The Steroid Binding Domain Influences Intracellular Solubility of the Baculovirus Overexpressed Glucocorticoid and Mineralocorticoid Receptors†

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ABSTRACT: The expression and formation of the oligomeric steroid binding form of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) were further examined in the baculovirus expression system. Analysis of the steroid binding ability and accumulation of the GR and MR in recombinant baculovirus-infected insect cells revealed that only 0.35% of the total expressed receptors are actually assembled into cytoplasmic oligomeric receptor complexes. The majority of the overexpressed GR and MR, which amounts to 50 pg/cell, appear to self-aggregate and form insoluble aggregates which fractionate with the nucleus. We believe that this large amount of receptors far exceeds the limited amounts of hsp90, hsp70, and other cellular factors which are required to assemble the heteromeric receptor complex. Attempts to assemble the GR and the MR *in vivo* by coexpression of the receptors with hsp90 or hsp70 failed to cause any increase in the formation of the steroid binding receptor complex and also in preventing the aggregation of the receptors. On the other hand, *in vitro* incubation of monomeric GR or MR partially purified from the insoluble receptor aggregates with reticulocyte lysate resulted in complete reconstitution of the oligomeric receptor complex with a concomitant restoration of full steroid binding ability. These data suggest that interaction of hsp90 with the GR or the MR is complex and highly regulated and requires the participation of other cellular factors which are limited in insect cells but can be supplied *in vitro* by reticulocyte lysate. Since hsp90 interacts with the GR and probably with the MR through the C-terminal steroid binding domain, we examined the possibility that this domain may cause the receptor to self-aggregate in the absence of hsp90 interaction. Deletion of the entire steroid binding domain resulted in overexpression of truncated GR and MR which did not form insoluble aggregates. The truncated GR and MR were associated with the nucleus as DNA binding proteins which can be extracted and solubilized with low concentrations of $MgCl_2$.

The glucocorticoid receptor (GR)[1] and the mineralocorticoid receptor (MR) are members of the steroid/thyroid hormone receptor superfamily of intracellular signal transducing transcriptional factors (Beato, 1989; Carson-Jurica et al., 1990; Evans, 1988; Yamamoto, 1985). Functional dissection of the steroid receptor sequences has led to assignment of several functional domains within their primary amino acid sequence. The DNA binding domain and the N-terminal transcriptional activation domain are important for binding to specific DNA sequences and transcriptional activation and interaction with other transcriptional factors (Beato, 1989; Hollenberg & Evans, 1988; Hollenberg et al., 1987). The ability to bind hormone is located within the C-terminal steroid binding domain which contains sequences important for interaction with hsp90 and also with other transcription factors (Giguére et al., 1986; Howard et al., 1990; Rusconi & Yamamoto, 1987; Webster et al., 1988). The GR and MR exist as soluble oligomeric receptor complexes which upon binding to hormone undergo an activation or transformation process that causes the dissociation of hsp90 and translocation of the receptors to the nucleus (Bodine & Litwack, 1990; Picard et al., 1988; Pratt, 1987; Rafestin-Oblin et al., 1989). So far, the mechanism of formation of the oligomeric receptor complex is not yet fully established. Interaction with hsp90 is believed to be the driving force for assembly of the steroid receptors into their oligomeric unactivated receptor complexes *in vivo* and *in vitro* (Pratt, 1990; Rexin et al., 1992; Scherrer et al., 1990; Smith et al., 1992). Recent studies have shown that, in the case of the progesterone receptor, hsp70 acting as a protein chaperone may be required for hsp90 binding to steroid receptors (Smith et al., 1992). In fact, there is some evidence that hsp70 and another p59 protein may be part of the unactivated oligomeric steroid receptor complex (Lebeau et al., 1992; Renoir et al., 1990; Rexin et al., 1992; Sanchez, 1990; Sanchez et al., 1990; Smith et al., 1992; Tai et al., 1986, 1992). However, the role of these proteins in the formation and maintenance of the oligomeric steroid receptor complex is not fully understood.

In this study we further examined the expression of the GR and MR in insect cells using the baculovirus expression system. Our data show that only a very small number of the total expressed receptors are actually assembled into the oligomeric steroid receptor complex probably due to limiting amounts of hsp70, hsp90, and other cellular factors. We suggest that the interaction of the steroid binding domain with hsp90 may be

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[1] Abbreviations: GR, glucocorticoid receptor; rGR, rat glucocorticoid receptor; MR, mineralocorticoid receptor; hMR, human renal mineralocorticoid receptor; hsp90, M_r = 90 kDa heat shock protein; hsp70, M_r = 70 kDa heat shock protein; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda* cells; TA, triamcinolone acetonide (9α-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)-bis(oxy)]pregna-1,4-diene-3,20-dione); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

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necessary to prevent the overexpressed receptor from self-associating and forming insoluble receptor aggregates.

MATERIALS AND METHODS

Materials. Untreated rabbit reticulocyte lysate was from Promega Corp. Creatine phosphate and creatine phosphokinase was from Sigma. All other materials and reagents used in this study have been previously described (Alnemri et al., 1991a,b).

Cell culture. Sf9 insect cells were grown, maintained, and infected with baculovirus as previously described (Alnemri et al., 1991a,b).

