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A Protein Kinase Copurified with Chick Oviduct Progesterone Receptor†

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ABSTRACT: A magnesium-dependent protein kinase activity was copurified with both the molybdate-stabilized 8S form of the chick oviduct progesterone receptor (PR) and its B subunit. In each case, purification was performed by hormonal affinity chromatography followed by ion-exchange chromatography. The K_m(app) values of the phosphorylation reaction for [γ-^{32}P]ATP and calf thymus histones were $\sim 1.3 \times 10^{-5}$ M and $\sim 1.6 \times 10^{-5}$ M, respectively, and only phosphorylated serine residues were found in protein substrates, including PR B subunit. Physicochemical parameters of the enzyme [p$I \sim 5.3$, Stokes radius ~ 7.2 nm, sedimentation coefficient ($s_{20,w}$) ~ 5.6 S, and $M_r \sim 200\,000$] were compared to those of purified forms of PR (B subunit, p$I \sim 5.3$, Stokes radius ~ 6.1 nm, and $M_r \sim 110\,000$; 8S form, Stokes radius ~ 7.7 nm and $M_r \sim 240\,000$). The results suggest that most of the protein kinase activity copurified with both oligomeric and monomeric forms of PR belongs to an enzyme distinct from currently known receptor components. Its physiological significance remains unknown.

Steroid receptors are phosphoproteins (Housley & Pratt, 1983; Grandics et al., 1984; Dougherty et al., 1982, 1984). Steroid receptor phosphorylation may be involved in the modulation of hormone binding (Migliaccio et al., 1982), in the change of receptor conformation (Housley et al., 1982), and in receptor transformation or activation (Barnett et al., 1980; Logeat et al., 1985; Garcia et al., 1986). In vitro phosphorylation of purified chick oviduct progesterone receptor (PR) by endogenous (Garcia et al., 1983) or exogenous (Weigel et al., 1981; Ghosh-Dastidar et al., 1984) protein kinases has been reported. However, the specific enzymes regulating the phosphorylation of steroid receptors in vivo are still unknown.

We have previously reported the copurification of two distinct protein kinase activities associated with purified chick oviduct PR preparations, corresponding to the B subunit of M_r 110 000 and to the 8S PR complex, respectively (Garcia et al., 1983). The B subunit preparation displayed Mg^{2+}-dependent activity and phosphorylated contaminants and histone substrates, as well as the B subunit itself. No effort was made to separate protein kinase activity from the PR B subunit. In the 8S PR extract, another "protein kinase activity", observed only in the presence of a high concentration of calcium, was described. It labeled specifically the hsp 90 component (Catelli et al., 1985), as observed after electrophoresis; however, subsequently we found that this labeling

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did not correspond to phosphate incorporation into amino acids.

The presence of protein kinase activities in purified preparations of glucocorticosteroid receptor was also reported by several groups, which suggested that it could possess intrinsic protein kinase activity (Kurl & Jacob, 1984; Singh & Moudgil, 1985; Schmidt & Litwak, 1985). However, recently, Sanchez and Pratt (1986) found that the L-cell glucocorticosteroid receptor is not a protein kinase. In the present work, we have restudied the Mg^{2+}-dependent protein kinase activity initially detected in the B subunit preparations. We know that this preparation contains the PR in a highly preserved state (Renoir et al., 1984b), not only binding the steroid, but also binding DNA characteristically (Von der Ahe et al., 1986). We found also in the purified 8S preparation (Renoir et al., 1984a) a protein kinase activity indistinguishable from that found in the purified B subunit preparations. This Mg^{2+}-dependent protein kinase activity is for the most part distinct from the presently known components of the PR.

Materials and Methods

Chemicals. [2,4,6,7-^{3}H]Progesterone ([^{3}H]P) (80–110 Ci/mmol) and [γ-^{32}P]ATP (0.5–3 Ci/mmol) were from the Radiochemical Centre (Amersham, Buckinghamshire, U.K.). Nonradioactive cortisol and progesterone were >95% pure and were obtained from Roussel-Uclaf (Romainville, France). DEAE-Sephacel was obtained from Pharmacia (Uppsala, Sweden). All chemicals used were reagent grade from usual sources except when noted.

