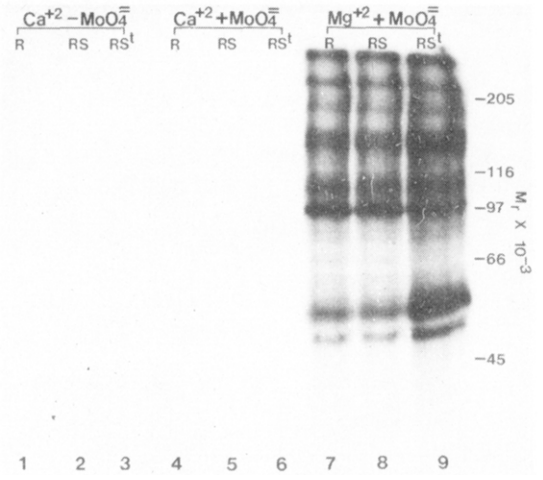

FIGURE 7: Effect of Ca^{2+} vs. Mg^{2+} on phosphorylation of 98K protein in immunoadsorbed pellets prepared with unbound receptors, steroid-bound untransformed receptor, and the transformed steroid–receptor complex. Aliquots (150 μL) of L-cell cytosol containing unbound receptors (R), receptors bound with dexamethasone (100 nM) but not transformed (RS), or temperature-transformed (25 °C, 1 h) steroid–receptor complexes (RS^t) were made 10 mM with respect to sodium molybdate (samples 4–9) or not (samples 1–3) as indicated at the top of the gel lanes. All samples were immunoadsorbed to protein A–Sepharose with 5% rabbit antiserum, incubated with [γ-^{32}P]ATP in the presence of 4 mM $CaCl_2$ (lanes 1–6) or 4 mM $MgCl_2$ (lanes 7–9) as indicated, and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

cubated with [γ-^{32}P]ATP in the presence of either Ca^{2+} or Mg^{2+}. Essentially no phosphorylation occurs if Ca^{2+} is present alone, and even when the gel is exposed for many days, no phosphorylation of the 90K or 98K bands is observed. It is clear from lanes 7–9 that the receptor is phosphorylated in the presence of Mg^{2+} regardless of whether it is unbound or bound with hormone or whether or not it was undergone previous transformation. The addition of Ca^{2+} and Mg^{2+} together yields the same pattern of phosphorylation that is seen with Mg^{2+} alone (data not shown). Thus, using an immunological technique rather than affinity chromatography for partially purifying the L-cell glucocorticoid receptor, we fail to confirm that either the receptor or the 90K receptor-associated protein has intrinsic protein kinase activity.

These are several possible explanations for the discrepancy between our observations and those of others. Although many protein kinases (including hormone receptors that are protein kinases)[1] undergo autophosphorylation when they are present in immune complexes, it is possible that the immunoadsorbed glucocorticoid receptor is blocked with regard to autophosphorylating activity. All of the published evidence for an association between protein kinase activity and the glucocorticoid receptor has been obtained with affinity-purified receptors from rat liver. Our work has been performed with receptors from cultured mouse fibroblasts, and it is possible that the source of the receptor is an important factor in demonstrating autophosphorylation. One difference could be that the mouse L-cell receptor is highly phosphorylated and it might be necessary to dephosphorylate the receptor before autophosphorylation would be evident. It has been shown, for example, that extensively purified calf uterine estrogen receptor is phosphorylated by a cytoplasmic tyrosine kinase only after it has been preincubated with a protein phosphatase to open up the phosphorylation sites (Migliaccio et al., 1984). It is interesting to note that in these experiments the purified estrogen receptor itself has not been reported to have intrinsic protein kinase activity. Addition of a partially purified cytoplasmic protein kinase is necesary for receptor phosphorylation.

In the work reported herein, we have shown that the immunoadsorbed glucocorticoid receptor is phosphorylated by an endogenous protein kinase from L-cell cytosol. The observation that receptor that has been immunoadsorbed to protein A–Sepharose with the monoclonal antibody is not phosphorylated (Figure 5) but can serve as a substrate for phosphorylation by an endogenous protein kinase (Figure 6, lane 5) is particularly useful. The receptor–monoclonal antibody immune complex can be used as a substrate for phosphorylation by L-cell cytosol that has been fractionated by conventional protein purification procedures, thus providing us with an assay system that should ultimately permit purification and characterization of the enzyme(s).

Registry No. Protein kinase, 9026-43-1; dexamethasone, 50-02-2.

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