Construction of Recombinant Transfer Vectors and Recombinant Baculoviruses. The strategy for constructing the recombinant baculoviruses AcNPV–rGR and AcNPV–hMR which encode the full-length rGR and hMR, respectively, has been described previously (Alnemri et al., 1991a,b). Similar strategies were also used to construct recombinant baculoviruses expressing the human hsp90 and hsp70, the truncated hMR (amino acids 1–719), and the truncated hGR (amino acids 1–532). Briefly, the hsp90 cDNA was excised from the pKN1-3 vector (Rebbe et al., 1987) using *Sal*I and subcloned in a *Sal*I-cut pGEMEX (Promega) vector to create *Bam*HI and *Eco*RI sites at the 5′ and 3′ ends, respectively, of the hsp90 cDNA. The entire hsp90 cDNA was then subcloned in a *Bam*HI/*Eco*RI-cut pVL1393 to generate the baculovirus transfer vector pVL1393–hsp90. The cDNA for the human hsp70 contained in the plasmid pH2.3 (Hunt & Morimoto, 1985) was excised using *Bam*HI at −64 and *Eco*RI at +2379 and subcloned in a *Bam*HI/*Eco*RI-cut pVL1393 to generate the baculovirus transfer vector pVL1393–hsp70. The cDNA for the truncated hGR lacking the entire steroid binding domain was excised from the HG3 vector (Kumar et al., 1987) using *Eco*RI and subcloned in an *Eco*RI-cut pVL1393 to generate the transfer vector pVL1393–HG3. The transfer vector pVL1393–HM2 encoding the truncated hMR which lacks the entire steroid binding domain was constructed from the transfer vector pVL1393–hMR (Alnemri et al., 1991b) by deleting the sequences 3′ to the *Hin*cII site at position +2157 relative to the ATG start site. The amber TAG termination codon present in the polylinker of pVL1393 as part of the *Xba*I restriction site was incorporated in frame with the hMR sequence 18 nucleotides downstream of the *Hin*cII site by employing several subcloning steps. This resulted in the destruction of the *Hin*cII site and the addition of the following extra amino acids at the C-terminus of the truncated MR: GIPGTF. The recombinant baculoviruses AcNPV–hsp90, AcNPV–hsp70, AcNPV–HG3, and AcNPV–HM2 containing the hsp90, the hsp70, the truncated hGR, and the truncated hMR sequences, respectively, under the transcriptional control of the polyhedrin promoter were produced by in vivo homologous recombination as previously described (Alnemri et al., 1991a; Summers & Smith, 1987).

Preparation of Cytosol, Nuclei, and Nuclear Extracts. Sf9 cells infected with the recombinant baculoviruses were harvested routinely 16–72 h postinfection. Cytosol was prepared in KP buffer (50 mM potassium phosphate, pH 7.0, 5 mM dithiothreitol, and 1 mM PMSF, with or without 20 mM molybdic acid) as previously described (Alnemri et al., 1991a,b). Nuclei were prepared by lysing the cells in HEPES/Nonidet P-40 buffer (50 mM HEPES, pH 7.4, 1 mM PMSF, and 1% Nonidet P-40) on ice for 10 min. The lysed cells were then centrifuged at 1000*g*, and the nuclear pellet was washed once in HEPES buffer without Nonidet P-40. Samples of nuclei were lysed in SDS–sample buffer and analyzed for protein content by SDS–PAGE. $MgCl_2$ nuclear extracts were prepared by extracting the nuclei in HEPES buffer containing 50 mM $MgCl_2$ on ice with occasional vortexing for 30 min. After extraction, the nuclei were removed by centrifugation at 1000*g*; the supernatant was collected, and samples were analyzed on SDS–polyacrylamide gel. Guanidine hydrochloride nuclear extracts were prepared by lysing the nuclei in HEPES buffer containing 6 M guanidine hydrochloride and 20 mM DTT. The lysate was then dialyzed against high-salt renaturation buffer A (50 mM HEPES, pH 7.4, 1 mM PMSF, 700 mM KCl, 0.1 mM $ZnCl_2$, and 5 mM DTT) overnight at 4 °C. The renatured receptors were then dialyzed against low-salt renaturation buffer B (50 mM HEPES, pH 7.4, 1 mM PMSF, 150 mM KCl, 0.1 mM $ZnCl_2$, and 5 mM DTT) for 4 h at 4 °C. The dialyzed receptor solution was clarified by centrifugation at 16000*g* and stored at −80 °C in 20% glycerol. The receptor concentrations were determined by Coomassie staining on SDS–polyacrylamide gels.

RESULTS AND DISCUSSION

Assembly of the MR and GR into Their Oligomeric Steroid Binding Receptor Complexes in an Overexpression System. In our earlier experiments we were following the expression of the GR and the MR in insect cells primarily by measuring steroid binding in the cytosol as a function of time after infection (Alnemri et al., 1991a,b). Although our immunofluorescence and Western blot analysis data indicated that the maximum amount of receptor is expressed at 48 h postinfection, steroid binding was maximal at 24 h post-infection. This kind of discrepancy between the receptor accumulation and the steroid binding prompted us to determine the fate of the receptor expressed after 24 h postinfection. One possible explanation was that the cellular machinery of insect cells may handle and assemble a limited amount of receptor into the oligomeric steroid binding form due to the limited amounts of other cellular components of the receptor (hsp90, hsp70, p59, modulator, etc.). When the receptor expression is in excess, the extra receptor molecules will not be able to assemble into steroid binding oligomeric complexes and they may either translocate to the nucleus and bind to the DNA or self-associate to form insoluble cytoplasmic or nuclear receptor particles which can be recovered with the nuclear pellet. To test these possibilities, insect cells infected with baculoviruses encoding either the GR or the MR were lysed at 16–72 h post-infection, and the receptor content in the cytosol was determined by steroid binding, whereas the receptor fractionating with the nuclear fraction was determined by SDS–PAGE and Coomassie staining. As shown in Figure 1A, both MR and GR acquire maximum cytosolic steroid binding ability at 24 h post-infection which deteriorates afterwards. When the amount of receptor was calculated on the basis of maximum specific steroid binding, we found that the receptor concentration in the cytosol is about 5–7 pmol/mg of protein, which is equivalent to $\sim 1.2 \times 10^6$ receptor molecules/Sf9 cell. This amount of receptor is undetectable by Coomassie staining when analyzed by SDS–PAGE. Interestingly, when the nuclei of the same samples shown in Figure 1A were lysed and electrophoresed in SDS–polyacrylamide gel followed by Coomassie staining, large amounts of GR and MR amounting to 30–50% of total nuclear proteins were detected at 48–72 h post-infection (Figure 1B, lanes 4, 5, 8, and 9). This large amount of receptor was not detected previously because it is apparently present as insoluble particles which remained with the nuclear pellets after cytosol preparations. It is estimated from the intensity of the Coomassie-stained receptor band that Sf9 cells express about 350 million

