Hinge and Amino-Terminal Sequences Contribute to Solution Dimerization of Human Progesterone Receptor

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We and others have shown previously that progesterone receptors (PR) form homodimers in solution in the absence of DNA and that dimers are the preferential form of receptor that binds with high affinity to target DNA. To determine the sequence regions involved in solution homodimerization, wild type PR and truncated PR proteins were expressed in an insect baculovirus system. The expression constructs included the ligand-binding domain [LBD, amino acids (aa) 688–933], the LBD plus hinge (hLBD, aa 634–933), the hLBD plus the DNA-binding domain (DhLBD, aa 538–933), and the full-length A and B isoforms of PR. PR-PR interactions were detected by three methods, coimmunoprecipitation of the PR fragments, pull-down of PR-polypeptides with polyhistidine-tagged versions of the same polypeptides immobilized to metal affinity columns and cooperative ligand-binding assays (Hill coefficient, \( n_H > 1 \) indicating PR-PR interaction). By all three assays, the LBD alone was not sufficient to mediate protein-protein interaction. However, the LBD did exhibit other properties ascribed to this domain, including binding to steroids with a relatively good affinity and specificity, ligand-induced conformational changes at the carboxyl-terminal tail and binding of heat shock protein 90 and its dissociation in response to hormone. Thus, failure of the expressed LBD to mediate dimerization does not appear to be due to an extensively misfolded or unstable polypeptide. The minimal carboxyl-terminal fragment capable of mediating PR-PR interaction was the hLBD construct. However, by immobilized metal affinity chromatography assay, self-association of PR-A was 3.5-fold more efficient than that of either the DhLBD or hLBD constructs. An expressed amino-terminal domain (aa 165–535) lacking the DNA-binding domain, hinge, and LBD was found to physically associate with PR-A or with another amino-terminal fragment lacking the LBD, but retaining the DNA-binding domain. These results provide evidence for direct amino-terminal interactions in the more efficient PR-PR interaction exhibited by wild-type PR-A, as compared with DhLBD and hLBD constructs. The overall results of this paper are consistent with the conclusion that the carboxyl-terminal LBD is not sufficient for mediating PR dimerization and that multiple regions, including the hinge and amino-terminal sequences, contribute either directly or indirectly to homodimerization of PR. (Molecular Endocrinology 11: 1114–1128, 1997)

INTRODUCTION

Members of the nuclear receptor superfamily of eukaryotic transcriptional activators can be classified according to their specific DNA-binding and dimerization properties (1–3). Receptors for nonsteroid hormones such as vitamin D, thyroid hormone, and retinoids bind preferentially as heterodimers with retinoic acid X receptor to direct repeat DNA sequence elements separated by a variable length nucleotide spacer. In contrast, the receptors for the classical steroid hormones bind as homodimers to partial palindromic hexanucleotide sequences that are separated by an invariant three-nucleotide spacer.
Two dimerization activities have been described for the steroid hormone subgroup of receptors. Within the DNA-binding domain (DBD), a short segment of amino acids near the carboxyl-terminal zinc finger (D-Box) has been shown by structural studies (4–6) and mutagenesis (7, 8) to function as an independent dimerization interface between two DBDs. Dimerization mediated by the D-box is dependent on binding to hormone response elements (HREs) and appears to be important for restricting receptor recognition to palindromic sequences separated by three intervening nucleotides (8). A second dimerization activity resides in a region(s) outside the DBD that is necessary for receptor dimerization in solution in the absence of DNA. Indeed, full-length receptors for estrogen (ER), progesterone (PR), and glucocorticoids (GR) have been shown to form stable dimers in solution. In contrast, the DBDs of most steroid receptors cannot form dimers in solution and have lower affinities for HREs than full-length receptors, suggesting that solution dimerization is important for high-affinity binding to target DNA sequences. A minimal sequence that directly mediates homodimerization in solution has not been well defined. The ligand binding domain (LBD) of ER has been reported to contain a strong hormone-dependent solution dimerization activity that is required for high-affinity binding of ER to estrogen response elements (9, 10). Moreover, a conserved heptad repeat of hydrophobic amino acids in the LBD was shown by mutagenesis to be required for efficient ER homodimerization (9). The LBD of rabbit PR was also reported to be required for solution dimerization, as detected in vivo by cotransfection oligomerization assays (11) and in vitro by coimmunoprecipitation (12). However, experiments with deletion mutants of GR and androgen receptor (AR) that retain the DBD have shown that both amino- and carboxyl-terminal sequences contribute to dimerization and maximal binding to specific DNA sequences (13–17), suggesting a role for amino-terminal sequences in solution dimerization.

Solution dimerization has been suggested to be a regulated step that occurs before DNA binding. As evidence to support this, several studies have shown that steroid hormone receptors bind preferentially to HREs as preformed dimers. For example, dimers of chicken PR separated from monomers by non-denaturing gel electrophoresis were shown to preferentially bind target DNA (18). Mixing experiments with full-length GR and its DBD revealed preferential binding of full-length GR with no evidence of a GR-DBD heterodimer bound to DNA (19). Additionally, kinetic and order of addition experiments with dilute and concentrated preparations of GR indicated that homodimerization is a rate-limiting step for high-affinity binding of GR to target DNA sequences (20).

Human PR is expressed as two different sized proteins from a single gene: full-length PR-B (120 kDa) and the amino-terminally truncated PR-A (94 kDa) (21). The two proteins are identical in sequence throughout the common amino-terminal region and in the DNA- and steroid-binding domains. Using antibodies against an epitope in the amino-terminal segment unique to PR-B, we have shown previously that PR-A can be coimmunoprecipitated with PR-B in the absence of DNA, indicating that human PR can form stable dimers in solution (22). In addition, we showed a correlation between the extent of solution dimerization between PR-A and PR-B and the ability of PR to bind to its target DNA sequences (22, 23). Dimerization of PR in solution has also been shown by positive cooperative ligand-binding studies (24–27).

In the present study, we investigated the contribution of different regions of human PR to solution dimerization in the absence of DNA. Full-length human PR and PR fragments were expressed in a baculovirus insect cell system, and protein-protein interactions were detected by three different methods: coimmunoprecipitation, pull-down of PR polypeptides by polyhistidine-tagged PR-polypeptides immobilized to metal affinity columns, and positive cooperative ligand binding. We found that while the LBD alone has sufficient structural information to bind ligands and heat shock protein 90 and to undergo ligand-induced conformational changes, it is not capable of mediating PR-PR interactions. Addition of hinge sequences was required to generate a minimal carboxyl terminus fragment capable of PR-PR interactions, whereas addition of sequences out to the amino terminus of wild type PR-A further enhanced these interactions. An expressed amino-terminal domain lacking the LBD and LBD was able to associate with wild type PR-A or with another amino-terminal fragment lacking the LBD. These results suggest a role for the hinge and amino-terminal sequences, either directly or indirectly, in mediating solution dimerization of PR.

