Evidence That Heat Shock Protein-70 Associated with Progesterone Receptors Is not Involved in Receptor-DNA Binding

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In the absence of hormone, human progesterone receptors (PR) are recovered in the cytosolic fraction of cell lysates as a multimeric complex containing the steroid-binding polypeptide, heat shock protein-90 (hsp90), and heat shock protein-70 (hsp70). Activated forms of human PR that acquire the ability to bind to DNA are dissociated from hsp90, but retain association with hsp70. The present study has examined whether associated hsp70 has a function in receptor-DNA binding. When activated PR was bound to specific target DNA in a gel shift assay, no hsp70 was detectable in the PR-DNA complex, as evidenced by the failure of several antibodies to hsp70 to affect the mobility or the amount of complexes. To determine whether hsp70 might indirectly influence DNA-binding activity, we have examined the effect of hsp70 dissociation on PR-DNA-binding activity. Dissociation was achieved either by treatment of immunoaffinity-purified immobilized PR complexes with ATP or by the binding of PR complexes to ATP-agarose, followed by elution with high salt. Under both conditions, dissociation from hsp70 neither enhanced nor impaired the ability of PR to bind to specific DNA. These results suggest that hsp70 is not involved in PR binding to DNA, either directly by participating in DNA binding or indirectly by modulating PR-DNA-binding activity. This implies that hsp70 functions at an earlier stage in the receptor activation pathway. Consistent with the known involvement of hsp70 in stabilizing unfolded states of other target proteins, we propose that hsp70 may assist in nuclear transport of PR or in assembly-disassembly of the 8–10S multimeric complex. (Molecular Endocrinology 5: 1993–2004, 1991)

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INTRODUCTION

Several different classes of the steroid family of receptors (1), most notably those receptors for glucocorticoids, progesterone, estrogen, and dioxin, have been shown to be recovered in the absence of ligand as 8–10S multimeric complexes composed of the receptor and heat shock protein-90 (hsp90) (Ref. 2 and references therein). There is good evidence that hsp90 associates with receptors not only *in vitro*, but also in intact cells (3–5), indicating that it plays a physiological role in receptor action. Studies have suggested two possible functions for hsp90: one to repress and maintain receptors in an inactive state in the absence of hormone (6–9), and the other to maintain the unoccupied receptor in a conformation competent to bind hormone (10, 11). The precise mechanism by which hsp90 affects receptor activity is not known.

In addition to hsp90, two other proteins have been commonly found to associate with steroid receptors. Proteins in the range of 55–59 kDa (p55–59) are associated with the 8–10S complex of several different steroid receptors (12–15). One of these may indeed be another hsp, hsp56, as recently reported by Sanchez (16). Studies with human progesterone receptors (PR) (17, 18), chick oviduct PR (19, 20), and mouse glucocorticoid receptors (GR) (21) have all identified receptor association with another hsp, hsp70. An earlier study reported a 72-kDa protein associated with purified preparations of activated rat GR. The identity of the 72-kDa protein was not made, but it may also be a form of hsp70 (22). Unlike hsp90 and p55–59, hsp70 is recovered with both unactivated and activated forms of receptors (17–21). This suggests that hsp70 associates with receptor through a different binding mechanism and may serve a functional role different from that of hsp90 and p55–59.

In the present study we have further characterized

the interaction of hsp70 with activated forms of human PR and have attempted to determine whether associated hsp70 either participates directly in receptor DNA binding or functions indirectly to modulate DNA-binding activity. Results suggest that hsp70 is not involved either directly or indirectly in PR-DNA binding, implying that it functions at an earlier stage of the receptor activation pathway. Possible actions for hsp70 in regulating receptor activity are discussed.

RESULTS

Both Unactivated and Activated Forms of Human PR are Associated with hsp70

Human PR are produced as two different sized proteins of 94 and 120 kDa, termed PR-A and PR-B, respectively (23). The A-receptor is a shortened version of B, missing N-terminal sequences. The two proteins are otherwise identical in their steroid- and DNA-binding domains (24). Although the molecular origin of PR-A and PR-B remains in doubt, the two receptor forms represent true cellular products synthesized in approximately equal amounts in T47D cells (23, 24). Figure 1 illustrates the association of both unactivated and activated forms of human PR with hsp70 by specific immune coisolation of PR and hsp70 from T47D cells metabolically labeled to steady state with [^{35}S]methionine. Cytosolic PR were prepared either in the presence of sodium molybdate to stabilize the unactivated 8–10S complex (19) or without molybdate and activated *in vitro* by hormone (R5020) binding, followed by treatment with 0.4 M NaCl. Because of the difficulty in separating the 94-kDa PR-A and hsp90 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; both proteins have nearly identical electrophoretic mobilities), the ^{35}S-labeled PR complex was immune isolated with a monoclonal antibody (MAb), B-30, that is specific for PR-B (17). In their unactivated states, the A- and B-forms of PR have each been shown to form separate multimeric complexes (19, 25). As shown by SDS-PAGE and autoradiography, molybdate-stabilized cytosol PR contains ^{35}S-labeled proteins corresponding to PR-B and three other predominant copurifying proteins of about 90, 70, and 55 kDa. The identity of the 90- and 70-kDa proteins as hsp90 and hsp70, respectively, was confirmed by parallel Western blot analysis of the immune isolated samples (Fig. 1, lane 5). The identity of the 55-kDa component is not known, but it is likely to be one of the protein(s) in the 55- to 59-kDa range found to be associated with unactivated forms of several other receptors (12–16). Because of the abundance of free hsp in the cell, we typically find that small amounts of hsp90 and hsp70 bind nonspecifically to control resins. However, the amounts were substantially less than those

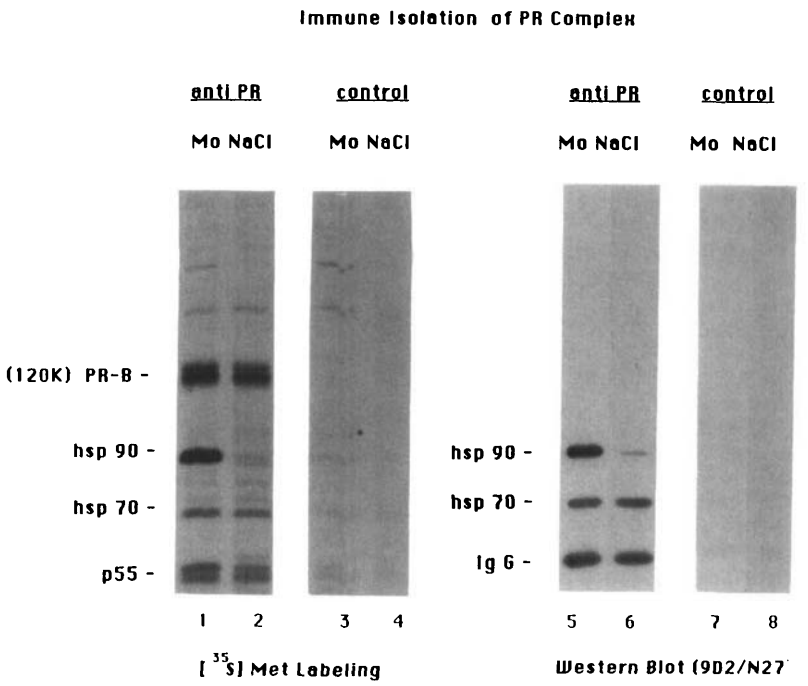

Fig. 1. Protein Composition of Unactivated and Activated Forms of Cytosolic Human PR