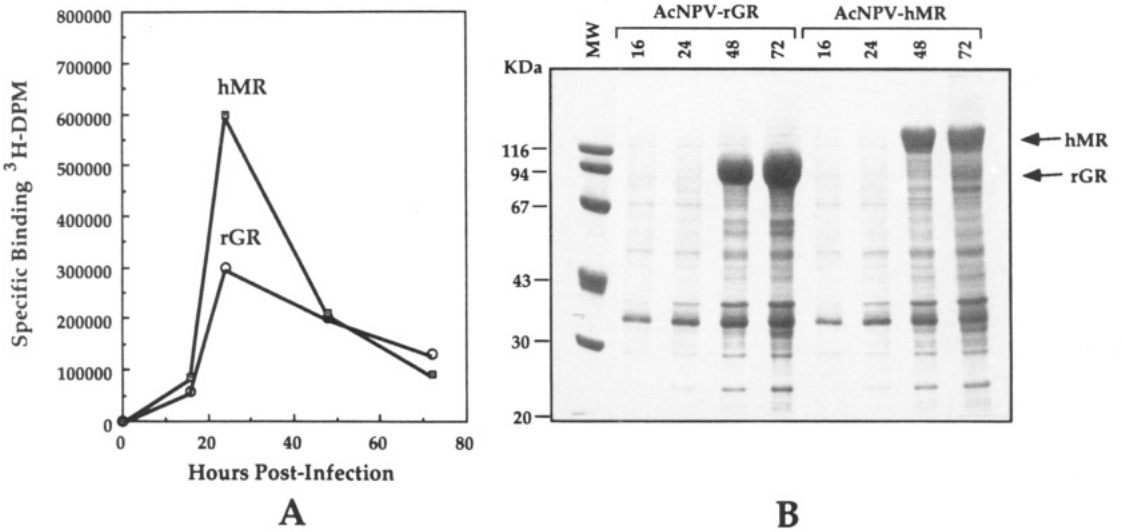

FIGURE 1: Time course analysis of the expression of recombinant rGR and hMR in baculovirus-infected Sf9 cells. Sf9 cells infected with either of the recombinant baculoviruses AcNPV-rGR or AcNPV-hMR were harvested at 16–72 h postinfection. Cells were fractionated into cytosolic (A) and nuclear (B) fractions as described under Materials and Methods. (A) Aliquots of the cytosolic fractions were incubated with 50 nM [^{3}H]TA in the case of rGR or with 100 nM [^{3}H]aldosterone in the case of hMR in the presence or absence of a 500-fold excess of nonradioactive steroid. Specific steroid binding was determined by the hydroxyapatite technique. Each point represents an average of two determinations per 2.5×10^6 cells. Nonspecific steroid binding was less than 5% of total steroid binding in all experiments. (B) Coomassie staining of total nuclear fraction proteins prepared from the same cells used in (A) to prepare the cytosol. The nuclei were lysed in SDS–sample buffer and analyzed on 10% SDS–polyacrylamide gel. Each sample is equivalent to 3×10^5 nuclei. Lanes 2–5 nuclear proteins from cells infected with AcNPV-rGR virus; lanes 6–9, nuclear proteins from cells infected with AcNPV-hMR virus. Molecular mass markers are shown in B (lane 1, MW). Time in hours is indicated at the top of each lane.

receptor molecules/cell. Based on the above estimations, only 0.35% of the expressed receptor is assembled into the soluble oligomeric steroid binding form which can be recovered with the cytosol. As we have shown previously (Alnemri et al., 1991a,b), the insect-expressed oligomeric steroid binding forms of both the MR and the GR are indistinguishable from their mammalian counterparts with respect to size, sedimentation coefficient, hormone binding and specificity, and *in vitro* activation mechanism. Our data suggest that insect cells, like mammalian cells, can assemble only limited amounts of receptor into the oligomeric steroid binding form depending on the cellular contents of other components of the receptor complex. The decline in steroid binding observed after 24 h postinfection (Figure 1A) is possibly due to the following reasons: first, destabilization of the oligomeric receptor complex due to virus-induced shutdown of cellular protein synthesis which is necessary for assembly and maintenance of the receptor complex and second, expression of large amounts of receptor, which may lead to competition between the receptor steroid binding domains and hsp90 for association with other steroid binding domains. As described below, the steroid binding domain is necessary for receptor self-association and aggregation. Pratt and co-workers have shown that when the GR is overexpressed in CHO cells, a large amount of the receptor translocates and resides in the nucleus (Sanchez et al., 1990) and the amounts of the oligomeric steroid binding form never exceed 9 pmol/mg of protein.