**RESULTS**

Expression of PR and Truncated PR Proteins in the Baculovirus Insect Cell System

Wild type human PR and the amino-terminal deletion mutants shown in Fig. 1 were expressed in Sf9 insect cells using the baculovirus system. The viral vectors were constructed as described in Materials and Methods by inserting human PR cDNAs into pBlueBacHis so that the recombinant proteins were expressed with an amino-terminal polyhistidine tag (6x his) and an enterokinase cleavage site. The three PR deletion constructs shown contain the following regions: DBD, hinge, and LBD (DhLBD); the hinge and LBD (hLBD); and the LBD alone (Fig. 1). The identities of wild type and truncated PRs were confirmed by Western blot of extracts prepared from infected Sf9 cells using region-specific PR monoclonal antibodies (28, 29). The detected molecular masses of ~90 kDa for PR-A (expected is 84 kDa),
44 kDa for DhLBD (expected is 41 kDa), 34 kDa for hLBD (expected is 31.5 kDa), and 28 kDa for LBD (expected is 25.7 kDa) were close to the expected values for each expressed protein (Fig. 2). It should be noted that the electrophoretic mobility of wild type PR and PR fragments on SDS-polyacrylamide gels is slightly slower than predicted from the molecular mass (21). The faint immunoreactive band that runs more slowly than PR-A is likely due to a readthrough product from the baculovirus expression vector. All PR fragments were detected by the carboxyl-terminal antibody C-262, while only the DhLBD was appropriately recognized by the N-559 antibody that binds to sequences immediately amino-terminal to the DBD [amino acids (aa) 551 to 564]. None of the PR fragments were detected by the amino-terminal antibody AB-52 (Fig. 2).

LBD and Extended Hinge are the Minimal Sequences Required to Form an Oligomeric Complex with Wild Type PR-A

Expressed PR fragments (See Fig. 1) were mixed with purified wild type PR-A, and samples were immunoprecipitated with the AB-52 monoclonal antibody (MAb) that recognizes an epitope present in the amino terminus of PR-A (28). The immunoprecipitates were analyzed for associated PR fragments by Western blot with a carboxyl-terminal antibody (C-262) that recognizes both PR-A and all the truncated PR proteins (29). As a positive control, PR-A association with PR-B was determined as described previously (22, 23) by coimmunoprecipitation with B-30, a MAb specific to the unique N terminus of PR-B (28).

As shown in Fig. 3 (lanes 1–3), the LBD did not coimmunoprecipitate with PR-A. In contrast, a significant amount of the LBD plus hinge (hLBD) and DhLBD truncated proteins were specifically coimmunoprecipitated with PR-A. To quantify the extent of association with PR-A, the ratio of truncated PR proteins to PR-A was determined by Phosphorimager analysis of the Western blots of the immunoprecipitates. A summary of the quantitative data from multiple experiments is presented in Fig. 4. No specific association between the LBD and PR-A was detected. However, the hLBD (hLBD:PR-A ratio = 0.32 ± 0.05) and DhLBD (DhLBD:PR-A ratio = 0.43 ± 0.04) each associated with PR-A
to a similar extent as the association of PR-A with PR-B (PR-A:PR-B ratio 50.42 ± 0.04) (Fig. 4). These results suggest that the LBD alone is not able to mediate dimerization in solution and that the minimal carboxyl-terminal fragment capable of mediating PR-PR interaction requires the LBD plus extended hinge sequences.

**Fig. 3. Coimmunoprecipitation of Truncated PR Proteins with PR-A**

Equal amounts (based on steroid-binding assays and Western blot analysis) of expressed LBD (lanes 1–3), hLBD (lanes 4–6), or DhLBD (lanes 7–9) were incubated in the absence or presence of PR-A and immunoprecipitated with AB-52 by absorption to protein A Sepharose. PR-A and PR truncated proteins were bound to R5020 before extraction from Sf9 cells. As a positive control (lanes 10–12), PR-A was incubated with PR-B and immunoprecipitated with B-30, a MAb that recognizes an epitope in the unique extended amino terminus of PR-B. The resins were washed with TEG containing 100 mM NaCl and extracted with SDS-sample buffer. Extracted samples were analyzed by Western blot using C-262 that detects all PR fragments as well as full-length PR (see Fig. 1). Western blot results were arranged in groups of three samples for each assay, which included the input of the PR polypeptide, nonspecific absorption of the PR fragment to protein A Sepharose in the absence of PR-A, and specific association in the presence of PR-A. The heavy chain of the MAb and secondary IgG also appear in the immunoprecipitates and are indicated by an arrow at ~ 50 kDa.

**Fig. 4. Quantification of Coimmunoprecipitation Results**

Results of multiple coimmunoprecipitation experiments, similar to that in Fig. 3, were quantitated as the ratio of PR fragment to PR-A in the immunoprecipitate after subtraction of the nonspecific binding of PR fragments to protein-A-Sepharose in the absence of PR-A. Western blot bands were quantified by direct scanning of Western blots for radioactivity (35S Protein A) by Phosphorimager analysis. Values are the mean ± SEM of the ratios of PR fragments to full-length PR in the immunoprecipitates: LBD/PR-A (n = 4), hLBD/PR-A (n = 8), DhLBD/PR-A (n = 5), and PR-A/PR-B (n = 14).

**Immobilized Metal Affinity Chromatography (IMAC) Assay with Polyhistidine-Tagged PR to Detect Hormone-Dependent PR-PR Interactions**

The coimmunoprecipitation assay is limited to detecting interactions between truncated and wild type PR and thus cannot distinguish whether PR-PR interactions are through the same sequences, as opposed to different sequence regions of the two interacting polypeptides. Therefore, to detect protein-protein interactions between similar PR fragments, we have developed an assay where non-histidine-tagged PR is pulled down with a polyhistidine-tagged version of the same PR-polypeptide immobilized to metal affinity resins. Wild type PR and truncated PR proteins were expressed from pBlueBacHis vectors as amino-terminal polyhistidine (his)-tagged proteins. Non-histidine-tagged versions of the same proteins were generated either by cloning cDNAs into the nonfusion pVL1392 transfer plasmid (PR-A and LBD) or by removal of amino-terminal polyhistidine sequences by enzymatic cleavage with enterokinase (hLBD and DhLBD).