T47D human breast cancer cells were labeled to steady state with [^{35}S]methionine, and cytosolic PR was prepared in low ionic strength buffer (TESH) in the presence or absence of 20 mM sodium molybdate (Mo). Cytosol PR in the absence of molybdate was further treated *in vitro* for 4 h at 4 C with 40 nM R5020, followed by 0.5 M NaCl for 1 h at 4 C (NaCl). PR were immune isolated with the anti-PR-specific MAb (B-30) coupled to Affigel-10 or with blank Affigel-10 resins as controls for nonspecific binding. Isolated PR complexes were analyzed by 7% SDS-PAGE and autoradiography (*left panel*) or by Western blot (*right panel*) with a mix of MAbs to hsp90 (9D2) and hsp70 (N27).

bound by receptor-specific MAbs (Fig. 1), indicating that the majority of hsp90 and hsp70 were copurified through specific association with receptors.

In separate studies (25) we have estimated molar ratios of hsp90, hsp70, and PR in the molybdate-stabilized cytosolic complex by quantifying ^{35}S counts incorporated into each component at steady state, as previously described by Mendel and Orti (26). After correction for the difference in methionine content for each protein, a molar ratio of 1.5 was found for hsp90/PR, and a ratio of 0.8:1 was found for hsp70/PR. Thus, hsp70 and PR are nearly stoichiometric, and hsp90 is present in approximately twice the amount of hsp70. It should be noted that hsp70 has half the methionine content as hsp90 and, thus, even when present in an equal amount will give a lower signal. Figure 1 (lane 1) is representative of this kind of analysis. Also shown in Fig. 1 (lane 2), [^{35}S]hsp90 is virtually undetectable in the salt-activated PR, while the relative amount of [^{35}S] hsp70 was essentially unchanged. A Western blot of the same samples with MAbs specific to hsp90 (9D2) or hsp70 (N27), showed the same relationship for the ratio hsp90/hsp70. Thus, the two methods detect hsp90 and hsp70 in similar relative amounts in the isolated PR complex. In subsequent studies we have used Western blot for analysis of hsp70/PR ratios in various treated and untreated PR preparations. The MAbs were used in excess and were, thus, saturating all antigen sites. Also, the sensitivities of anti-PR (AB-52) and anti-hsp70 MAbs (N27) against serial dilutions of their respective antigens were similar (not shown). Therefore, Western blot analysis is a reliable estimate of the relative abundance of hsp70 and PR in immune isolated receptor complexes.

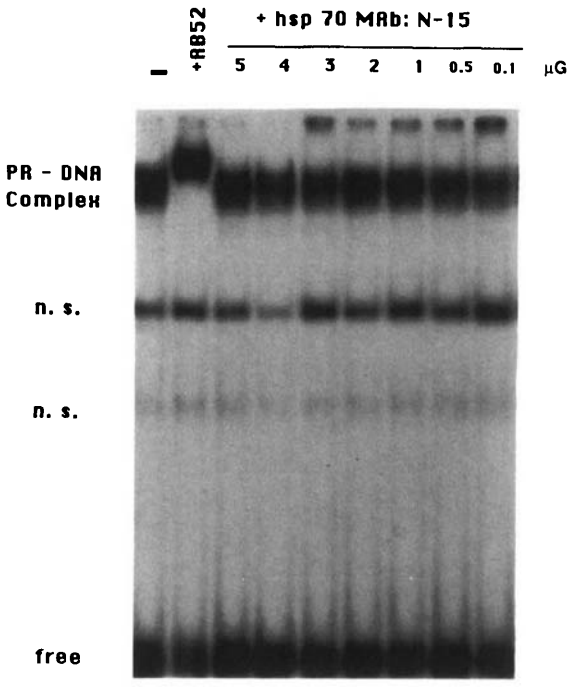

Fig. 2. Effects of MAb to hsp70 on PR-DNA Binding

Nuclear PR (60 fmol) activated *in vivo* was incubated for 30 min at 25 C with 0.3 ng of a ^{32}P end-labeled PRE oligonucleotide and electrophoresed on a 5% nondenaturing polyacrylamide gel. The presence of PR in the slowest mobility DNA complex is shown by the further mobility shift produced by the addition of the receptor-specific MAb AB-52 (1 μg). The other lanes show the effect of adding a MAb (N15 F3-6) to hsp70 in varying amounts (0.1-5 μg). Five other MAbs to hsp70 (N27-F3-4, N21-F3-6, H7F4-2, C92-F3-6, and C96F3-3) and a purified rabbit antiserum to hsp70 also had no effect on the amount or mobility of PR-DNA complexes (not shown).

Antibodies to hsp70 Do not Affect PR-DNA Complexes in a Gel Shift Assay

Since salt-activated PR retained association with hsp70, we sought to determine whether associated hsp70 might directly participate in PR-DNA binding. To address this question, human PR activated *in vivo* was incubated with a ^{32}P end-labeled progesterone response element (PRE) oligonucleotide, and receptor-DNA complexes were separated from free DNA by nondenaturing gel electrophoresis, as outlined in *Materials and Methods*. As shown in Fig. 2, this resulted in the formation of reduced mobility [^{32}P]DNA complexes that contained PR, as evidenced by the further upshift produced by addition of the MAb, AB-52 (PR-A and PR-B specific). If activated PR bound to DNA were to contain hsp70, then MAbs specific for hsp70 would also be expected to affect the electrophoretic mobility of the receptor-DNA complex. When gel shift assays were performed in the presence of varying amounts of a MAb to hsp70 (N15), no effect was observed on either the mobility or total amount of receptor-DNA complexes formed, even when antibody was added in excess (Fig. 2). Since it is possible that the epitope recognized by this particular MAb may be unavailable in the receptor-DNA complex, we have tested six other antibodies to hsp70 for effects on PR-DNA binding. None of five different MAbs nor a polyclonal rabbit antibody against hsp70 had any effect on the mobility or on the total amount of receptor-DNA complexes obtained (not shown). Figure 2 shows results with nuclear PR activated *in vivo*. The same results were obtained with cytosolic PR activated *in vitro* (not shown). This lack of effect with hsp70 antibodies suggests that hsp70 is not associated with the specific PR-DNA complex.

Dissociation of Activated PR from hsp70 Does not Affect Its DNA-Binding Activity

We next asked whether associated hsp70 might be involved indirectly in modulating PR-DNA-binding activity, perhaps by transiently stabilizing a conformational state necessary for DNA binding. If so, one might expect that receptor dissociation from hsp70 would adversely affect its ability to recognize and bind to specific DNA sequences of target genes. Kost and colleagues (19) have shown that ATP treatment of purified chick oviduct PR immobilized to MAb affinity resins resulted in hsp70 dissociation from receptors.