Effect of Coexpression of hsp90 and GR or MR on Their Solubility and Steroid Binding Ability. Most steroid receptors have been shown to contain a dimer of hsp90 as part of their oligomeric steroid binding form (Carson-Jurica et al., 1990; Pratt, 1990). Therefore, it is possible that the failure of insect or mammalian cells to assemble all synthesized receptor monomers into oligomeric steroid binding complexes may be due to the limited amounts of free hsp90. To test this possibility, we have overexpressed the human hsp90 in insect cells. When Sf9 cells are infected with the recombinant baculovirus AcNPV–hsp90, hsp90 is overexpressed as a soluble protein to almost 50% of total cytoplasmic proteins (Figure 2A, lane 5). The expression of hsp90 has also exceeded the expression of the polyhedrin protein in the wild type virus-infected Sf9 cells (Figure 2A, lane 2). No hsp90 was detected in the nucleus of AcNPV-hsp90 recombinant virus-infected cells (Figure 2A, lane 4). Unfortunately, when hsp90 and GR or MR were coexpressed in insect cells, there was no increase in the assembly of the receptor monomers into the steroid binding complex, as determined by steroid binding studies (Table I) and by SDS–PAGE (Figure 2B). Table I shows that, at 48 h postinfection, the steroid binding of GR or MR coexpressed with hsp90 was only 10% higher than the binding of GR or MR in the absence of hsp90 coexpression. The same samples shown in Table I were further divided into cytoplasmic and nuclear fractions and analyzed by SDS–PAGE. The results of this experiment for the MR are shown in Figure 2B. At 24 h postinfection, there was no Coomassie stain-detectable expression of either hMR or hsp90 in both the cytosolic (lanes 2 and 3) or the nuclear fractions (lanes 6 and 7). However, at 48 h postinfection, hsp90 was detected in the cytosolic fraction (lane 5) and hMR expression was detectable in the nuclear fraction (lane 8 and 9). Both hsp90 and hMR are expressed to almost the same extent as determined in total cellular lysates (lane 10). It is evident that there was no detectable increase in the soluble cytoplasmic receptor at 48 h after coexpression with hsp90 (lane 5). Also, hsp90 coexpression was unable to prevent the accumulation of the insoluble particulate receptor at 48 h postinfection (Figure 2B, lane 9). Similar results were obtained with the GR (data not shown).

Our data suggest that hsp90 may not be the only factor which determines the formation of the active oligomeric steroid binding receptor form and that additional factors, which are necessary for correct assembly and formation of active steroid binding complex, are still limited. Similar observations were previously obtained by us using the GR in a reticulocyte lysate translation system (Daniel et al., 1991) and by Toft and co-workers using the progesterone receptor in an *in vitro* assembly