The IMAC pull-down assay involves incubating the polyhistidine-tagged PR with the nontagged version of the same PR polypeptide. The receptor complexes were then bound to a metal ion affinity resin (Talon, CLONTECH, Palo Alto, CA), which has a high affinity for multiple sequential histidine residues. After washing extensively with buffer containing 100 mM NaCl and 15 mM imidazole to remove nonspecifically bound
proteins, proteins that remained bound were eluted and analyzed by Western blot. Because the polyhistidine tag and enterokinase cleavage site adds 29 amino acids to the amino terminus, the polyhistidine-tagged proteins can be distinguished easily from non-fusion proteins on Western blots by an approximate 3 kDa difference in molecular mass. The presence or absence of the polyhistidine sequences was also confirmed by Western blot analysis with an antibody to the polyhistidine tag leader sequence (data not shown).

The results of a representative IMAC pull-down experiment comparing the ability of truncated PRs and wild type PR-A to self-associate are shown in Fig. 5. Little or no interaction was detected between LBD and LBDhis, whereas a significant amount of hLBD, DhLBD, and PR-A were each pulled down in a specific manner by hLBDhis, DhLBDhis, an d PR-Ahis, respectively. To quantitate the extent of these protein-protein interactions, the ratios of non-histidine- to polyhistidine-tagged PR polypeptides specifically bound to Talon resins were determined by PhosphorImager analysis of the Western blots from multiple independent IMAC assays. The results given in Table 1 confirm that no specific LBD-LBDhis interaction was measurable. In contrast, hLBD and DhLBD constructs both exhibited substantial self-association, the extent of which was similar for both. Interestingly, the efficiency of self-association of PR-A was 3.5-fold higher than that obtained with either DhLBD or hLBD (P < 0.05). The self-association of wild type PR-A and PR fragments shown in Fig. 5 and summarized in Table 1 was observed in the presence of the synthetic progestin agonist, R5020. When similar experiments were done in the absence of ligand, little to no specific interactions were detected (data not shown), indicating that these PR-PR interactions measured by IMAC pull-down assay are hormone-dependent.

It has been suggested that contaminating DNA in cell extracts can stabilize protein dimerization, and that protein-protein interactions may only appear to be DNA-independent (30). To determine whether PR-PR interactions detected were in fact DNA independent or not, micrococcal nuclease was added to Sf9 extracts used in IMAC pull-down assays under conditions determined in control reactions to give complete digestion to test plasmid DNA (see Materials and Methods). These results suggest that PR-PR interactions detected are not dependent on contaminating DNA in cell extracts.

Separately Expressed PR Amino Terminus Interactions

Self-association of wild type PR-A was more efficient than that of the truncated PR proteins, suggesting that amino-terminal sequences have a role in homodimerization of PR. To determine whether amino-terminal sequences affect homodimerization directly or indirectly, we expressed the amino-terminus of PR-A in baculovirus as a polyhistidine-tagged polypeptide. This construct extends from the extreme amino terminus of PR-A to the amino-terminal side of the DBD (aa 165 to 535) and thus lacks the DBD, hinge, and LBD

### Table 1. Quantification of PR-PR Protein Interactions by IMAC Pull-Down Assays

<table>
<thead>
<tr>
<th>Construct</th>
<th>Ratio of Nonfusion PR: PPHis (Mean ± SEM)</th>
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<tbody>
<tr>
<td>LBD</td>
<td>0.02 ± 0.01 (n = 4)</td>
</tr>
<tr>
<td>hLBD</td>
<td>0.13 ± 0.04 (n = 4)</td>
</tr>
<tr>
<td>DhLBD</td>
<td>0.17 ± 0.08 (n = 4)</td>
</tr>
<tr>
<td>PR-A</td>
<td>0.57 ± 0.05 (n = 6)</td>
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</table>

Multiple IMAC pull-down assays were quantified by determining the ratio of nonfusion to histidine-tagged PR polypeptides specifically bound to Talon resins. Values (mean ± SEM) were determined by direct PhosphorImager scanning of Western blots for radioactivity (bound [35S]Protein A) in the receptor bands.
(see schematic in Fig. 6). This amino-terminal domain polypeptide, expressed with a polyhistidine tag (PR-ANhis), was observed by IMAC pull-down to interact efficiently with non-histidine-tagged PR-A (bound to hormone) as shown by the Western blot results in Fig. 6 (left panel), where binding of PR-A to Talon resins was dependent on the presence of the ANhis PR fragment. Similarly, the ANhis PR fragment specifically pulled down a non-histidine-tagged amino-terminal fragment of PR-A that contains the DBD but lacks all of the LBD (ANDBD) (Fig. 6). Multiple independent protein-protein interaction assays were done with these amino-terminal PR fragments, and the results were quantitated by Phosphorimager analysis of the Western blots to determine the ratios of ANhis associated with non-histidine-tagged PR-A or the ANDBD fragment. The efficiency of interaction of the amino-terminal fragment (ANhis) with PR-A (PR-A:ANhis ratio = 0.47 ± 0.09; n = 4) was greater than that of the self-association observed between carboxyl-terminal fragments (DhLBD and hLBD), but equal to or less than that of interactions between PR-A and PR-A. The interaction between two amino-terminal fragments (ANDBD:ANhis ratio = 0.17 ± 0.03; n = 4) was similar quantitatively to the self-interactions of the carboxyl terminal fragment (see Table 1). These results indicate that the amino terminus provides direct protein-protein contacts that contribute to PR homodimerization and that these interactions in combination with C-terminal interactions account, in part, for the more efficient dimerization of full-length PR-A. The more efficient interaction of the amino-terminal fragment with PR-A than with another amino-terminal fragment lacking the LBD, further suggests that there is an amino-terminal-carboxyl-terminal interaction that contributes to PR dimerization.

**Hinge Sequences are Required for Positive Cooperative Binding of Progesterone to the LBD**

As an independent approach for detecting dimerization in solution, saturation progesterone-binding experiments were performed with wild type PR-A and truncated PR proteins, and the data were analyzed by Scatchard plot. As determined by the limiting slope of the Scatchard plot (Fig. 7), no difference was detected in the affinity of PR-A, DhLBD, and hLBD for [³H]progesterone; the values obtained for association constants (Kᵋ) were between 0.45 and 0.65 nM⁻¹ (Table 2). In contrast, the affinity of the LBD for [³H]progesterone was lower by approximately 7-fold; 0.07 nM⁻¹ (p < 0.05, Table 2). Also shown in Fig. 7, the Scatchard plots for the binding of [³H]progesterone to PR-A, the DhLBD, and the hLBD were convex, which is con-

**Fig. 6. Direct Interactions between Amino-Terminal Domains**

*Top*, Schematic of non-histidine-tagged PR-A (aa 165 to 933) and amino-terminal domains of PR-A: ANDBD that contains the amino terminus, DBD, and hinge region of PR-A (aa 165 to 688) in which the polyhistidine tag has been removed by cleavage with enterokinase; and ANhis that contains the amino terminus only of PR-A (165 to 535) with a polyhistidine tail at the N terminus. *Bottom*, ANhis was incubated for 30 min at 4°C with either full-length PR-A or ANDBD. Samples were then bound to Talon resins, eluted, and analyzed by Western blot with AB-52, which recognizes an epitope in the amino terminus common to all three polypeptides. Also included are Western blots of input PR-A, ANDBD, ANhis, and nonspecific binding of non-histidine-tagged PR-A and ANDBD alone.