Following similar procedures, we have immunoaffinity purified activated forms of human PR, and the PR-hsp70 complexes immobilized to protein-A-Sepharose were then treated with ATP. As with chick PR studies (19), ATP was found to be more effective in the presence of $MnCl_2$ than with other divalent cations (not shown). Relative amounts of hsp70 and PR, before and after treatment with ATP, were assessed by Western blot with AB-52 (anti-PR) and N27 (anti-hsp70). As shown in Fig. 3A, PR (both A- and B-forms) were specifically associated with hsp70 in the absence of ATP (lane 1). ATP treatment resulted in a reduction in the ratio of hsp70/PR, indicating hsp70 dissociation. The effect was dose dependent, with maximal dissociation occurring with 10^{-3} M ATP. We also determined the DNA-binding activity of these same immobilized receptor preparations by directly incubating the immobilized PR complexes with a [^{32}P]PRE oligonucleotide, as outlined in *Materials and Methods*. As shown in Fig. 3B, ATP had no significant effect on the total amount of [^{32}P]PRE specifically bound to immobilized receptors, even at the highest concentration of ATP that was the most effective in dissociating hsp70 (Fig. 3B).

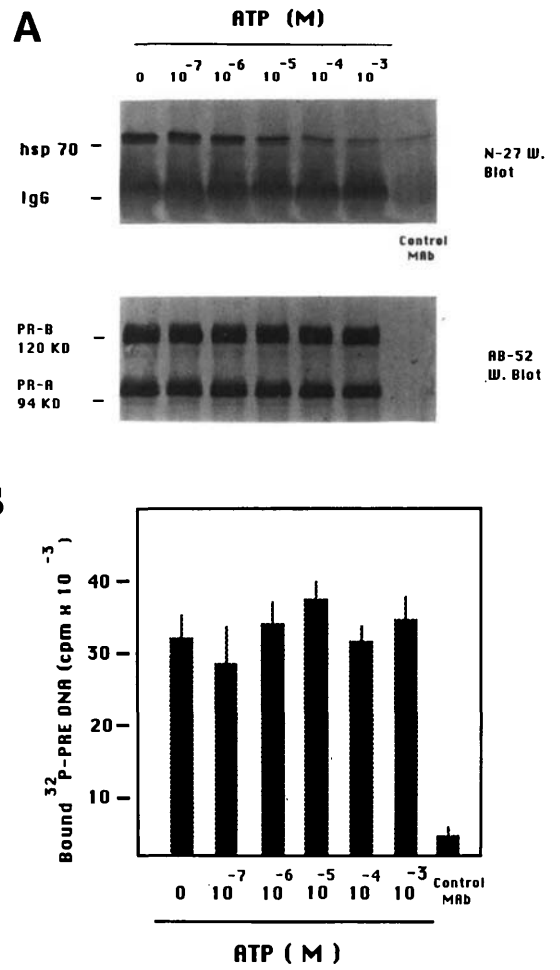

Fig. 3. ATP Dissociation of hsp70 from PR Complexes Immobilized to MAb Beads

A, Nuclear PR (30 pmol) activated by hormone *in vivo* was immune isolated and immobilized to MAb protein-A-Sepharose, as described in *Materials and Methods*. The immobilized PR complex was then incubated with or without the concentrations of ATP indicated, washed, extracted with SDS-sample buffer, and Western blotted with AB-52 and N27. To determine levels of nonspecific binding, immune isolation was performed with a control antibody. B, DNA-binding activity of immobilized PR. Aliquots of immobilized PR (1.5 pmol) were incubated with the ^{32}P end-labeled PRE oligonucleotide (7.5 pmol), as described in *Materials and Methods*. Free DNA was removed by washing and centrifugation of protein-A-Sepharose. The amount of [^{32}P]PRE bound was measured by Cerenkov counting of protein-A-Sepharose beads. Values represent average bound counts from duplicate incubations.

Since PR was immobilized to MAbs, which potentially can have the effect of stabilizing receptor DNA-binding activity (27), we have devised methods to dissociate the activated PR complex from hsp70 in solution. Earlier studies with chick oviduct PR had shown that activated receptors will bind to ATP-agarose and can be partially purified by elution from ATP-agarose columns (28). ATP-agarose affinity chromatography is also the principle step used in purification of hsp70 since hsp70 is an ATP binding protein (29). If the activated PR-hsp70 complex were to bind to ATP-agarose primarily through hsp70, we thought it might be possible to differentially elute PR and hsp70 from ATP-agarose and thereby separate PR from hsp70 in solution. *In vivo* activated nuclear PR was fractionated by the scheme shown in Fig. 4A. PR bound to DEAE was eluted with a NaCl gradient as a single peak of bound [^{3}H]R5020 (Fig. 4B). The pooled PR fractions eluted from DEAE were then bound to ATP-agarose and eluted sequentially, first by NaCl gradient and then in batch with 3 mM ATP. As shown in Fig. 4C, PR was bound to ATP-agarose and eluted with NaCL as a single peak of hormone-binding activity. Analysis of protein fractions by SDS-PAGE and silver staining showed that a number of other proteins were also absorbed and eluted by NaCl from ATP-agarose (Fig. 5A). However, essentially a single protein of 70 kDa remained bound to ATP-agarose in the presence of high NaCl and was eluted with ATP. This was identified as hsp70 by Western blot with an anti-hsp70 MAb (not shown). The hsp70 can be visualized as a stained band even in crude cell extracts, and as shown, the bulk of cellular hsp70 was bound to ATP-agarose (Fig. 5A). No PR was detectable by either silver stain or Western blot in the ATP eluate (not shown). Aliquots of pooled fractions (nuclear extract, DEAE elute, ATP-agarose flow-through, and ATP-agarose salt eluate) were immune isolated with receptor-specific MAbs, and the relative amounts of hsp70/PR in the isolated complexes were assessed by Western blot. As shown in Fig. 5B, PR in the starting nuclear extract and eluted from DEAE remained associated with the same relative amount of hsp70. Receptors that were bound (nearly all were bound since none was detected in flow-through) and eluted from ATP-agarose by NaCl were recovered essentially free of hsp70 (Fig. 5B, lane 4). These same receptor fractions were measured for DNA-binding activity by gel mobility shift assay using increas-