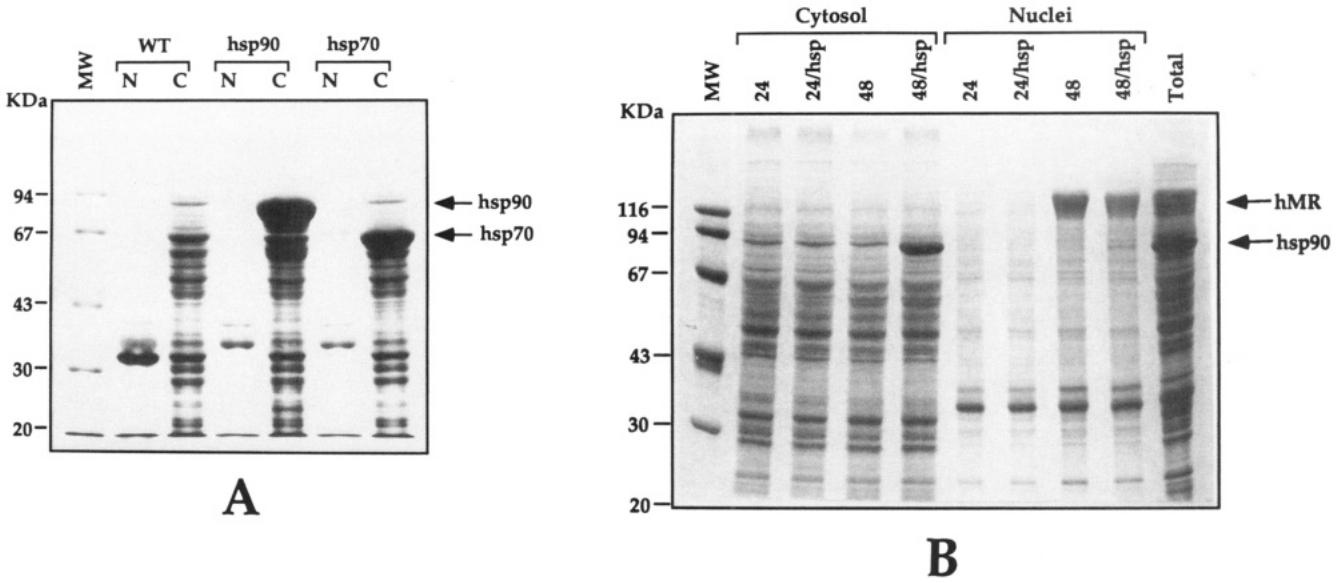

FIGURE 2: Coexpression of hsp90 with hMR in Sf9 cells. (A) Sf9 cells infected with either the wild-type baculovirus (WT, lanes 2 and 3) or the recombinant baculoviruses AcNPV-hsp90 (hsp90, lanes 4 and 5) or AcNPV-hsp70 (hsp70, lanes 6 and 7) were harvested at 48 h postinfection. Cells were fractionated into cytosolic (C) and nuclear (N) fractions. Aliquots of the nuclear and cytosolic fractions were separated on 10% SDS–polyacrylamide gel and visualized by Coomassie staining. Each sample is equivalent to 3×10^5 cells or nuclei. (B) Sf9 cells either infected with AcNPV-hMR alone (lanes 2, 4, 6 & 8) or coinfected with the recombinant baculoviruses AcNPV-hMR and AcNPV-hsp90 (lanes 3, 5, 7, and 9) were harvested at 24 and 48 h postinfection. Cells were fractionated into cytosolic (Cytosol) and nuclear (Nuclei) fractions and analyzed as above and as described in footnote *a* of Table I. Lane 10 contains total cell lysate from cells coinfected with hMR and hsp90 baculoviruses and harvested at 48 h postinfection. Molecular mass markers are shown in A and B (lane 1, MW). Time in hours is indicated at the top of each lane.

Table I: Steroid Binding Activity of rGR and hMR Coexpressed with hsp90[a]

	rGR (dpm)		hMR (dpm)	
condition	24 h	48 h	24 h	48 h
–hsp90	103 103	59 063	161 409	85 847
+hsp90	103 222	66 911	100 206	97 188

[a] Cytosols from Sf9 cells infected with either AcNPV-rGR or AcNPV-hMR (–hsp90) alone or coinfected with the recombinant baculoviruses AcNPV-rGR and AcNPV-hsp90 (+hsp90) or AcNPV-hMR and AcNPV-hsp90 (+hsp90) were prepared at 24 and 48 h postinfection. Steroid binding was determined as described in the caption to Figure 1. The nuclear pellet proteins from the same cells were analyzed by SDS–PAGE as described in the caption to Figure 2.

system (Smith et al., 1992). In the latter study with the assembly of the progesterone receptor, Toft and co-workers have suggested that hsp70 may also be required as a protein chaperone in order for hsp90 to bind to PR, and this process may also require ATP and divalent cations.

Although the probability of efficient coexpression of two proteins in the same cell using two recombinant baculoviruses is more than 95% (Miller, 1988; O'Reilly & Miller, 1988; St. Angelo et al., 1987), coexpression of three or more recombinant viruses may not be controllable or feasible. Therefore, to address the question of assembly of steroid receptors *in vivo*, it is necessary to coexpress all components of the receptor, which may prove to be difficult. The other alternative is to overexpress all receptor components individually and then study the receptor assembly *in vitro*. We have already overexpressed the human hsp70 in our laboratory (Figure 2A). Coexpression of hsp70 and GR or MR also failed to increase the formation of the oligomeric steroid binding complex *in vivo* (data not shown).

In Vitro Assembly of GR and MR. To determine whether the overexpressed insoluble GR or MR can be assembled into the oligomeric steroid binding form *in vitro*, nuclei from Sf9 cells infected with the GR or MR baculoviruses were solubilized in 6 M guanidine hydrochloride. The solubilized materials were dialyzed against a renaturation buffer, which resulted in the precipitation of many insoluble nuclear proteins and the recovery of the receptors as soluble 85% pure proteins. The renatured receptors can bind glucocorticoid-responsive elements with high specificity and affinity (data not shown). The partially purified and renatured receptors were then incubated for 40 min at room temperature with reticulocyte lysate supplemented with an energy regeneration system. Figure 3A shows a dose–response histogram of steroid binding in reticulocyte lysate as a function of increasing concentrations of added GR. The ability of reticulocyte lysate to assemble the receptor into a steroid binding form was reflected by a dose-dependent increase in steroid binding as a function of the amounts of added monomeric GR. A linear increase in steroid binding was observed at concentrations of GR below 0.4 μg/25 μL of reticulocyte lysate. At GR concentrations higher than 0.8 μg/25 μL of reticulocyte lysate there was a decline in the assembly of the receptor. The inhibitory effect of GR at higher concentrations may reflect the limited ability of reticulocyte lysate to assemble a limited amount of receptors. This effect may be due to the competition between the added receptor molecules for a limited amount of hsp90 or hsp70 which may result in the formation of intermediate steroid nonbinding forms. Similar results were obtained with the MR. Since it is well known that the unactivated oligomeric steroid binding form of the GR or the MR elutes from an anion exchanger such as DEAE-cellulose at salt concentrations of 200–250 mM, compared with the activated form, which elutes at 50–100 mM, we decided to analyze the elution profile of the *in vitro* assembled GR on DEAE-cellulose. As expected, the *in vitro* assembled GR eluted at 200–250 mM potassium phosphate (Figure 3B), thus confirming the reverse transformation of the monomeric form of the GR to the oligomeric form. Although Toft's (Smith et al., 1992) and Pratt's (Hutchison et al., 1992) laboratories were first to demonstrate the possibility of conversion of the activated progesterone