**Fig. 7. Positive Cooperative Binding of Progesterone as an Independent Assay for PR Dimerization**

Extracts containing PR-A and PR truncation proteins, expressed in Sf9 insect cells, were incubated in the presence of varying concentrations of [³H]progesterone in TEDG buffer (pH 7.4) at 0 C for 2 h and specific progesterone binding to PR was determined by DCC assay. Shown are the Scatchard plots of representative experiments with PR-A (●), DhLBD (□), hLBD (○), and LBD (▲). The corresponding Hill coefficients (nᵢ) and number of binding sites (Bₘₐₓ) for the individual experiments shown are nᵢ = 1.25 ± 0.001 for PR-A (Bₘₐₓ = 1.37 nM); nᵢ = 1.42 ± 0.14 for the DhLBD (Bₘₐₓ = 2.19 nM); nᵢ = 1.33 ± 0.12 for the hLBD (Bₘₐₓ = 1.89 nM); and nᵢ = 0.88 ± 0.12 for the LBD (Bₘₐₓ = 1.29 nM).
Hill coefficients were calculated by nonlinear regression analysis of bound vs. free $[^3H]$progesterone. The association constants ($K_a$) were calculated from the limiting slope of the Scatchard plot.

The Hill coefficients ($n_H$) of binding $[^3H]$progesterone to DhLBD and hLBD were indistinguishable from that of PR-A (Table 2). Additionally, the values were similar to that previously reported for human PR-A, $n_H = 1.34$ (25), and to the maximal value reported for the bovine uterine progesterone receptor, $n_H = 1.2$ (24, 25, 27). Moreover, these values are consistent with a moderately positive cooperative binding mechanism. In contrast, the Hill coefficient of binding $[^3H]$progesterone to the LBD was significantly reduced, $n_H = 0.89$ ($P < 0.05$; Table 2). Thus, the positive cooperative progesterone binding detected with hLBD and the noncooperative binding of the LBD provide further evidence that the hinge is essential for PR homodimerization and that the LBD alone is unable to mediate protein-protein interaction.

**The LBD is Sufficient for Supporting Other Functions Ascribed to this Domain**

Because the LBD was not able to mediate protein-protein interaction, we investigated whether it has sufficient structural information to mediate other properties ascribed to this domain. As shown in Fig. 7, the LBD is capable of binding progesterone, albeit with a 7-fold lower affinity than wild type PR-A (Table 2). In addition, as determined by competitive inhibition bind-

![Table 2. Binding of $[^3H]$Progesterone to PR-A and PR Fragments](https://example.com/table2.png)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Hill Coefficient ($n_H$) (mean ± SEM)</th>
<th>Affinity ($K_a$ nM$^{-1}$) (mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>LBD</td>
<td>$0.89 ± 0.03^a$</td>
<td>$0.07 ± 0.01^a$ (n = 7)</td>
</tr>
<tr>
<td>hLBD</td>
<td>$1.23 ± 0.05$</td>
<td>$0.45 ± 0.05$ (n = 7)</td>
</tr>
<tr>
<td>DhLBD</td>
<td>$1.33 ± 0.04$</td>
<td>$0.68 ± 0.06$ (n = 5)</td>
</tr>
<tr>
<td>PR-A</td>
<td>$1.27 ± 0.05$</td>
<td>$0.49 ± 0.05$ (n = 13)</td>
</tr>
</tbody>
</table>

Hill coefficients were calculated by nonlinear regression analysis of bound vs. free $[^3H]$progesterone. The association constants ($K_a$) were calculated from the limiting slope of the Scatchard plot.

$^a$ The $n_H$ and $K_a$ values for LBD were significantly lower than that of other PR constructs. ($P < 0.05$).

consistent with a cooperative binding mechanism. In contrast, the Scatchard plot of the binding of $[^3H]$progesterone to the LBD was nearly linear, which is consistent with a noncooperative mechanism.

The cooperativity of binding $[^3H]$progesterone was further investigated by determining the Hill coefficient from the saturation binding data. A Hill coefficient greater than 1 is indicative of a positive cooperative binding mechanism, in which the binding of ligand to one site increases the binding affinity for a second site. Because PR has only one steroid binding site per polypeptide, positive cooperativity requires protein-protein interaction and therefore is also a measure of dimerization. A Hill coefficient near 1 is described as noncooperative and indicates that either the protein is monomeric or that site-site interactions do not occur (31).

The Hill coefficients ($n_H$) of binding $[^3H]$progesterone to DhLBD and hLBD were indistinguishable from that of PR-A (Table 2). Additionally, the values were similar to that previously reported for human PR-A, $n_H = 1.34$ (25), and to the maximal value reported for the bovine uterine progesterone receptor, $n_H = 1.2$ (24, 25, 27). Moreover, these values are consistent with a moderately positive cooperative binding mechanism. In contrast, the Hill coefficient of binding $[^3H]$progesterone to the LBD was significantly reduced, $n_H = 0.89$ ($P < 0.05$; Table 2). Thus, the positive cooperative progesterone binding detected with hLBD and the noncooperative binding of the LBD provide further evidence that the hinge is essential for PR homodimerization and that the LBD alone is unable to mediate protein-protein interaction.

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As reported previously, the carboxyl-terminal tail of human PR undergoes a conformational change in response to binding progestin agonists that is distinct from that induced by antagonists. This was detected by partial proteolytic digestion assay (32) and by differential recognition by the carboxyl-terminal C-262 MAb (29). As confirmed in Fig. 8B, wild type PR-A was efficiently recognized and immunoprecipitated by C-262 when bound to the antagonist $[^3H]$RU486, but not when bound to the agonist $[^3H]$R5020. The aminoterminal MAb AB-52 did not exhibit this differential reactivity and efficiently immunoprecipitated both agonist- and antagonist-bound PR-A. Baculo virus-expressed LBD (without hinge sequences), bound to agonist or antagonist, displayed the same differential recognition by the C-262 MAb as wild type PR-A (Fig. 8B). Thus, the LBD is capable of undergoing differential ligand-induced conformational changes at the carboxyl-terminal tail in a manner similar to that of PR-A.