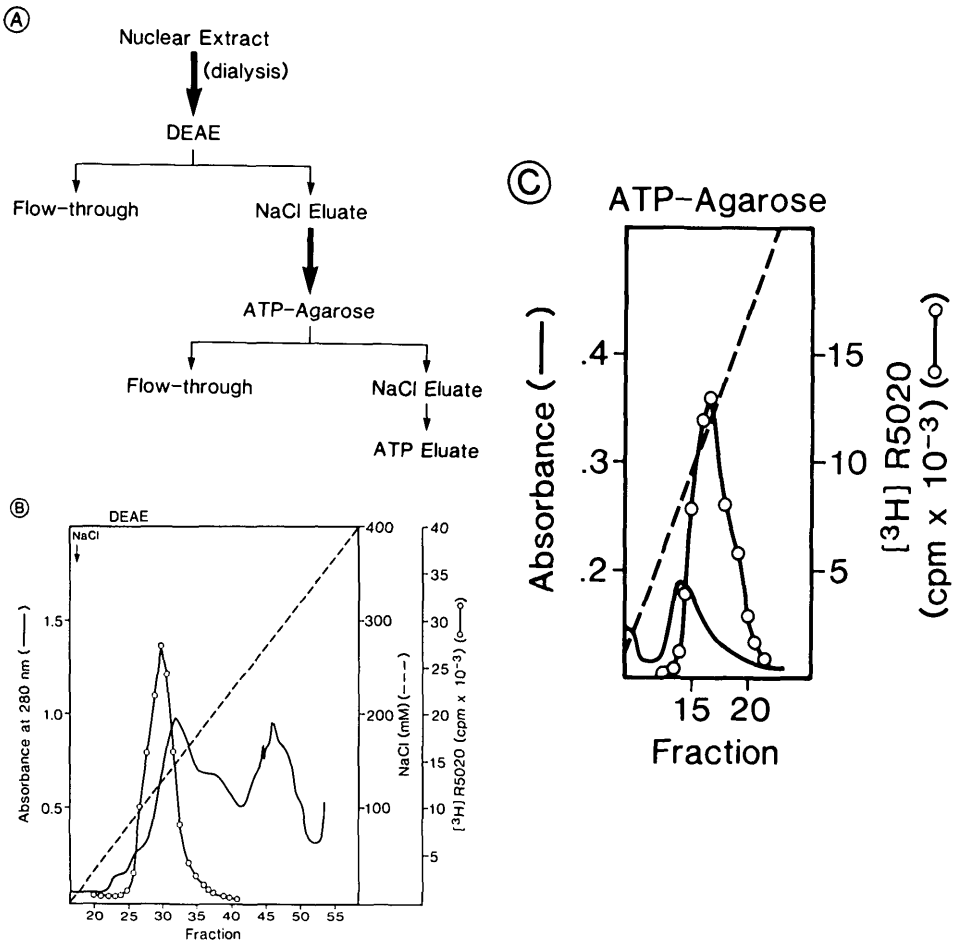

Fig. 4. Fractionation of the Activated PR Complex to Dissociate hsp70 in Solution

A, Fractionation scheme. B, Nuclear PR, activated by hormone binding *in vivo*, was labeled by exchange *in vitro* with [^{3}H]R5020. Samples were dialyzed to reduce the NaCl concentration and bound to DEAE. Bound PR was eluted by a NaCl gradient (20–400 mM NaCl), 1.5-ml fractions were collected, and aliquots (100 μl) were counted for [^{3}H]R5020. C, Eluted PR fractions were pooled, dialyzed against TEDG, and incubated in batch for 2 h at 4 C with ATP-agarose. Receptors bound to ATP-agarose were eluted by a NaCl (20–500 mM) gradient (*dashed line*), and aliquots (100 μl) of fractions (1.5 ml) collected were counted for [^{3}H]R5020. Residual protein that remained bound to ATP-agarose was eluted with 3 mM ATP (not shown). DEAE and ATP-agarose fractions were continuously monitored for protein by absorbance at 280 nm.

ing amounts of PR and a constant amount of ^{32}P-labeled PRE. As shown in Fig. 6, the DNA-binding activities of receptors that retained high ratios of hsp70/PR (nuclear extract and DEAE) showed little difference from that of receptors eluted from ATP-agarose where the hsp70/PR ratio was reduced to near zero. Although the total amount of PR-DNA binding obtained with ATP-agarose-eluted PR was slightly less at each receptor concentration, scanning of the gels to quantitate bound [^{32}P]DNA indicated that the affinity for DNA was not substantially different for any of these receptor preparations (not shown). The slightly lower total DNA binding in Fig. 6C probably reflects inaccuracies in our estimates of receptor numbers by steroid-binding assay, which is more prone to error after several column chromatography steps. Dissociation of essentially all hsp70 did not result in loss of PR-DNA-binding activity. Thus, similar to results obtained with purified immobilized PR, dissociation from hsp70 in solution had little or no effect on the ability of receptors to bind to specific target DNA. It should also be noted that PR eluted from ATP-agarose (essentially free of associated hsp70) exhibited the same electrophoretic mobility when bound to DNA as PR associated with the higher starting amounts of hsp70 (Fig. 6). This further supports the conclusion that hsp70 does not directly participate in PR-DNA binding.

PR Activated *in Vivo* Is Partially Dissociated from hsp70 and Exhibits a Higher DNA-Binding Activity Than PR Activated *in Vitro*

We have previously observed that nuclear PR activated *in vivo* exhibits a higher DNA-binding activity for specific DNA than does cytosolic PR activated *in vitro*, despite the fact that both forms of PR were dissociated from hsp90 (8). This suggests that hormone *in vivo* produces

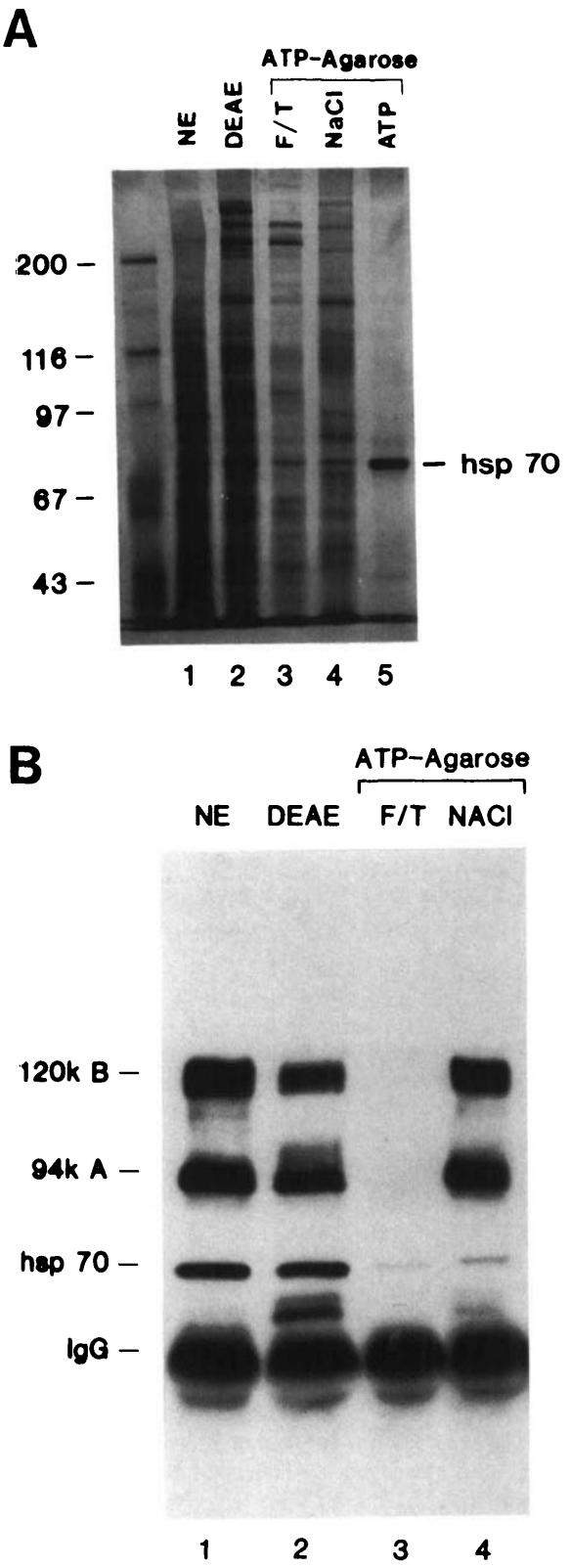

Fig. 5. Analysis of Fractionated Protein and Receptors

A, Analysis of column fractions from Fig. 4 by SDS-PAGE and silver staining. B, Immune isolation of PR from pooled column fractions, followed by Western blot of the isolated PR complex with a mixture of MAbs to PR (AB-52) and hsp70 (N27). Lane 1, Unfractionated PR in nuclear extracts (NE); lane 2, pooled receptor peaks eluted from DEAE; lane 3, ATP-agarose flow-through; lane 4, pooled receptor peaks eluted by NaCl from ATP-agarose.