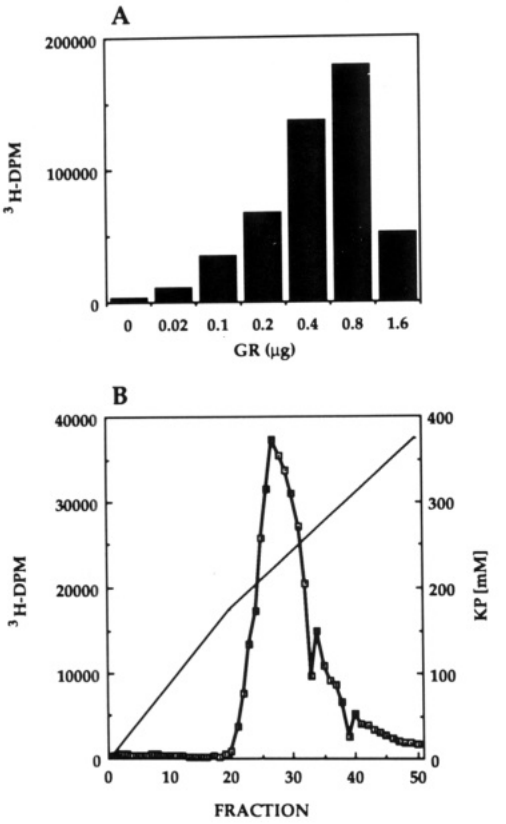

FIGURE 3: Assembly of GR in reticulocyte lysate. Partially purified GR (85% homogeneity) was incubated with reticulocyte lysate in the presence of an ATP regeneration system at room temperature for 40 min. The lysate was diluted with 50 mM KP buffer containing 20 mM sodium molybdate and 5 mM DTT and then labeled with [^{3}H]-TA for 2 h on ice. In (A), different concentrations of GR (4 μL of each sample) were mixed with 25 μL of reticulocyte lysate and incubated as described above. Steroid binding was determined as described in the caption to Figure 1. In (B), 3 μg of GR was assembled in 100 μL of reticulocyte lysate as described above. The lysate was then loaded onto a 3-mL DEAE–cellulose column, washed with 30 mL of 5 mM KP, and eluted with a 50-mL linear gradient (5–500 mM KP and 10 mM molybdate, pH 7.4). One-milliliter fractions were collected, and 0.5-mL aliquots of each fraction were counted by liquid scintillation.

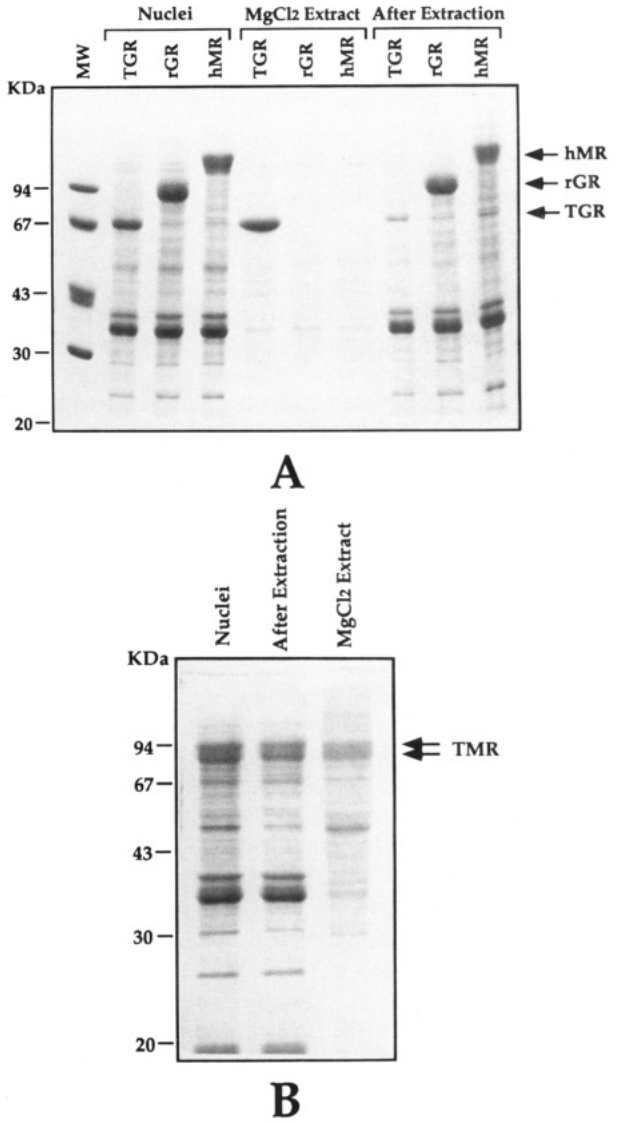

FIGURE 4: Deletion of the steroid binding domain results in expression of extractable truncated GR and MR. Sf9 cells infected with truncated GR (TGR, lanes 2, 5, and 8 in panel A), rGR (lanes 3, 6, and 9 in panel A), hMR (lanes 4, 7, and 10 in panel A), or truncated MR (TMR, lanes 1, 2, and 3 in panel B) recombinant baculoviruses were harvested at 48 h postinfection. Cells were lysed, and nuclei were prepared, as described under Materials and Methods. The nuclei were extracted with 50 mM $MgCl_2$, and aliquots of nuclei, $MgCl_2$ nuclear extracts, and extracted nuclei were separated on SDS–polyacrylamide gel and visualized by Coomassie staining. Each sample is equivalent to 3×10^5 nuclei. Molecular mass markers are shown in A (lane 1, MW).

receptor and GR to the oligomeric unactivated forms in reticulocyte lysate, their systems involved immunopurification and immobilization of the receptors on an antibody. Our data demonstrate for the first time that it is possible to convert or reverse transform the GR or the MR to the oligomeric form in solution. These data suggest that the GR or the MR can self-renature and refold properly into an active DNA binding protein and further be converted by association with other cellular components into an oligomeric steroid binding form. Also, the receptor does not need to be anchored or immobilized onto a static structure in order for it to be assembled into the oligomeric form. This system should allow detailed analysis of the mechanism of assembly, such as involvement of intermediate forms, effect of phosphorylation/dephosphorylation, and rate-limiting components of the assembly system. Work is underway to study the assembly of the GR and the MR *in vitro* using overexpressed receptor monomers, hsp90, hsp70, modulator, and fractionated mammalian cytosol to supply other receptor components such as p59 (Lebeau et al., 1992; Renoir et al., 1990; Tai et al., 1986, 1992).