Buffers. Buffer A was 10 mM sodium phosphate, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 10% (w/v) glycerol, and 12 mM 1α-thioglycerol, pH 7.4 at 25 °C. Buffer B was buffer A + 20 mM Na_2MoO_4. Buffer C was 10 mM KH_2PO_4 and 12.5% methanol (v/v), pH 3 at 25 °C.

Purification of Chick Oviduct Progesterone Receptor. The details of the purification procedure have been published (Renoir et al., 1982, 1984a,b). Briefly, the cytosol fraction of the estrogen-stimulated chick oviduct tissue (300–400 mL) was prepared either in buffer A, and treated by incubation with 0.3 M KCl for 2 h at 0–4 °C to obtain the "4S" form, or in buffer B, containing sodium molybdate, to obtain the "8S" form. The cytosol was then loaded on the affinity chromatography gel (NADAc-Sepharose 4B), and after appropriate washing, the receptor was eluted with 1 μM [^{3}H]P (5–20 Ci/mmol). The affinity gel eluates were loaded on a DEAE-Sephacel column, and the elution was carried out either with a 0.08 M KCl elution followed by a linear 0.08–0.5 M KCl gradient (B subunit purification) or with a linear 0–0.5 M KCl gradient in buffer B to obtain the molybdate-stabilized 8S PR forms.

The radioactivity of receptor-bound [^{3}H]progesterone was determined in aliquots mixed with 7 mL of Scintimix (0.4% in toluene) by counting in a Packard liquid scintillation spectrometer.

Determination of Protein Kinase Activity. Samples (40–90 μL) in buffer A or B were incubated in the presence of 6 mM $MgCl_2$, 20–50 μg of histones (calf thymus, HL4, Worthington), and 30 μM of [γ-^{32}P]ATP (0.5–3 Ci/mmol) in a final volume of 100 μL at 25 °C for 30 min. We have verified that at this point the incorporation of ^{32}P into histones was linear as a function of time. The reaction was stopped by adding concentrated electrophoresis sample buffer (Laemmli, 1970) and by boiling the samples for 1–2 min. After electrophoresis, determination of the amount of incorporated ^{32}P was accomplished by cutting out the appropriate regions of the stained and dried gel and directly counting the Cerenkov radiation.

Analytical Electrofocusing. The electrofocusing was performed at 2–4 °C by using a 110-mL column (LKB) in which a pH 5–8 gradient was preestablished overnight with 0.1% ampholines. One milliliter of purified PR B subunit containing the protein kinase activity was introduced into the gradient and focused during 8 h at 800 V. After focusing, 1-mL fractions were collected (flow rate of ~1.5 mL/min), and aliquots were used to determine the receptor and the protein kinase profiles.

Analytical Gel Filtration [High-Performance Liquid Chromatography (HPLC)]. Samples (100 μL) were analyzed on a TSK G 3000 column (LKB), equilibrated at 4 °C with buffer B, using a HPLC system (liquid chromatography 1082B, Hewlett-Packard) (Pavlik et al., 1982). Flow rate was 1 mL/min, and calibration was carried out with the following proteins: thyroglobulin, R_s = 8.6 nm; cytosol 8S PR, R_s = 7.7 nm; β-galactosidase, R_s = 6.9 nm; catalase, R_s = 5.2 nm; human transferrin, R_s = 3.6 nm. Void volume (V_0) was determined with Dextran blue and total volume (V_t) with tryptophan. Standard curves were plotted according to Porath (1963): $K_D^{1/3}$ against R_s, where $K_D = (V_e - V_0)/(V_t - V_0)$ and V_e is the elution volume of the protein studied.

Ultracentrifugation Analysis. Sucrose gradients (5–20% w/v) were prepared in buffer A, and samples (300 μL) containing the purified protein kinase were layered on the top of the preformed gradients. A tube containing the marker enzymes, horseradish peroxidase ($s_{20,w}$ = 3.6 S, P-Sigma type VI) and fungal glucose oxidase ($s_{20,w}$ = 7.9 S, Boehringer Mannheim, Mannheim, FRG), was run in parallel. The tubes were centrifuged in a SW 60 rotor (Beckman) at 0–2 °C at 250000g for 16 h. Fractions were collected, and sedimentation coefficients were determined according to Martin and Ames (1961).