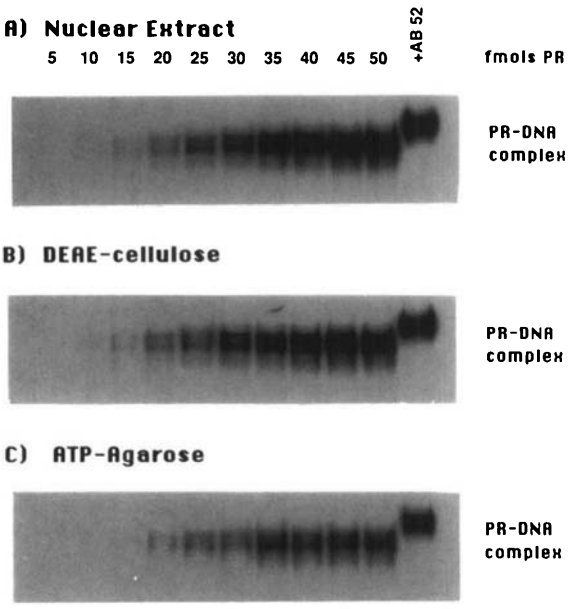

Fig. 6. DNA-Binding Activity of Fractionated PR

Varying amounts of PR (5–50 fmol) in crude nuclear extracts (A), eluted from DEAE-cellulose (B), or eluted by NaCl from ATP-agarose (C) were analyzed for their ability to bind to a [^{32}P]PRE oligonucleotide by gel mobility shift assay. The figure shows *insets* from the region of the gels containing the PR-DNA complex. The presence of PR in the DNA complex is illustrated by the further mobility shift produced by addition of the MAb, AB-52.

a modification of PR, in addition to dissociation from hsp90, that is not fully mimicked *in vitro*. *In vitro* and *in vivo* activated PR, therefore, were compared for relative amounts of associated hsp70 and were found to differ in this regard. As shown by immune isolation of PR complexes and Western blot, unactivated forms of cytosolic PR prepared in low ionic strength buffer in the presence or absence of sodium molybdate, were associated with both hsp90 and hsp70, while *in vitro* (cytosol plus 0.5 M NaCl) and *in vivo* (nuclear) activated forms of PR were dissociated from hsp90 and remained associated with hsp70 (Fig. 7A). However, the two activated receptor forms were associated with different relative amounts of hsp70; there was about a 50% reduction in the hsp70/PR ratio with nuclear receptors activated *in vivo* compared with cytosolic receptors activated *in vitro*. When the above receptor preparations were analyzed by gel shift assay, the two cytosolic forms of PR associated with hsp90 and hsp70 failed to bind to DNA (Fig. 7B). DNA binding was obtained only with salt-activated cytosolic and *in vivo* activated nuclear PR that were both dissociated from hsp90, and as reported previously, *in vivo* activated PR has a higher DNA-binding activity. The fact that *in vivo* activated nuclear PR also showed a reduction in the hsp70/PR ratio suggested the possibility that additional dissociation from hsp70 may be required for maximal receptor DNA-binding activity.

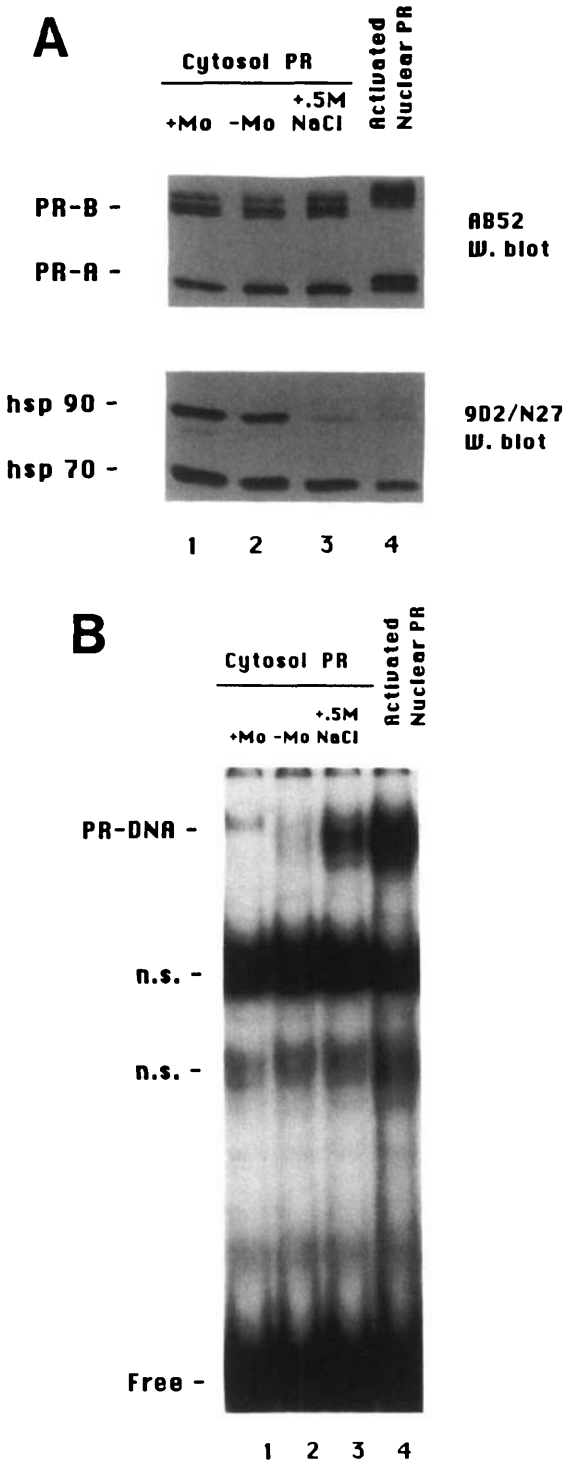

Fig. 7. Comparison of Different PR Forms for Associated hsps and DNA-Binding Activity

A, Protein composition of immune isolated PR complexes. Cytosol PR was prepared from nonhormone-treated cells in low ionic strength buffer (TEDG) in the presence of 20 mM sodium molybdate (+Mo) or in the absence of molybdate (−Mo). Cytosol PR in the absence of molybdate was activated *in vitro* by further treatment with hormone and 0.5 M NaCl, as described in the text. Nuclear PR activated by hormone *in vivo* was extracted from nuclei with 0.5 M NaCl. Each PR preparation, containing equal numbers of receptors (20 pmol), was immune isolated with AB-52 and then Western blotted with anti-PR MAb AB-52 (*top panel*) or a mixture of MAbs to hsp90, 9D2, and hsp70, N27 (*bottom panel*). B, DNA-binding activity of the different forms of PR above. Aliquots containing 60 fmol of PR were incubated with the [^{32}P]PRE oligonucleotide and submitted to gel mobility shift assay.