Deletion of the Steroid Binding Domain Results in the Expression of Extractable Truncated Receptors. In an overexpression system where hsp90, hsp70, and other cellular factors are limiting, association of the receptor with the nuclear fraction may be due to one of two possibilities. First, the extra receptor molecules may behave as activated receptor monomers and therefore translocate and bind to the nuclear chromatin. Alternatively, in the absence of hsp90, interaction with the steroid binding domain and the high hydrophobicity of this domain may cause the GR and MR monomers to self-associate through the steroid binding domain and form insoluble receptor particles or aggregates which copurify with the nuclear fraction. To test the first possibility, we used a hypotonic buffer containing 50 mM $MgCl_2$ to extract the GR and the MR from the nuclear fractions. Low concentrations of $MgCl_2$ have been shown to disrupt the DNA binding of GR and to result in the extraction of GR from mammalian nuclei and from DNA–cellulose (Stevens et al., 1978; Wrange et al., 1984). As shown in Figure 4A, lanes 6 and 7, there was no

detectable GR or MR in the nuclear extracts, and all receptor apparently remained associated with the nuclear pellets (lanes 9 and 10). The GR and the MR remained associated with the nuclear fractions even after sucrose gradient ultracentrifugation in 1.6 or 2 M sucrose cushions, suggesting that the receptors have the same density as the nuclei or were physically tightly associated with the nuclei (data not shown). In addition, extractions with high salt failed to solubilize the particulate receptors. As we mentioned above, 6 M guanidine hydrochloride was the only reagent we used that resulted in complete solubilization of the particulate receptor. We were able to purify the guanidine hydrochloride solubilized MR and GR to more than 90% homogeniety with a single gel filtration step. Complete renaturation and recovery of full DNA binding activity was achieved after removal of guanidine hydrochloride (data not shown). Similar observations were reported for the androgen receptor (Xie et al., 1992). Interestingly, in insect Sf9 cells, the minor cytoplasmic oligomeric steroid binding forms of the GR or MR can be activated *in vivo* by treatment with steroid, which results in the translocation of the receptor to the nucleus (Alnemri et al., 1991a; Alnemri et al., 1991b). In contrast to the major insoluble particulate receptor, the minor steroid-activated nuclear form of the receptor can be extracted from the nuclei by $MgCl_2$, suggesting that those forms of receptor are bound to the nuclear chromatin (data not shown). Our data suggest that the majority of the overexpressed GR and MR are not bound to the chromatin as activated receptors but are self-associated probably through the hydrophobic steroid binding domains, to form insoluble particulate receptor proteins.

To address the role of the steroid binding domain in the self-association and formation of insoluble GR or MR, we overexpressed truncated GR and MR, lacking the steroid binding domain. The overexpressed truncated GR and MR are both associated with the nuclear fraction (Figure 4A, lane 2, and Figure 4B, lane 1). Similar to our previous observations with the full-length MR (Alnemri et al., 1991b), the truncated MR lacking the steroid binding domain is seen to migrate in SDS–gel as multiple isoforms ranging in molecular mass from 85 to 90 kDa, suggesting that the posttranslational modifications are localized in the N-terminus. As predicted, deletion of the steroid binding domain resulted in the expression of truncated receptors which are probably bound to the nuclear chromatin. In contrast to the full-length receptors, the overexpressed truncated GR and MR can be extracted by hypotonic buffer containing 50 mM $MgCl_2$. Figure 4A, lane 5, shows that more than 90% of the truncated GR can be extracted from the nuclei by $MgCl_2$. The ability of $MgCl_2$ to extract the truncated receptors suggests that these receptors are associated with the nucleus as DNA binding proteins. The lack of the hydrophobic steroid binding domain may have allowed the truncated receptors to translocate to the nucleus as activated DNA binding proteins. In fact, the truncated GR lacking the steroid binding domain has been shown to act as a constitutive steroid-independent nuclear DNA binding transactivator (Hollenberg et al., 1987; Kumar et al., 1987). Similar results were obtained with the truncated MR, although the amount of extractable truncated MR is only 40–50% of the total nuclear receptor (Figure 4B, lane 3). This could be attributed to the higher molecular mass of the truncated MR (85–90 kDa) compared to the truncated GR (65 kDa) or to the higher hydrophobicity of the MR N-terminus which may reduce its extractability with $MgCl_2$. Our data suggest that the physiological significance of hsp90 association with the hydrophobic steroid binding domain of steroid receptors may be not only to keep the receptors in a docking complex ready to bind steroid but also to prevent the receptors from self-association or aggregation. It is also possible that association of the receptor with hsp90 is necessary to prevent the receptor from binding to cytoskeletal proteins such as actin and tubulin. In fact, while this paper was in revision, Scherrer and Pratt (1992) showed that the monomeric transformed GR can be converted to a particulate form through association with cytoskeletal proteins and that this process requires the presence of the steroid binding domain. Consequently, aggregation of the full-length GR or MR is due to the absence of proper association with hsp90, hsp70, and other cellular components. Therefore, in the absence of the steroid binding domain the receptors are relieved of self-interaction or association with cytoskeletal proteins and are expressed in a soluble form. It is worth mentioning that the truncated GR and to a lesser extent the truncated MR can be purified from the Sf9 nuclei to at least 80% purity in a single step. These two forms of the receptor can bind the GRE in a gel shift assay (data not shown). However, it appears that GR binds much tighter than MR and can compete with MR for binding to the GREs (E. S. Alnemri and G. Litwack, manuscript in preparation). This suggests that the MR may have specific MREs, which are different from GREs. Further work on the specificity and kinetics of interactions of the MR and the GR with GREs is underway.