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Slab Gel Electrophoresis. Electrophoresis was performed according to Laemmli (1970) using 7.5–15% gradients of polyacrylamide slab gels containing 0.1% SDS. Molecular weight values were determined by the method of Weber and Osborn (1969) by reference to a calibration curve obtained with the following standard proteins: myosin, M_r 205 000; β-galactosidase, M_r 130 000; phosphorylase B, M_r 97 000; bovine albumin, M_r 66 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 29 000; (HMWSM kit SDS-6H, Sigma) plus cytochrome c, M_r 12 600.

Phosphoamino Acid Analysis. Phosphorylation of a mixture of calf thymus histones or of purified PR B subunit was performed in a 0.1–0.5-mL reaction volume containing 6 mM $MgCl_2$ and 30 μM [γ-^{32}P]ATP. Samples were incubated at 25 °C for 30 min, and the reaction was stopped by the addition of 10% trichloroacetic acid (TCA) and cold ATP (1 mM). Samples were kept at 0 °C for 30 min, and the precipitates were washed twice with 1 mL of 10% TCA containing 1 mM ATP. The phosphorylated histones were then hydrolyzed in 6 N HCl at 110 °C for 90 min, lyophilized, and resuspended in buffer C containing the phosphoamino acid markers (phosphothreonine, 0.5 mM; phosphoserine, 0.5 mM; phosphotyrosine, 25 μM, Sigma). The phosphoamino acids were separated on a Partisil-10 SAX anion-exchange resin (Whatman), equilibrated with buffer C, by using an HPLC system (Yang et al., 1982). The elution was performed at a flow rate of 1 mL/min, and the markers were detected by spectrophotometry at 260 nm (sensitivity: 8×10^{-4}/cm^2). Fractions of 0.5 mL were collected and counted for Cerenkov radiation.

Results

DEAE-Sephacel Chromatography and Electrofocusing.

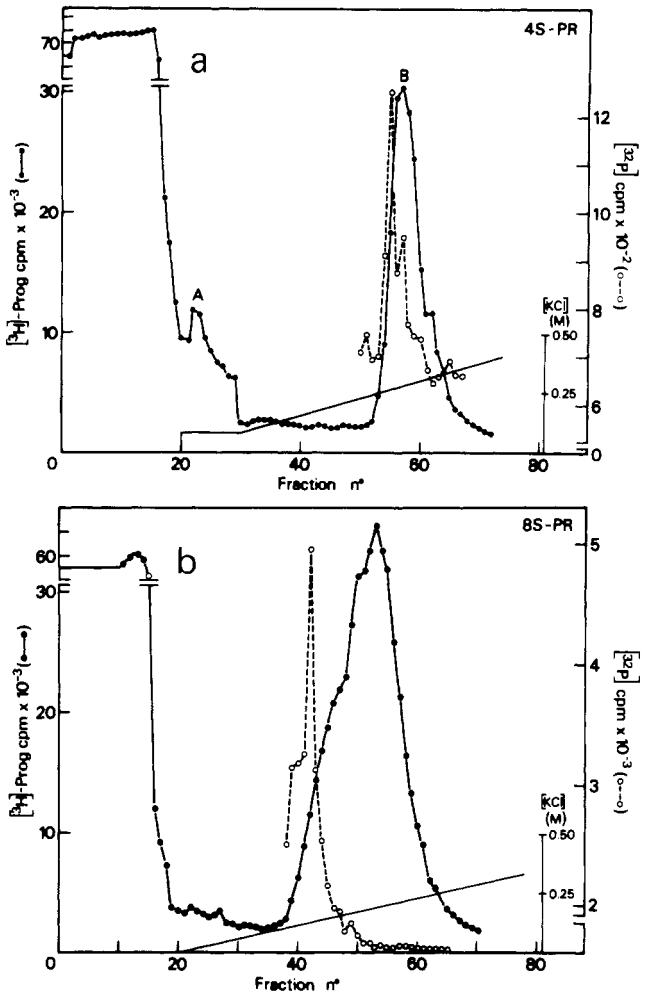

FIGURE 1: DEAE-Sephacel chromatography of purified PR preparations containing the protein kinase activity. The affinity chromatography eluates of the 0.3 M KCl treated cytosol (a) or of the 20 mM molybdate prepared cytosol (b) were analyzed on a DEAE-Sephacel column (see Materials and Methods). Fractions (1.2 mL) were collected, 30-μL portions were assayed for radioactivity (●), and 40-μL portions were used for the protein kinase assay (○).