Dissociation of hsp70 from *in Vitro* Activated Cytosol PR Does not Increase DNA Binding to that of *in Vivo* Activated Nuclear PR

To test whether dissociation of activated cytosolic PR from hsp70 might increase its DNA-binding activity to the level of *in vivo* activated nuclear PR, we have fractionated cytosolic PR sequentially on DEAE- and ATP-agarose, as described in Fig. 4. As shown by Western blot of PR complexes immune isolated from different fractions, hsp70 was recovered with the starting cytosol receptor and with receptors eluted from DEAE (Fig. 8A) in approximately the same relative amounts. Half of DEAE-eluted receptors were bound to ATP-agarose (note PR in the ATP-agarose flow-through) and when eluted with NaCl displayed a reduced hsp70/PR ratio that was nearly the same as that of the *in vivo* activated nuclear PR. Binding of each receptor fraction to the [^{32}P]PRE oligonucleotide by gel mobility shift assay is shown in Fig. 8B. Partial dissociation of cytosolic PR from hsp70 after elution from ATP-agarose did not increase DNA-binding activity compared with that of untreated or DEAE-eluted PR that retained higher relative amounts of hsp70. Also shown for comparison is the higher DNA-binding activity of unfractionated *in vivo* activated nuclear PR (Fig. 8B, lane 5). Thus, further dissociation of cytosolic PR from hsp70 in solution did not increase DNA-binding activity to the level obtained with *in vivo* activated nuclear PR. Similar results were obtained when activated cytosolic PR were depleted of associated hsp70 by either ATP treatment of the purified complex immobilized to MAb resins or treatment of cytosolic PR in solution with high doses of ATP (not shown). It should also be noted that *in vivo* activated nuclear PR has a reduced electrophoretic mobility (both PR-A and PR-B) on SDS-PAGE, which does not occur with *in vitro* activated cytosolic receptors (Fig. 8A). This is a distinctive feature of hormone-dependent covalent modification of PR by phosphorylation (23). Thus, the higher DNA binding of *in vivo* activated PR compared with that of *in vitro* activated PR correlates better with the phosphorylation status of PR than with relative amounts of associated hsp70.

DISCUSSION

The present study was prompted by the fact that both unactivated and activated forms of human progesterone have been found to be associated with hsp70 (17–21). This suggests that hsp70 associates with PR by a

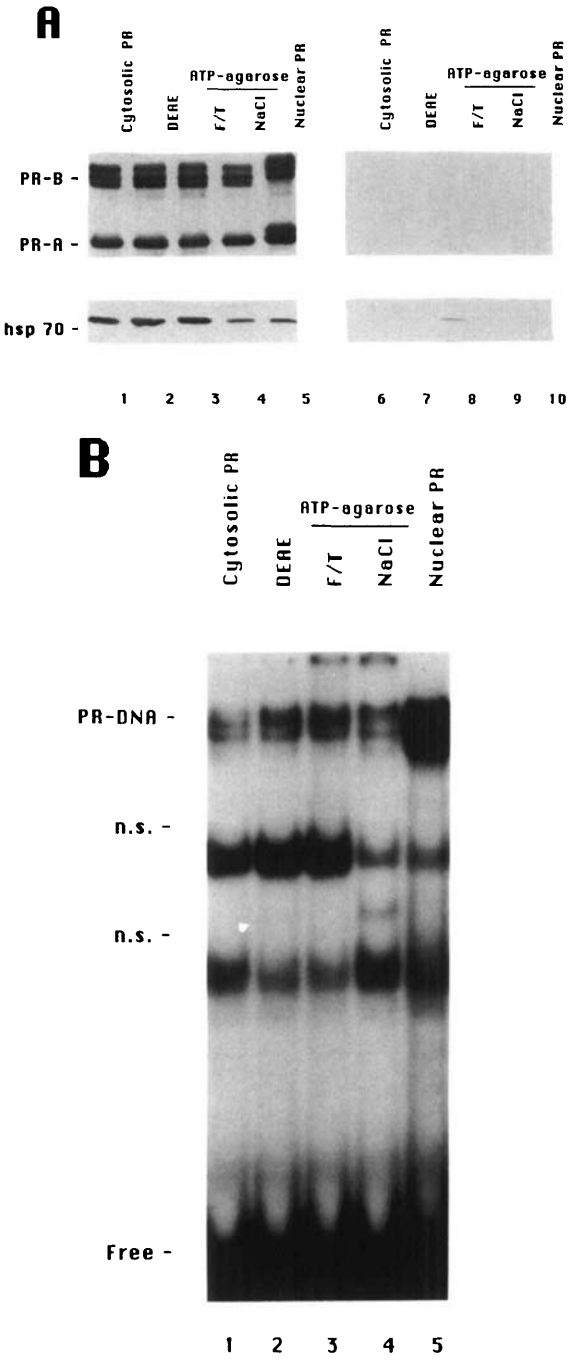

Fig. 8. Fractionation of *in Vitro* Activated Cytosolic PR on DEAE and ATP-Agarose to Dissociate hsp70 in Solution

Cytosolic PR, prepared in low ionic strength buffer (TEDG), was activated *in vitro* by binding [^{3}H]R5020 (40 nM) for 4 h at 4 C, followed by 0.5 M NaCl for 1 h at 4 C. Samples were dialyzed against TEDG to reduce NaCl and applied to the same fractionation scheme as that in Fig. 4. A, Immune isolation of PR from protein fractions and analysis by Western blotting with antireceptor MAb, AB-52 (*top panel*), or anti-hsp70 MAb, N27 (*bottom panel*). Nuclear PR activated by hormone *in vivo* were immune isolated for comparison (lane 5). Samples were also immune isolated with a control antibody (lanes 6–10) to determine the level of nonspecific binding of PR and hsp70. B, Gel mobility shift assay of the PR preparations described above. Equal numbers of receptors (60 fmol) in each fraction were incubated with the [^{32}P]PRE oligonucleotide and submitted to gel mobility shift assay.

different mechanism than hsp90 and may also have a different function. We sought to determine whether associated hsp70 might be involved in PR-DNA binding, either directly by participating in DNA binding or indirectly by modulating PR-DNA-binding activity, perhaps by transiently stabilizing PR in a conformation competent for DNA binding. If hsp70 were to participate in PR-DNA binding, this would imply that it might be involved in stabilizing PR-DNA interactions or in mediating transcriptional activity of receptors once they were bound to DNA. These seemed plausible hypotheses, since hsp70 has been observed to either colocalize in the nucleus or form stable complexes with other DNA-binding proteins, including *myc*, p53, adenovirus EIA proteins, SV-40 large T-antigen, and polyoma middle T-antigen (30–32). It has been speculated that hsp70 might function to facilitate the action of these other proteins by assisting in their DNA binding, transactivation, or replicative functions (30). However, the present findings that the mobility of specific PR-DNA complexes by electrophoretic gel shift assay was unaffected by the addition of antibodies to hsp70 and by dissociation of hsp70 from PR before gel shift assay provides good evidence that hsp70 does not directly participate in PR-DNA binding.