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REFERENCES

Alnemri, E. S., Maksymowych, A. B., Robertson, N. M., & Litwack, G. (1991a) *J. Biol. Chem. 266*, 3925–3936.
Alnemri, E. S., Maksymowych, A. B., Robertson, N. M., & Litwack, G. (1991b) *J. Biol. Chem. 266*, 18072–18081.
Beato, M. (1989) *Cell 56*, 335–344.
Bodine, P. V., & Litwack, G. (1990) *Receptor 1*, 83–119.
Carson-Jurica, M. A., Schrader, W. T., & O'Malley, B. W. (1990) *Endocr. Rev. 11*, 201–220.
Daniel, V., Maksymowych, A. B., Alnemri, E. S., & Litwack, G. (1991) *J. Biol. Chem. 66*, 1320–1325.
Evans, R. M. (1988) *Science 240*, 889–895.
Giguére, V., Hollenberg, S. M., Rosenfeld, M. G., & Evans, R. M. (1986) *Cell 46*, 645–652.
Hollenberg, S. M., & Evans, R. M. (1988) *Cell 55*, 899–906.
Hollenberg, S. M., Giguére, V., Segui, P., & Evans, R. M. (1987) *Cell 49*, 39–46.
Howard, K. J., Holley, S. J., Yamamoto, K. R., & Distelhorst, C. W. (1990) *J. Biol. Chem. 265*, 11928–11935.
Hunt, C., & Morimoto, R. I. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6455–6459.
Hutchison, K. A., Czar, M. J., Scherrer, L. C., & Pratt, W. B. (1992) *J. Biol. Chem. 267*, 14047–14053.
Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R., & Chambon, P. (1987) *Cell 51*, 941–951.
Lebeau, M.-C., Massol, N., Herrick, J., Faber, L., Renoir, J.-M., Radanyi, C., & Baulieu, E.-E. (1992) *J. Biol. Chem. 267*, 4281–4284.
Miller, L. K. (1988) *Annu. Rev. Microbiol. 2*, 177–199.
O'Reilly, D. R., & Miller, L. K. (1988) *J. Virol. 62*, 3109–3119.
Picard, D., Salser, S. J., & Yamamoto, K. R. (1988) *Cell 54*, 1073–1080.
Pratt, W. B. (1987) *J. Cell. Biochem. 35*, 51–68.
Pratt, W. B. (1990) *Mol. Cell. Endocrinol. 74*, C69–C76.

Rafestin-Oblin, M.-E., Couette, B., Radanyi, C., Lombes, M., & Baulieu, E.-E. (1989) *J. Biol. Chem. 264*, 9304–9309.
Rebbe, N. F., Ware, J., Bertina, R. M., Modrich, P., & Stafford, D. W. (1987) *Gene 53*, 235–245.
Renoir, J.-M., Radanyi, C., Faber, L., & Baulieu, E.-E. (1990) *J. Biol. Chem. 265*, 10740–10745.
Rexin, M., Busch, W., Segnitz, B., & Gehring, U. (1992) *J. Biol. Chem. 267*, 9619–9621.
Rusconi, S., & Yamamoto, K. R. (1987) *EMBO J. 6*, 1309–1315.
Sanchez, E. R. (1990) *J. Biol. Chem. 265*, 22067–22070.
Sanchez, E. R., Hirst, M., Scherrer, L. C., Tang, H.-Y., Welsh, M. J., Harmon, J. M., Simons, S. S., Jr., Ringold, G. M., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 20123–20130.
Scherrer, L. C., & Pratt, W. B. (1992) *Biochemistry 31*, 10879–10886.
Scherrer, L. C., Dalman, F. C., Massa, E., Meshinchi, S., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 21397–21400.
Smith, D. F., Stensgard, B. A., Welch, W. G., & Toft, D. O. (1992) *J. Biol. Chem. 267*, 1350–1356.
St. Angelo, C. S., Smith, G. E., Summers, M. D., & Krug, R. M. (1987) *J. Virol. 61*, 361–365.
Stevens, J., Stevens, Y.-W., Rhodes, J., & Steiner, G. (1978) *J. Natl. Cancer Inst. 61*, 1477–1485.
Summers, M. D., & Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures* (Summers, M. D., & Smith, G. E., Eds.) Texas Experimental Station Bulletin No. 1555, Texas A&M University, College Station, TX.
Tai, P.-K., Maeda, Y., Nakao, K., Wakim, N. G., Duhring, J. L., & Faber, L. E. (1986) *Biochemistry 25*, 5269–5275.
Tai, P.-K. K., Albers, M. W., Chang, H., Faber, L. E., & Schreiber, S. L. (1992) *Science 256*, 1315–1318.
Webster, N. J. G., Green, S., Jin, J. R., & Chambon, P. (1988) *Cell 54*, 199–207.
Wrange, O., Okret, S., Radojcic, M., Carlstedt-Duke, J., & Gustafsson, J.-A. (1984) *J. Biol. Chem. 259*, 4534–4541.
Xie, Y.-B., Sui, Y.-P., Shan, L.-X., Palvimo, J. J., Phillips, D. M., & Janne, O. A. (1992) *J. Biol. Chem. 267*, 4939–4948.
Yamamoto, K. R. (1985) *Annu. Rev. Genet. 19*, 209–252.