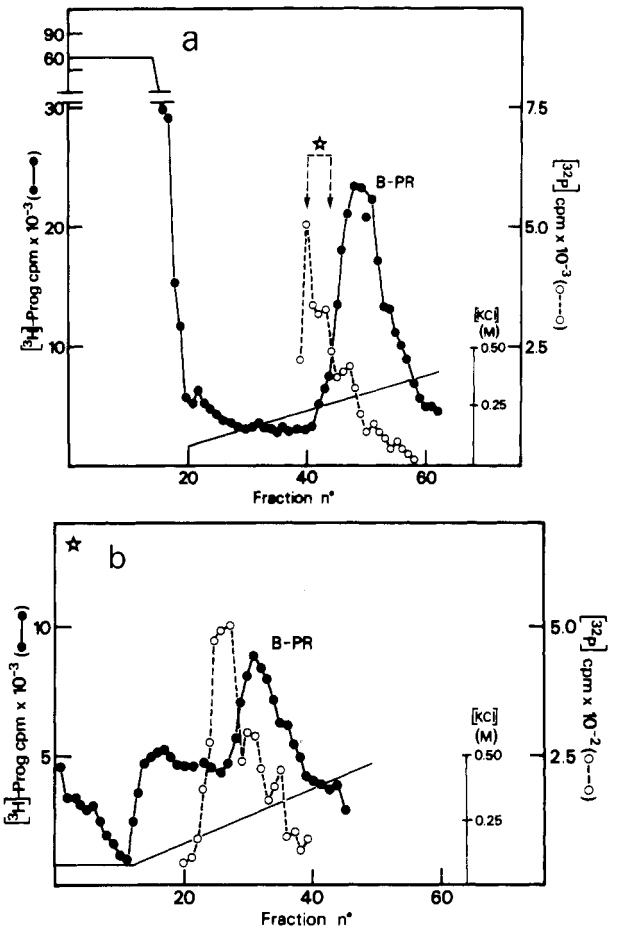

FIGURE 2: Two-step DEAE-Sephacel chromatography of purified B subunit containing the protein kinase activity. (a) The affinity chromatography eluate of a 0.3 M KCl treated cytosol was analyzed on a DEAE-Sephacel column, and fractions were collected. Thirty- and forty-microliter portions were respectively assayed for radioactivity (●) and for protein kinase activity (○). (b) The fractions containing both activities (☆) were pooled, rechromatographed in the same system, and assayed for radioactivity (●) and for protein kinase activity (○).

When purification started from 0.3 M KCl treated cytosol, the affinity column eluate chromatographed on DEAE-Sephacel resin gave a main peak of receptor-bound radioactivity at ~0.25 M KCl, corresponding to the PR B subunit (Renoir et al., 1984b). When aliquots of the DEAE-Sephacel fractions were tested for protein kinase activity, a single peak eluting at ~0.2 M KCl was observed (Figure 1a). No protein kinase activity was detected in the fractions containing the PR A subunit (data not shown).

When the purification started from a 20 mM molybdate containing cytosol, the DEAE-Sephacel chromatography gave a broad peak of receptor-bound radioactivity corresponding to 8S PR eluted at ~0.15 M KCl (Renoir et al., (1982, 1984a). In this case, a peak of protein kinase activity was also detected, but it was eluted at ~0.10 M KCl (Figure 1b).

To compare the enzymatic activities present in these two distinct PR preparations, DEAE-Sephacel fractions containing the receptor-bound radioactivity of an 8S PR peak and the copurified protein kinase (obtained as reported in Figure 1b) were pooled, diluted to decrease the ionic strength, and loaded on a second DEAE-Sephacel column. Under these conditions, the 8S receptor was transformed to 4S PR by intensive washing of the gel with buffer A. Upon elution, the profiles of receptor-bound radioactivity and of protein kinase activity were identical with those obtained in Figure 1a. The different behavior of the enzyme in the two distinct conditions can be ascribed to the presence of molybdate in the case of the 8S PR preparations.