Members of the hsp70 family are known to form stable complexes with a number of target proteins. As a primary function, hsp70 has been proposed to stabilize transition states of unfolded proteins during their assembly into oligomeric complexes or their translocation across intracellular membranes (30–33 and references therein). With these functional properties in mind, it seemed plausible as an alternative, that hsp70 might modulate PR-DNA-binding activity indirectly by transiently maintaining receptors in a conformational state competent for binding to DNA. However, when different forms of activated PR were either partially or totally dissociated from hsp70, this neither impaired nor enhanced binding of PR to specific DNA. Thus, we conclude based on the presently available evidence that hsp70 associated with activated PR does not function to maintain the receptor in a conformational state suitable for DNA binding. Earlier studies reported that a 72-kDa protein, which itself did not bind DNA, associated with activated purified GR and GR-DNA complexes on glycerol gradients (22). Addition of an enriched 72-kDa protein fraction to purified activated GR with a low GR/72-kDa protein ratio was reported to increase binding of GR to specific DNA (34). These data, therefore, suggest a direct role for the 72-kDa protein in modulating GR-DNA binding. The reason for the apparent discrepancy with the present results is not known. Assuming that this 72-kDa protein also represents hsp70, which was not determined, it is possible that the increased GR-DNA-binding activity obtained was due to other factors, since mixing experiments were not performed with purified 72-kDa protein. We have been unable to take PR stripped of hsp and rebind hsp70 by simply adding the highly purified preparations of cellular hsp70 shown in Fig. 5, nor have we been able to

generate effects on PR-DNA-binding activity by addition of purified hsp70 (not shown). Smith *et al.* (35) reported that purified cellular hsp90 and hsp70 will not rebind to chicken PR stripped of hsp. Reconstitution of hsp binding was achieved by incubation of purified PR with a crude rabbit reticulocyte lysate in a temperature-dependent manner (35).

The presence of accessory proteins that facilitate binding of steroid receptors to DNA have been demonstrated for thyroid receptors (36, 37), estrogen receptor (38), vitamin D receptors (39), and GR (40) and, thus, may be a common feature of receptor action. The identity of these accessory proteins is not yet known. In an earlier study we reported the presence of a factor in nuclear extracts that increased the binding of activated cytosolic PR to specific DNA (41) that could account in part for the higher DNA-binding activity of *in vivo* activated nuclear PR compared with that of *in vitro* activated cytosolic PR. Results from the present study would indicate that the factor is not hsp70. *In vivo* activated PR contains a lower hsp70/PR ratio than *in vitro* activated cytosol PR, and receptor dissociation from hsp70 had no effect on DNA-binding activity.

The fact that we were unable to demonstrate a role for associated hsp70 in receptor-DNA binding implies that hsp70 functions at an earlier stage in the receptor activation pathway. Other possible functions are suggested from studies of hsp70 interactions with other target proteins. Possibilities are an involvement of hsp70 in facilitating nuclear translocation of receptors or oligomerization of PR. Most members of the steroid receptor family, including PR, reside in the nucleus in the absence of hormone and contain nuclear translocation sequences (42, 43). The mechanism for nuclear translocation of steroid receptors is not known, but is thought to be a facilitated process. Since hsp70 has been shown to be involved in facilitating the import of certain proteins into organelles (44, 45), hsp70 could, in an analogous manner, assist in the translocation of steroid receptors across nuclear membranes. Sanchez *et al.* (21) have made the interesting observation that GR overexpressed in Chinese hamster ovary cells were localized in nuclei in the absence of hormone and were found associated with hsp70. On the other hand, endogenous GR in mouse L-cells were predominantly cytoplasmic in the absence of ligand and were not associated with hsp70. Based on this correlation between nuclear localization and association with hsp70, Sanchez *et al.* (21) proposed that hsp70 may be involved in facilitating nuclear transport of steroid receptors. Alternatively, hsp70 could function transiently to stabilize PR in an unfolded state required for assembly-disassembly of the 8–10S multimeric complex or could stabilize the transition state between receptor monomers and active dimers. In support of this, we can find no *in vitro* conditions in which PR retains association with hsp90 in the absence of associated hsp70 (25). The reverse, however, clearly occurs. Smith and colleagues (35) have recently shown that chicken PR, stripped of associated proteins, will rebind hsp90 when rabbit reticulocyte lysates are used as the source for hsp90 and other factors required for reconstitution of the receptor-hsp90 complex. Similar reconstitution of mouse GR-hsp90 complexes has also been achieved (46). Since reassociation of hsp90 with GR and PR was found to also result in rebinding of hsp70, it was proposed that reconstitution may be due to an unfolding of the receptor assisted in part by hsp70 in the rabbit reticulocyte system (35, 46). hsp70 has also been implicated in targeting of proteins for lysosomal degradation (47), which raises the possibility that hsp70 could also be involved in deactivation of receptors upon their completion of transcriptional enhancement and release from DNA.

In summary, we propose that hsp70 does not affect the receptor activation steps of DNA binding or transcriptional enhancement after receptors are bound to specific DNA sites. Instead, hsp70 would appear to be involved in regulating receptor activity at an earlier stage in the receptor activation pathway. Much is to be learned about the nature and functional consequences of steroid receptor interaction with hsp70.

MATERIALS AND METHODS

Materials

[^{3}H]R5020 (17α-[*methyl*-^{3}H]promegestone; 17,21-dimethyl-19-norpregna-4,9-diene-3,20-one; 87 Ci/mmol) and unlabeled R5020 were obtained from DuPont/New England Nuclear Research Products (Boston, MA). ATP-agarose (ATP-agarose attached through C-8 with a six-carbon spacer arm) was obtained from Sigma (St. Louis, MO). L-[^{35}S]Methionine (1000–1200 Ci/mmol), [^{32}P]αdATP, and [^{32}P]αdCTP (3000 Ci/mmol) were purchased from ICN (Irvine, CA). [^{35}S]Protein-A (2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). MAb AB-52 and B-30 (mouse immunoglobulin G_1) were prepared against purified human PR, as previously described (17). MAb N27F3–4, N21F3–6, C92F3–6, C96F3–3, H7F4–2, and N15 F3–6 were prepared by W. J. Welch against constitutive and inducible forms of hsp70 isolated from HeLa cells (48). Rat MAb 9D2 against human hsp90 was prepared by Lai *et al.* (49). Antibodies were purified from mouse ascites fluids (17).