Studies with other steroid hormone receptors indicate that heat shock protein 90 (hsp90) binds to sequences within the LBD and functions to assist in protein folding to maintain receptor in a proper conformation in the absence of hormone (33–35). To determine whether the LBD of human PR is sufficient to bind hsp90, an LBD construct (lacking the hinge) was synthesized in vitro in rabbit reticulocyte lysates in the presence of $[^35S]$methionine. In rabbit reticulocyte lysates, newly synthesized GR (34) and chick PR (35) bind efficiently to hsp90, which can be detected by coimmunoprecipitation of the radiolabeled receptor with a MAb, 3G3, to hsp90 (36). Furthermore, this interaction can be dissociated by hormone in the presence of ATP and elevated temperature (34, 35). As shown in Fig. 8C, the synthesized $[^35S]$LBD of human PR was specifically coimmunoprecipitated by the MAb 3G3, but was not pulled down by a control antibody (rabbit anti-mouse, RAM). Addition of R5020 in the presence of ATP and elevated temperature (30 C) resulted in LBD dissociation from hsp90 as indicated by the loss of immunoprecipitation of $[^35S]$LBD by 3G3. It should also be noted in Fig. 8C, that the addition of R5020 to the LBD resulted in loss of specific reactivity with the PR carboxyl-terminal MAb C-262, consistent with the immunoprecipitation data in Fig. 8B. Thus the LBD, expressed independently of other receptor domains, assumes the conformation required for binding and dissociation from hsp90 in a ligand-dependent manner.

**DISCUSSION**

The present study has shown by three independent assays, coimmunoprecipitation, IMAC pull-down, and
positive cooperative ligand binding, that the LBD of PR is not capable of mediating protein-protein interaction. Furthermore, the LBD exhibited a somewhat lower binding affinity for progesterone (7-fold) than wild type PR. This suggests that this domain does not contain sufficient structural information and/or sequences for mediating homodimerization and for wild type affinity for ligand. In contrast, the LBD was capable of carrying out other properties ascribed to this domain in a manner similar to that of wild type PR-A, including ligand-binding specificity, ligand-induced conformational changes, and hormone-dissociable binding of hsp90. These data strongly suggest that this domain alone can assume the appropriate conformation required for these other properties. Thus, the failure of the LBD to mediate PR-PR interaction does not appear to be due...
to extensive misfolding or destabilization of the expressed polypeptide in the absence of other receptor sequences. The minimal carboxy-terminal construct capable of mediating PR-PR interaction was the LBD with upstream hinge region sequences (hLBD). The hLBD interacted with PR-A by communoprecipitation and mediated self-association as detected by IMAC pull-down and positive cooperative ligand-binding assays. Furthermore, the hLBD exhibited high affinity binding of progesterone similar to wild type PR. We conclude from these results that the hinge of human PR has an important role in mediating homodimerization and high-affinity ligand binding.

The hinge region of nuclear receptors has generally been thought to provide a flexible link between the LBD and DBD and not to have other functional roles. This belief in part is due to the fact that the hinge region is generally a hydrophilic, poorly conserved sequence region among superfamilly members (37). However, there is growing evidence that the hinge does have other important functional roles. For example, nuclear localization sequences have been mapped to the hinge region of certain steroid receptors (11, 38). More recently it has been shown that the hinge region of retinoid acid (RAR) and thyroid receptors (TR) has conserved residues that form a binding site for corepressor molecules (39, 40). In addition, the binding site for estrogen receptor interaction with the TATA-binding protein-associated factor TAF$_{30}$ was mapped to the hinge region (41). An alternative function has been suggested by studies with the ROR$_{a}$ orphan nuclear receptor, where the hinge was shown to be essential for proper alignment of the DBD to produce maximal bending of target DNA (42). In the present study, we show that the hinge is also involved in homodimerization of PR in solution, suggesting another function for this region. However, it is not known whether this function of the PR hinge is direct by providing interfaces for protein-protein contact or indirect by conferring the appropriate conformation for protein-protein interaction through other more carboxy-terminal sequences in the LBD. Our studies with human PR also show that the hinge influences the affinity of the LBD for progesterone. The expressed LBD alone bound progesterone with a 7-fold lower affinity than full-length PR, whereas the LBD expressed with the extended hinge sequences bound ligand with the same affinity as full-length PR.

Among different members of the nuclear receptor family the hinge region has been reported to have a variable influence on ligand-binding affinity. In the case of ER, there appears to be little or no requirement of the hinge for the LBD to bind estradiol with high affinity (43, 44). Indeed, the purified LBD alone of human ER, expressed in bacteria, bound estradiol stoichiometrically and with an affinity equal to that of full-length receptor (44). Studies with GR and mineralocorticoid receptor (MR) are complicated by the fact that ligand binding is more dependent on association with hsp90 than it is with other steroid receptors. However, the LBD of MR (lacking hinge sequences), expressed in bacteria and reconstituted to bind hsp90 from rabbit reticulocyte lysates, bound hormone with the same affinity as wild type MR, suggesting that the hinge of MR is not required for the LBD to bind ligand (45). In the case of GR, the influence of the hinge on steroid binding is less clear. When an LBD construct of rat GR with the extended hinge region sequences was synthesized in vitro in rabbit reticulocyte lysates, it associated with hsp90 and bound hormone with high affinity (46). In other studies with rat GR, the LBD alone (aa 537 to 795) expressed in mammalian cells was highly unstable resulting in no detectable protein or steroid binding. However, fusion of the rat GR LBD to other unrelated proteins ($\beta$-galactosidase and dihydrofolate reductase) yielded stable polypeptides that bound ligand with high affinity approaching that of native GR. These findings suggest that the LBD of GR is not functionally independent and requires the influence of other receptor sequences on tertiary structure for its ligand-binding activity (47). Whether the hinge region could substitute for the fusion protein sequences was not investigated (47). In apparent contrast to most reports with the classical steroid receptors, hinge region sequences have been observed to be essential for ligand binding of two nonsteroid nuclear receptors. No ligand binding was detectable with the expressed LBDs of chicken or human thyroid hormone receptors (TR) and retinoic acid receptor-$\alpha$ (RAR$_{a}$) unless these domains were expressed with either the carboxyl-terminal half of the hinge or the entire hinge region (48–50). Interestingly, the expressed LBD of RAR$_{a}$ with extended hinge sequences, bound all-trans-retinoic acid similar to that of full-length RAR$_{a}$ with a strong positive cooperative mechanism ($n_{h}$ = 2.0), which was correlated with the formation of RAR$_{a}$ homodimers (50).