In order to determine whether the receptor-bound radioactivity coeluting with the kinase activity in the KCl gradient represented an enzymatically active isoform of the PR B subunit, a second DEAE-Sephacel chromatography of these fractions was performed (Figure 2a). The same elution pattern of receptor-bound radioactivity and of protein kinase activity was observed (Figure 2b), implying that the majority of kinase activity is distinct from the steroid binding activity. However, the rechromatography experiment suggests multiple forms of kinase activity. There is even a substantial amount that still coelutes with the B protein and thus remains of unknown significance.

Fractions eluted from DEAE-Sephacel columns during purification of the PR B subunit that contained the protein kinase activity were pooled and used in further experiments ($s_{20,w}$ and phosphoamino acid analysis) as a source of enriched enzyme. The isoelectric points of the purified B subunit and of the copurified protein kinase were compared and found close to each other in the pH region of about 5.3 (Figure 3).

Determination of Hydrodynamic Parameters. Purified preparations of PR B subunit and 8S PR, both containing the protein kinase activity, were analyzed by gel filtration (HPLC). In this system the Stokes radius of the enzyme was estimated

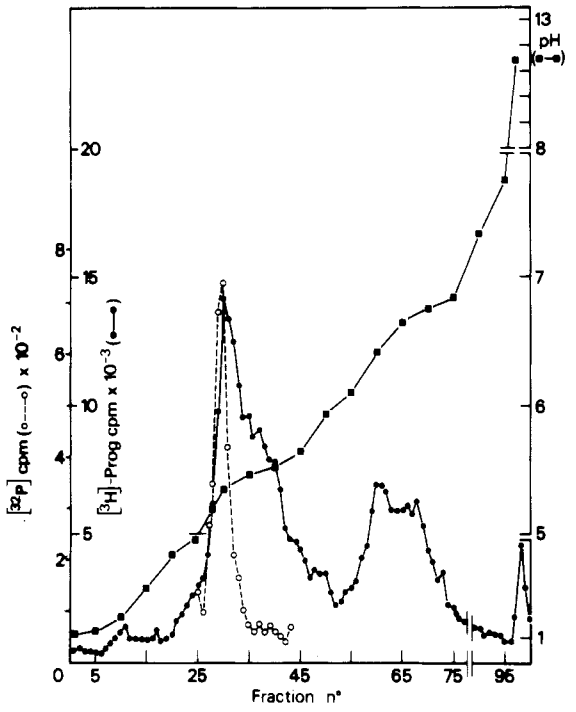

FIGURE 3: Purified B subunit and protein kinase electrofocusing. Purified PR B subunit (~10 μg) containing the protein kinase activity was focused in a pH 5–8 gradient (see Materials and Methods). Fractions (1 mL) were collected, 100-μL portions were assayed for radioactivity (●), 90-μL portions were used for the protein kinase assay (○), and the pH in each fraction (■) was measured.

at 7.2 nm, a value intermediate between those of purified 8S PR (R_s = 7.7 nm) and PR B subunit (R_s = 6.1 nm) analyzed under the same conditions (Figure 4).

The sedimentation coefficient of the enzyme was determined by using enriched protein kinase preparations obtained from DEAE-Sephacel columns as described. The enzyme migration corresponded to a sedimentation coefficient of ~6.5 S (Figure 5). This value was clearly distinct from those found in our laboratory for the nontransformed PR ($s_{20,w}$ ~ 8 S; Wolfson et al., 1980) and for the purified PR B subunit ($s_{20,w}$ ~ 4 S; Renoir et al., 1984b).

From these determinations, the molecular weight of the kinase was estimated according to Siegel and Monty (1966) and Sherman et al. (1983): $M_r = S_{20,w}$ (S) × R (Å) × 422.6 ~ 200 000.