Cell Cultures, Metabolic Labeling, and Receptor Preparations

T47D human breast cancer cells were cultured as previously described (17, 42). For labeling with [^{35}S]methionine, near-confluent cultures in 175-cm^2 flasks (Falcon, Oxnard, CA) were labeled to steady state by incubation for 48 h at 37 C with 25 μCi/ml [^{35}S]methionine in 5% fetal serum and growth medium (Minimum Essential Medium) containing normal methionine concentrations. Harvested cell pellets were lysed in TEDG [10 mM Tris-base (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol] or TESH [10 mM Tris-base (pH 7.4), 1 mM EDTA, and 10 mM monothioglycerol] in the presence of a cocktail of protease inhibitors (17). Sodium molybdate (20 mM) was added for stabilization of the 8–10S unactivated cytosol PR. Cytosols were prepared by centrifugation of cell lysates at 105,000 $\times$ *g* at 4 C for 30 min. Nuclear receptors were prepared by centrifugation of cell lysates at 800 $\times$ *g* for 20 min at 4 C and extraction of pelleted nuclei with 0.5 M NaCl (in TEDG), followed by centrifugation at 105,000 $\times$ *g* for 30 min to yield a salt-soluble nuclear extract (50).

In vitro activation of cytosolic PR was performed by incubation of samples with 40 nM R5020 for 4 h at 4 C, followed by 1 h at 4 C with 0.5 M NaCl. Samples were then dialyzed against TEDG to reduce NaCl concentrations. *In vivo* activation of PR was performed by incubation of intact cells for 1 h at 37 C with 40 nM R5020. Receptors were extracted from nuclear pellets with 0.5 M NaCl and dialyzed against TEDG. To determine the number of steroid receptor-binding sites, samples were incubated for 16 h at 4 C with 20 nM [^{3}H]R5020 in the presence or absence of a 200-fold excess of unlabeled R5020, and binding was measured by a dextran-coated charcoal method (17).

Immune Isolation of PR

Two methods were used for immune isolation of PR. For analysis of [^{35}S]methionine-labeled PR, cytosol extracts (1.0 ml) were incubated for 4 h at 4 C with a 100-μl suspension of Affigel-10 (Bio-Rad, Richmond, CA) coupled with receptor-specific MAbs at a substitution of 4.5 mg MAb/ml resin. As controls to determine the level of nonspecific binding, blank Affigel-10 (inactivated with 1 M ethanolamine) was substituted for MAb resins. For Western blot analysis of receptor complexes, protein-A-Sepharose was precoated with two receptor MAbs, B-30 and AB-52, and used as an immunoabsorbent. To determine the level of nonspecific binding, protein-A-Sepharose was precoated with an unrelated rabbit antimouse immunoglobulin G. MAb resins (both Affigel-10 and protein-A-Sepharose) were washed, as previously described (8), by centrifugation in TEG buffer containing 0.3 M NaCl and 0.2% Tween-20. Sodium molybdate (20 mM) was also included in all washes to stabilize PR complexes once bound.

SDS-PAGE and Western Immunoblotting

Proteins bound to MAb resins were eluted with 2% SDS-sample buffer and applied directly to either 7.0% or 7.5% discontinuous SDS-polyacrylamide gels. Electrophoresis and Western immunoblotting of PR was carried out by methods previously described, using [^{35}S]protein-A and autoradiography as the detection method (17, 42). Similar procedures were used for immunoblotting of hsp, except that electrotransfer from SDS-PAGE to nitrocellulose was performed with a different buffer [20 mM Tris-base (pH 7.4), 150 mM glycine, and 20% methanol]. Proteins metabolically labeled with [^{35}S]methionine were detected by autofluorographic enhancement of SDS-gels. Gels were fixed for 1 h in 40% methanol-10% acetic acid, treated with Amplify (Amersham), dried under vacuum, and exposed to preflashed X-Omat film (Eastman Kodak, Rochester, NY). Prestained mol wt markers (Bethesda Research Laboratories, Gaithersburg, MD) were used for both Western and ^{35}S-labeled gels (myosin, 224,330; phosphorylase-B, 109,100; BSA, 71,830; ovalbumin, 45,830; carbonic anhydrase, 28,500).

Gel Mobility Shift Assay

A 32-bp double stranded synthetic oligonucleotide, corresponding to the distal most (−189 to −162) PRE/glucocorticoid response element of mouse mammary tumor virus, was end labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP by Klenow polymerase fill-in. PR in the range of 5–100 fmol (based on steroid binding assay) was incubated with 0.3 ng of the [^{32}P] PRE oligonucleotide for 30 min at 25 C. The DNA-binding buffer and conditions for gel electrophoresis have been previously described (50). In experiments in which MAbs were tested for effects on PR-DNA binding, 1 μg of purified MAb (unless otherwise indicated) was added at the completion of the 30-min reaction and allowed to incubate for another 20 min at 25 C.

ATP Dissociation of hsp70 from Immobilized PR Complexes

Approximately 30 pmol of cytosolic or nuclear PR (determined by hormone binding) were immobilized to MAb protein-A-Sepharose as described above, except that the resins were washed in a DNA-binding buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 10 mM monothioglycerol, and 60 mM NaCl; TTE_{N60}] and then resuspended in 1 ml TTE_{N60} containing 5 mM $MnCl_2$ and the final concentrations of ATP indicated in the figure legends. Immobilized receptors were then incubated in suspension for 16 h at 4 C and washed twice by centrifugation in TTE_{N60}. To measure binding of the [^{32}P]PRE oligonucleotide to immobilized PR, aliquots of the above washed protein-A-Sepharose (containing 1.5 pmol PR) were incubated as a suspension for 2 h at 4 C with 7.5 pmol of the ^{32}P end-labeled PRE oligonucleotide in the presence of 1 μg competitor poly(dA·dT-dA·dT). After 2-h incubation, protein-A-Sepharose was washed four times by centrifugation with 1 ml TTE_{N60}. Bound [^{32}P]DNA was then measured by Cerenkov counting.

Receptor Fractionation on DEAE and ATP-Agarose

Cytosols (20 ml) were prepared from nonhormone-treated T47D cells (8×10^8) and 0.5 M NaCl nuclear extracts (20 ml) from hormone-treated (R5020 for 1 h at 37 C) T47D cells (8×10^8). To follow PR fractionation, samples were preincubated *in vitro* for 8 h at 4 C with [^{3}H]R5020 (20 nM). Afterward, cytosol PR was further treated for 1 h at 4 C with 0.5 M NaCl. Receptor-[^{3}H]R5020 complexes were then dialyzed at 4 C against TEDG to reduce NaCl concentrations and bound to a DEAE (DE-52, Whatman, Clifton, NJ) ion exchange resin (15 ml packed bed volume) equilibrated in TEDG. The column was washed with TEDG and eluted with a NaCl gradient (20–400 mM NaCl). Aliquots of collected fractions were counted to determine the elution position of the [^{3}H]R5020-PR complex, and peak fractions were pooled and dialyzed against TEDG. After dialysis, PR were bound in batch to a 1.5-ml suspension of ATP-agarose equilibrated in TEG. After incubation for 2 h at 4 C, ATP-agarose was pelleted by centrifugation, washed in TEG, and eluted sequentially in a column by NaCl gradient (20–500 mM), followed by batch elution with 3 mM ATP (in the presence of 5 mM $MnCl_2$). The number of receptors in collected fractions was estimated by [^{3}H]R5020 binding. For nuclear PR that was occupied by unlabeled R5020 *in vivo*, we estimated that only half of the unlabeled R5020 exchanges with [^{3}H] R5020 and have accordingly adjusted the specific activity of bound [^{3}H]R5020.

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