To examine the ability of like PR polypeptides to mediate protein-protein interaction, we used an IMAC pull-down assay instead of the more widely used assay with glutathione-S-transferase (GST) fusion proteins immobilized to glutathione resins. The large GST fusion (27 kDa) is more likely to affect PR structure and function than the much shorter polyhistidine/entero-kinase fusion sequence (3 kDa). In addition, GST itself has been reported to mediate dimerization (51, 52). By comparing ligand-binding and DNA-binding properties of nonfusion and polyhistidine-tagged PR, we have determined that the polyhistidine fusion sequences at the amino terminus have no effect on these PR functions (data not shown). Immobilized polyhistidine fusion proteins have been used previously to study protein-protein interactions between heterodimer subunits of human immunodeficiency virus-reverse transcriptase (53), homodimerization of the upstream stimulatory transcription factor of the major late adenovirus promoter (54), and the physical association between bacterial heat shock proteins and the heat shock transcription factor (55). To our knowledge, this is the first study to use immobilized polyhistidine-
tagged steroid receptors to investigate receptor dimerization. In addition, this assay should be amenable to studying steroid receptor interactions with other proteins.

Although the IMAC, coimmunoprecipitation, and cooperative binding assays were consistent in detecting the hLBD as the minimal carboxyl-terminus fragment capable of mediating PR-PR interactions, there was a quantitative discrepancy between IMAC and other assays when comparing the efficiency of dimerization of the different PR constructs. Coimmunoprecipitation and cooperative steroid binding results indicated that protein-protein interactions mediated by PR-A and DhLBD were no greater than that of the minimal carboxyl-terminal hLBD construct. However, by IMAC assay, self-association of PR-A was more efficient than that of either hLBD or DhLBD. The reason for this apparent discrepancy is not known. One possibility is that cooperative binding assays may not be capable of detecting this level of quantitative difference. The difference between coimmunoprecipitation and IMAC results likely stems from the fact that PR-A association with PR-B was used as the 100% control for dimerization interface that is regulated by phosphorylation (57). As further evidence that homodimerization of ER is mediated predominantly by carboxyl-terminal interactions within the LBD, the expressed LBD is capable of spontaneously dimerizing in solution (58–60). This clearly contrasts with our results with the LBD of PR, which was not able to homodimerize. Several studies have also suggested that GR and AR may use different sequence regions than ER for homodimerization in solution. Analysis of various deletion mutants of AR and GR have shown that the amino terminus has a strong influence on homodimerization and binding affinity for GREs, perhaps more so than the carboxyl terminus (14, 16, 17, 19). Also, deletion of the conserved heptad repeat hydrophobic repeats in AR, which are important for ER homodimerization in solution, had little effect on AR dimerization and DNA-binding affinity (15). However, another study showed that the expressed LBD of AR was able to form stable homodimers in solution (52). Thus, it has been suggested that the amino- and carboxyl termini of AR/GR work in synergy to mediate homodimerization (16, 17). As further evidence that homodimerization of ER may be different than other steroid receptors, ER dimers in solution appear to be more stable than PR and GR dimers (26, 61, 62). In a direct comparison, ER was determined to form dimers at a lower concentration than PR, suggesting that ER has a higher dimerization constant than PR (26).

Similar to studies with AR and GR, the present results indicate that amino-terminal sequences in human PR also contribute to solution dimerization. Although the hLBD construct was the minimal carboxyl-terminal fragment to mediate PR-PR interaction, full-length PR-A showed a 3.5-fold more efficient self-association.
than either the DhLBD or hLBD constructs (Table 1). Additionally, two amino-terminal fragments lacking the LBD were able to physically associate with each other, suggesting that the amino terminus contributes directly to homodimerization of PR (Fig. 6). These results are consistent with protein contacts between two amino-termini accounting, at least in part, for the higher efficiency of PR-A homodimerization as compared with that of the carboxyl-terminal hLBD and DhLBD constructs. Our results differed from a coimmunoprecipitation study with rabbit PR that showed an amino-terminal mutant lacking the hinge and LBD was not able to self-associate. It was concluded from this study with rabbit PR that the LBD was required for homodimerization and that the amino terminus was not involved (12). The reason for the apparent discrepancy between the results with rabbit PR (12) and our present findings is not known. One possibility is the use of different methodologies to analyze PR-PR interactions. Alternatively, this may be due to a mechanistic difference between human and rabbit PR. Rabbit PR is expressed only as the larger B isoform, and thus coimmunoprecipitation was done with amino-terminal fragments containing sequences in the extended amino terminus of PR-B. Our studies detected amino-terminal PR-PR interactions with constructs lacking the amino-terminal extension of PR-B. We have observed previously by cooperative ligand binding (25) and IMAC pull-down assays (M. J. Tetel, M. Altman, and D. Edwards, unpublished), that the unique amino-terminal segment of PR-B has a repressive effect on solution dimerization of PR. As further evidence that amino-terminal sequences contribute to PR homodimerization, we have analyzed the PR fragments used in this study that contain the DBD for relative binding affinity for target DNA sequences. As determined from saturation binding analysis by electrophoretic gel mobility shift assay, amino-terminal fragments lacking the LBD (ANDBD) bound to DNA as dimers with almost the same affinity as full length PR dimers. In contrast, the carboxyl terminal fragment (DhLBD) had a significantly lower binding affinity suggesting that amino-terminal sequences have a stronger influence on dimerization and DNA binding affinity than carboxyl-terminal sequences (V. Melvin and D. Edwards, unpublished).

Recent mammalian cell two-hybrid assays with separately expressed domains of AR have suggested a somewhat different role for the amino terminus in homodimerization. Separately expressed carboxy-terminal LBD constructs of AR did not make measurable protein-protein interactions in vivo (63). However, an interaction was detected between separately expressed amino-terminal domains, and a stronger hormone-dependent interaction was detected between amino and carboxy-terminal constructs (63). From these results the authors suggested that AR may form homodimers through an antiparallel interaction between the amino and carboxy-terminal. In a similar study, the separately expressed amino- and carboxy-terminal domains of ER were shown to functionally interact in vivo (64). Our finding that the separately expressed amino-terminal domain of PR interacted more strongly with full-length PR-A than it did with another expressed amino-terminal fragment lacking the LBD, suggests that human PR may also exhibit an interaction between its amino- and carboxyl-terminal domains. Whether these interactions might contribute to PR homodimerization, to an intramolecular interaction within a PR monomer, or both, remains to be determined.

In summary, our results are consistent with the concept that homodimerization of PR in solution is not mediated by a single discrete domain, but that multiple regions, including the hinge and amino terminus, contribute to PR dimerization. Further studies will be required to determine which regions contribute directly to the dimerization interface or contribute indirectly by affecting structural conformation.