Kinetic Parameters of the Kinase Activity. Protein kinase preparations obtained by DEAE-Sephacel chromatography during PR purification were stored for several weeks at −20 °C without significant loss of activity. The kinase required >5 mM Mg^{2+} in order to activate the transfer of phosphate from ATP to histone substrate. Magnesium ions could not be replaced by calcium, and the enzymatic activity was not enhanced by addition of cAMP or phospholipids. At 15 °C, the phosphorylation reaction was linear for at least 50 min. The apparent K_m [K_m(app)] of the phosphorylation reaction with respect to ATP was ~1.3 × 10^{-5} M when the enzyme copurified with a PR B subunit preparation was used and ~1.5 × 10^{-5} M when the enzyme came from an 8S PR preparation. The K_m(app) value was ~1.6 × 10^{-5} M when a histone mixture was used and an average molecular weight of 15 000 was assumed or ~1.1 × 10^{-5} M when casein was used. The double-reciprocal plots were always linear and consistent with the possibility that a single enzyme was responsible for the ^{32}P incorporation into protein substrate.

Phosphoamino Acid Analysis. Whether the substrate for phosphorylation was histones, casein, or purified PR B subunit,

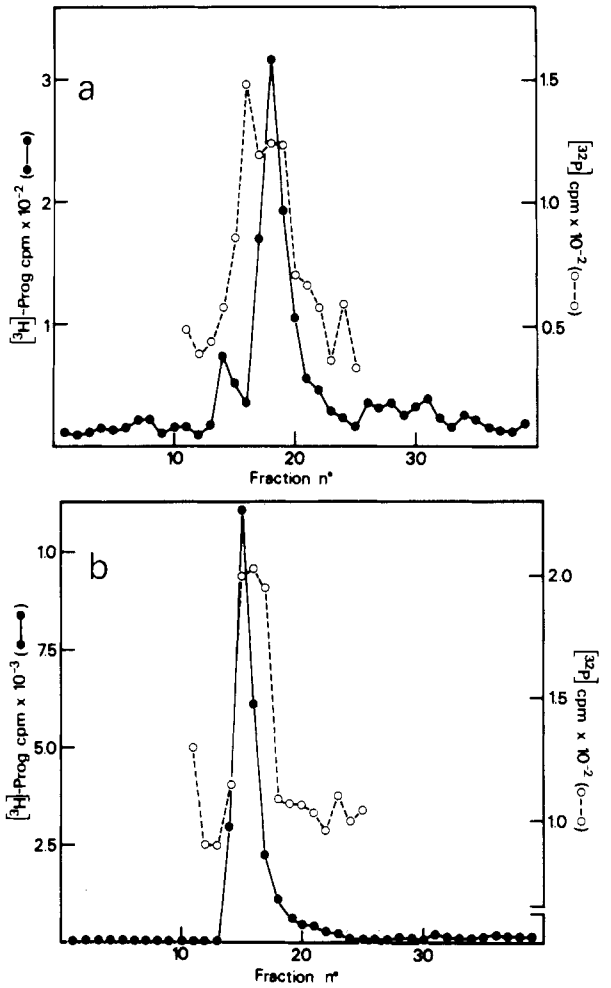

FIGURE 4: Gel filtration analysis of purified PR preparations containing the protein kinase activity. An aliquot (100 μL) of the pooled fractions from the DEAE-Sephacel column, containing purified PR B subunit (a) or 8S PR (b), were analyzed on a TSK G 3000 gel filtration column (HPLC). Fractions (0.5 mL) were collected at a flow rate of 1 mL/min, 30-μL portions were assayed for radioactivity (●), and 90-μL portions were used for the protein kinase assay (○).

analysis of ^{32}P-labeled phosphoamino acids always showed a single radioactive peak corresponding to phosphoserine residues. No phosphothreonine- or phosphotyrosine-associated radioactivity was ever detected (Figure 6).

DISCUSSION

Purifications of the molybdate-stabilized 8S form and of the B subunit of chick oviduct PR were achieved by using a previously described hormonal affinity chromatography system (Renoir et al., 1982, 1984a,b). The 8S receptor preparations were found to contain a calcium-dependent "protein kinase" activity (Garcia et al., 1983), but further experiments demonstrated that there was no formation of phosphorylated amino acids, and the labeling observed after electrophoresis is probably ascribable to a nonenzymatic effect. Both B subunit and 8S preparations were found to contain Mg^{2+}-dependent protein kinase activity, which is further studied here. Recent reports have described the copurification of protein kinase activities with the glucocorticosteroid receptor (Kurl & Jacob, 1984; Schmidt & Litwack, 1985; Singh & Mougdil, 1985). However, the work of Sanchez et al. (1986) with the L-cell glucocorticosteroid receptor fails to indicate that this receptor possesses intrinsic enzymatic activity. It is presently difficult to assess the results of Weigel et al. (1981), who did not find intrinsic protein kinase activity in a chick oviduct PR preparation that is known not to be composed for the most part by