MATERIALS AND METHODS

Materials

$[^3H]R5020$ (promegestone: [17α-methyl-$^3H$]17α, 21-dimethyloxy-19-norpregna-4,9-diene-3, 20-one; 87 Ci/mmol) and unlabeled R5020 were obtained from Dupont/New England Nuclear Products (Boston, MA). Unlabeled RU486 (Mifepristone, 17-hydroxy-11[4-dimethylaminophenyl]-17-progynyl-estra-4, 5 diene-3-one) was a gift from Roussel-UCLAF (Romainville, France). [1,2-$^3H$(N)]Progestosterone (50.0–51.5 Ci/mmol; 850.0–1, 905.5 GBq/mmol) was obtained from DuPont/NEN Research product (Boston, MA). Unlabeled steroids (progesterone, estradiol, dexamethasone, and dihydrotestosterone) were purchased from Sigma Chemical Company (St. Louis, MO). Metal ion affinity resins (Talon) were obtained from CLONTECH (Palo Alto, CA). Monoclonal antibodies against human PR include AB-52, which recognizes both the A and B isoforms and B-30, which recognizes only PR-B (28). C-262 is a monoclonal antibody directed against the last 14 amino acids of the carboxy-terminal end of PR (29). The MAbs H-130 and N-559 were generated against amino acids 669–682 of the hinge region and amino acids 551–564 of the DBD of human PR, respectively. The 3G3 MAAb to hsp90 was provided by Gary Perdew (36).

Construction of Recombinant Baculovirus Vectors for PR and PR Fragments

Recombinant viral vectors expressing full-length PR-A or PR-B as non-fusion proteins were constructed using the transfer plasmids pVL1392 and pVL1393 (Invitrogen, San Diego, CA) as described previously (65). Viral vectors, which generate amino-terminal polyhistidine (6x) sequences (N. L. Weigel, B. W. O’Malley, M. J. Tetel, and D. P. Edwards, unpublished), were constructed by insertion of PR-A or PR-B cDNAs into BamHI sites of pBlueBacHis (Invitrogen). A baculovirus vector expressing the DhLBD fragment of PR (the entire DBD, hinge region, and LBD of PR) was constructed by restriction digest of the plasmid phPR-A obtained from Donald McDonnell (66) with Accl, filling in of the 5′ overhang by Klenow DNA polymerase, and restriction digestion with EcoRI yielding a DNA fragment encoding aa 538–933 of human PR. The Accl/EcoRI fragment was then gel purified.

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and inserted between the BamHI (after filling in to blunt end) and EcoRI sites in pBlueBacHis2A. A baculovirus vector expressing the LBD (aa 165–535) of the PR-A was obtained from Ming Tsai and Bert O’Malley (Baylor College of Medicine, Dallas, TX) (32) with Styl and EcoRI to yield a fragment encoding amino acid residues of PR from 634 to 933. After blunt ending, this restriction fragment was gel purified and inserted into BamHI and HindIII sites of the multiple cloning cassette of pBlueBacHis C. A baculovirus vector that expresses the LBD of PR as a nonfusion protein, was constructed by restriction digestion of the plasmid pT7hPR-A by RsrII and BclI to drop out 1,554 bp of PR cDNA spanning just 3′ of the ATG translation start site to the 5′ boundary of the LBD (nucleotide +1 to 1156). The RsrII and BclI sites were ligated, linking the LBD (aa 688–933) in frame with the ATG start site at aa 165–168. The entire LRBD linked to the ATG start site of PR-A was excised with Ncol and EcoRI and cloned into the BglII and EcoRI sites of the baculovirus transfer plasmid, pVL1382 (Invitrogen). A baculovirus vector expressing the PR LBDA was constructed by restriction digestion of pT7hPR-A with BclI and EcoRI, to yield a PR cDNA encoding aa residues 688 to 933. This DNA fragment was gel purified and inserted between the HindIII and BamHI sites of the multiple cloning cassette of pBlueBacHis C.

A baculovirus vector expressing the ANhis (the amino terminus of PR-A that lacks the DBD, hinge, and LBD, as an N-terminal polyhistidine-tagged fusion protein) was constructed by restriction digestion of the PR-A plasmid by EcoNI to drop out bp 1779–2671 of PR-A cDNA. The EcoNI ends were made blunt by digestion with Mung Bean nuclelease and then religated resulting in a cDNA encoding a PR fragment aa 165–535. A baculovirus vector expressing the ANDBDhis (the amino terminus of PR-A including the DBD and hinge, but lacking the LBD, as an N-terminal polyhistidine-tagged fusion protein) was constructed by restriction digestion of the plasmid pT7hPR-A with BclI and EcoRI, to yield a PR cDNA encoding aa residues 688 to 933. This DNA fragment was gel purified and inserted between the HindIII and BamHI sites of the multiple cloning cassette of pBlueBacHis C.

Baculovirus Expression of PR Constructs

Sf9 insect cells were cotransfected with the appropriate recombinant transfer plasmids above and wild type AcNPV baculovirus DNA as described previously (65). Recombinant viruses, which were formed by homologous recombination, were then isolated by plaque-purification (65). For production of PR or PR fragments, Spodoptera frugiperda (Sf9) insect cells were grown in spinner vessels containing expressed PR constructs. A mixture of protease inhibitors (28) was added immediately, data from that experiment were discarded.

Coimmunoprecipitation of PR and PR Fragments

To prepare whole-cell extracts, Sf9 cells were lysed in TEDG buffer (10 mM Tris-base, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 10% glycerol), containing 0.5 M NaCl and a mixture of protease inhibitors (28). Cell lysates were centrifuged at 100,000 × g for 30 min to yield a soluble supernatant and then dialyzed against the lysis buffer containing no NaCl. Protein A Sepharose was precoated noncovalently with receptor-specific MAbS and used as an immunoabsorbent as described previously (23). Resins were prebound to rabbit antimonute IgG (Cappel, Durham, N.C.), used as a bridging antibody. Receptor-specific MAbS were then bound to the immobilized rabbit antimonute IgG. Sf9 cell extracts containing PR or PR fragments were mixed together in siliconized microcentrifuge tubes and incubated on ice for 30 min. Equal amounts of receptors were added to each assay as determined by hormone-binding assay and Western blot analysis. MAb-coated protein A-Sepharose beads (100 μl) were added to each tube and incubated at 4 C for 3 h on an end-over-end rotator. Resins were washed four times by centrifugation in TE buffer (TEDG minus dithiothreitol) containing 100 mM NaCl, transferred to a new microcentrifuge tube, and washed twice more. Immobilized proteins were eluted with 2% SDS loading buffer and then analyzed by Western Blot, using [35S]protein A and autoradiography as the detection method. Dried nitrocellulose blots were scanned directly for [35S] in protein bands with a series 400 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cooperative Ligand-Binding Assays

Cell pellets were homogenized on ice using a Potter-Elvehjem tissue grinder in 40 mM Tris/5 mM EDTA/10% (vol/vol) glycerol, pH 7.4 (TEDG buffer) with 10 mM sodium molybdate, 20 mg/ml ovalbumin, and 0.2 mM phenylmethylsulfonyl fluoride. Final concentrations of 47 mg/ml leupetin and 1 mg/ml pepstatin were added, and the cell homogenate was centrifuged at 100,000 × g for 30 min at 4 C. The supernatant (cytosol) containing the expressed receptor was used for equilibrium binding experiments.