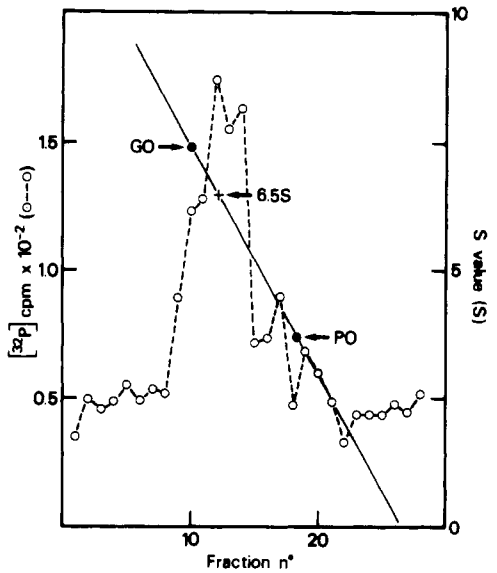

FIGURE 5: Sucrose gradient centrifugation analysis of the copurified protein kinase. Three hundred microliters of pooled DEAE-Sephacel fractions from a PR B subunit purification containing the protein kinase activity was deposited onto a sucrose (5–20%) gradient in buffer A. A tube containing the marker enzymes (GO, glucose oxidase; PO, peroxidase) was run in parallel (see Materials and Methods). After centrifugation, gradients were collected, and 90 μL of each fraction was used for the protein kinase assay. Sedimentation was from right to left.

the actual PR (Weigel et al., 1986). The phosphorylation experiments of Ghosh-Dastidar et al. (1984) used epidermal growth factor receptor as a source of tyrosine protein kinase and are therefore essentially different from those reported here.

We have used both 8S PR and PR B subunit preparations purified by hormonal affinity chromatography. When these preparations were submitted to DEAE-Sephacel chromatography, most protein kinase activity was found in a peak eluted slightly before the progesterone binding components. When purified 8S PR was subsequently transformed and analyzed on a DEAE-Sephacel column, the receptor and enzyme elution profiles were similar to those of the originally activated receptor, suggesting that the same enzyme was copurified with the two distinct receptor preparations. In agreement with this, the kinetic parameters of the phosphorylation reaction with respect to ATP and histone substrates were the same with both sources of the enzyme activity.

To examine the possibility that the coelution of a fraction of progesterone binding material with protein kinase activity may have been due to a minor isoform of PR displaying enzymatic activity, eluates from DEAE-Sephacel containing the two activities were pooled and rechromatographed. Again, the progesterone binding material and the protein kinase activity were only partially superimposed, suggesting that they were most likely present in two distinct proteins. However, this ion-exchange system did not completely resolve the two activities, due to the very similar isoelectric points of the enzyme and of the PR B subunit. According to the pattern obtained by two successive chromatographies (Figure 2), multiplicity of protein kinases cannot be excluded, including the possibility of an enzymatic activity associated to the B protein. It remains that whether we confirm protein kinase activity in the purified B subunit preparation (Garcia et al., 1983), we found that most activity likely belongs to an enzyme distinct from the PR B subunit.

The enzyme was further characterized by using gel filtration and sucrose gradient centrifugation. On the basis of the Stokes radius (~7.2 nm) and sedimentation coefficient (~6.5 S)

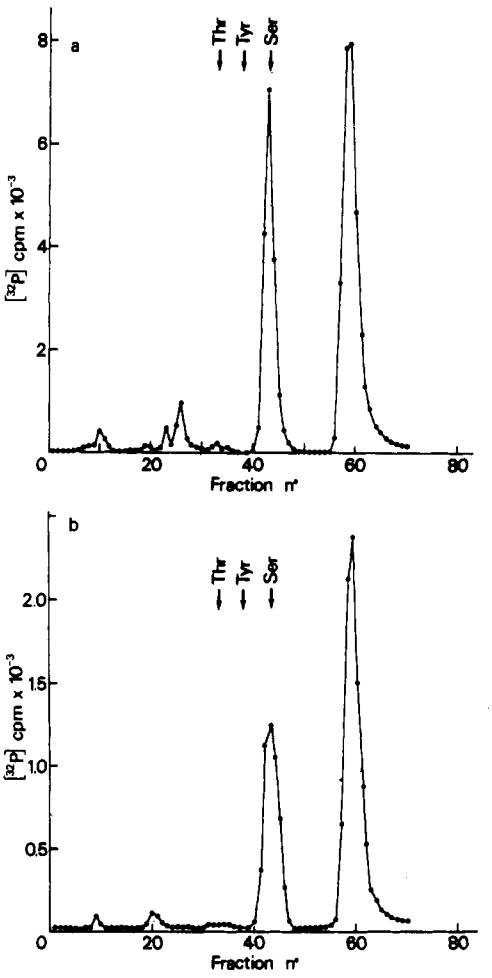

FIGURE 6: Phosphoamino acid analysis. (a) Calf thymus histones (100 μg) were phosphorylated by using 90 μL of PR-copurified kinase in the presence of 30 μM [γ-^{32}P]ATP and 6 mM $MgCl_2$. (b) Purified PR B subunit (~10 μg) containing the protein kinase activity was phosphorylated in the presence of 30 μM [γ-^{32}P]ATP and 6 mM $MgCl_2$. The ^{32}P-labeled proteins were prcipitated, hydrolyzed, and lyophilized prior to HPLC analysis (see Materials and Methods). Marker phosphoamino acids (Thr, phosphothreonine; Tyr, phosphotyrosine; Ser, phosphoserine) were detected by spectrophotometry. Fractions (500 μL) were collected and directly counted for Cerenkov radiation.

values, a molecular weight of ~200000 was estimated. Taken together, these parameters indicate that this enzyme is probably different from the PR B subunit and from the previously described components of the 8S PR (subunit A or B and hsp 90; Renoir et al., 1984a; Catelli et al., 1985).

The in vitro phosphorylation of tyrosine (Gosh-Dastidar et al., 1984) or serine (Weigel et al., 1980) residues in PR preparation is difficult to assess for the reason indicated above. As far as the present results are concerned, we observed that the PR-copurified protein kinase phosphorylated exclusively serine residues in both histones and the B subunit of the PR. Under in vivo conditions, phosphorylation of chick oviduct PR components occurs also only on serine residues (Dougherty et al., 1982, 1984).

Specific enzymes may be involved in regulating phosphorylation of steroid receptors. Characterizations of a cytosol protein kinase (Migliaccio et al., 1982) and of a nuclear phosphatase (Auricchio et al., 1981) involved in the estrogen receptor phosphorylation have been reported. Several recent reports have described the copurification of protein kinases with the chick oviduct PR (Garcia et al., 1983) and with the glucocorticosteroid receptor (Schmidt & Litwack, 1985; Kurl & Jacob, 1984; Singh & Mougdil, 1985). Whether these

enzymes interact with steroid receptors in the intact target cells is presently unknown, and no convincing evidence for substrate specificity has been provided. It may even be argued that this copurification can be due to contamination by abundant and unrelated cytosol enzymes. The PR preparations obtained in our laboratory contain up to 5% of contaminating proteins (Renoir et al., 1984a,b), and a contaminant in very small amount could be detected by its enzymatic activity.

However, it is intriguing that apparently a single protein kinase, out of the many present in the cell, is copurified in affinity chromatography of the chick oviduct PR, since experimental data such as isoelectric focusing and enzyme kinetics are consistent with the presence of a unique enzyme in the PR preparations. We cannot exclude the possibility that this particular kinase is in fact associated with the PR in vivo. The association is not very strong (separation occurs on ion-exchange chromatography) and may not be stoichiometric as the amount of the enzyme seems small in molecular terms. It is of course possible that most of the putative PR-associated kinase was removed during affinity chromatography, where drastic washing conditions are employed. The physiological role of this PR-copurified enzyme is unknown, and further studies are needed to answer the question of whether such complexes are related to the PR phosphorylation in vivo.

Registry No. 5′-ATP, 56-65-5; protein kinase, 9026-43-1; progesterone, 57-83-0.

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