A aliquots of the cytosol (200 μl) were incubated in duplicate for 2 h in an ice water bath with concentrations of [3H]progesterone between 0.5 nM and 40 nM. The nonspecific binding was measured by a parallel incubation of the receptor with [3H]progesterone in the presence of a 200-fold molar excess of unlabeled progesterone. At the completion of the incubation, 50 μl of each incubation mixture were removed for determination of its total [3H]progesterone concentration. Then 100 μl of 1% (wt/vol) charcoal/0.1% (wt/vol) dextran/T-500 in TDE buffer was added to each tube. The suspension was incubated for 10 min at 0 C for adsorption of unbound progesterone. The tubes were centrifuged at 4,000 × g for 5 min, and 100 μl of the supernatant were removed for measurement of bound [3H]progesterone by liquid scintillation counting. An incubation period of 2 h at 0 C was shown to be sufficient for equilibrium binding of [3H]progesterone to each PR construct (results not shown).

To assess the stability of the receptor during each experiment, the amount of specifically bound [3H]progesterone at a saturating concentration of ligand was measured in cytosol to which ligand was added immediately and in cytosol which was kept at 0 C for 2 h before the addition of ligand. This measured the degree of inactivation of the hormone-free receptor during the incubation conditions. If the amount of receptor-binding sites in the cytosol that had been kept at 0 C for 2 h before the addition of ligand was less than 90% of the amount of receptor in the cytosol to which ligand had been added immediately, data from that experiment were discarded.

Enzymatic Cleavage of the Polyhistidine Tag

Extracts of Sf9 cells expressing hLBDhis, DHlBDhis, or PR-Ahis were bound to metal affinity resins (Talon, Clontech) in TG buffer, pH 8.0, containing 350 mM NaCl and 5 mM
imidazole in a siliconized 15-ml tube. The resin was washed twice in the same buffer and twice in the same buffer containing 0.1 M NaCl and then transferred to a siliconized microcentrifuge tube. EnterokinaseMax (Invitrogen) was added (2 U/µg protein) to the immobilized polyhistidine-tagged protein and incubated at 4 °C for 16 h on an end-over-end rotator. The final suspension was brought to 0.5 M NaCl and incubated for another 30 min at 4 °C. The supernatant with the cleaved receptor was collected by centrifugation at 1,500 rpm. The resin was washed four times with 0.5 ml of TG buffer containing 0.5 M NaCl to collect any residual cleaved protein. EnterokinaseMax enzyme was removed by incubating the cleaved receptor with soybean trypsin inhibitor affinity resins (Sigma) at 4 °C for 2 h on an end-over-end rotator. The enterokinase-free cleaved receptor was dialyzed against TG buffer and analyzed by Western blot and silver-stained SDS-gel electrophoresis.

**IMAC Pull-Down Assays to Detect PR-PR Interactions**

Whole-cell extracts were prepared as described above for the coimmunoprecipitation assays except that the lysis buffer contained no EDTA or DTT, which inhibits binding to the metal ion resin. Whole-cell extracts containing PR polypeptides were mixed with polyhistidine-tagged versions of the same polypeptides in siliconized microcentrifuge tubes for 30 min on ice. Each reaction was then brought to a total volume of 100 µl with buffer containing 20 mM Tris, pH 8.0, and 10% glycerol (TG) and then TG buffer containing 45 mM imidazole and 300 mM NaCl was added to bring the final imidazole concentration to 15 mM and NaCl to 100 mM. One hundred microliters of a 1:1 suspension of Talon metal affinity resin (Clontech), was added to each tube. Samples were incubated at 4 °C for 1 h on an end-over-end rotator followed by washing of the resins four times by centrifugation in TG buffer containing 15 mM imidazole and 100 mM NaCl. Resins were transferred to a new microcentrifuge tube and washed twice more. Bound proteins were extracted with 2% SDS sample buffer and analyzed by Western blot with the PR MAb to hsp90 as described by Perdew et al. (36) and radiolabeled LBD was detected by SDS gel electrophoresis and autoradiography.

**SDS-PAGE and Western Blotting**

PR and PR fragments were electrophoresed on 10 or 7.5% polyacrylamide SDS gels as previously described (23, 28, 67). Separated proteins were transferred to nitrocellulose paper and detected by Western blot assays with receptor-specific MAb (B-30, AB-52, N-559, or C-262) using either immunoblot and detected by Western blot assays with receptor-specific polyacrylamide SDS gels as previously described (23, 28, 67). PR and PR fragments were electrophoresed on 10 or 7.5% polyacrylamide SDS gels as previously described (23, 28, 67). PR and PR fragments were electrophoresed on 10 or 7.5% polyacrylamide SDS gels as previously described (23, 28, 67).

**Coimmunoprecipitation of in Vitro Synthesized LBD with a Monoclonal Antibody to hsp90**

The LBD of PR was synthesized in vitro by a coupled transcription/translation assay using the Promega TNT kit. The plasmid for in vitro synthesis was pT7phLBD for the LBD of PR (aa 668–933). The LBD was transcribed by T7 RNA polymerase and RNA was translated in nuclease treated rabbit reticulocyte lysates in the presence of [35S]methionine (32). To detect hsp90 complexes, translated LBD was coimmunoprecipitated with the 3G3 MAb to hsp90 as described by Perdew et al. (36) and radiolabeled LBD was detected by SDS gel electrophoresis and autoradiography.

**Data Analysis**

For the results from coimmunoprecipitation and IMAC pull-down assays, comparison was done by ANOVA using Excel 5.0 (Microsoft, Redmond, WA) to determine whether there was a significant difference among groups. Bound and free concentrations of [3H]progesterone were calculated using Quattro Pro (Borland International Inc., Scotts Valley, CA). The receptor concentration (Bmax) and Hill coefficient were calculated by nonlinear regression using Enzfitter (Elsevier-Biosoft, Cambridge, UK). The association constant (Kd) was calculated from the limiting slope of the Scatchard plot (68). The results for the different PR constructs were compared by ANOVA followed by Bonferroni’s test for all pairwise comparisons using SigmaStat (v 1.0, Jandel Scientific, San Rafael, CA). Positive results were statistically significant at a probability of less than 0.05.

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