J. Steroid Biochem. Molec. Biol. Vol. 37, No. 4, pp. 481–490, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

THE STEROID-BINDING PROPERTIES OF RECOMBINANT GLUCOCORTICOID RECEPTOR: A PUTATIVE ROLE FOR HEAT SHOCK PROTEIN hsp90

YUKO OHARA-NEMOTO,* PER-ERIK STRÖMSTEDT, KARIN DAHLMAN-WRIGHT, TAKAYUKI NEMOTO,* JAN-ÅKE GUSTAFSSON and JAN CARLSTEDT-DUKE †

Department of Medical Nutrition and Center for Biotechnology, Karolinska Institutet, Huddinge University Hospital F60, Novum, S-141 86 Huddinge, Sweden

(Received 5 June 1990)

Summary—The steroid-binding domain of the human glucocorticoid receptor was expressed in *Escherichia coli* either as a fusion protein with protein A or under control of the T7 RNA polymerase promoter. The recombinant proteins were found to bind steroids with the normal specificity for a glucocorticoid receptor but with reduced affinity (K_d for triamcinolone acetonide ~ 70 nM). Glycerol gradient analysis of the *E. coli* lysate containing the recombinant protein indicated no interaction between the glucocorticoid receptor fragment and heat shock proteins. However, synthesis of the corresponding fragments of glucocorticoid receptor *in vitro* using rabbit reticulocyte lysate resulted in the formation of proteins that bound triamcinolone acetonide with high affinity (K_d 2 nM). Glycerol gradient analysis of these proteins, with and without molybdate, indicated that the *in vitro* synthesised receptor fragments formed complexes with hsp90 as previously shown for the full-length rat glucocorticoid receptor. Radiosequence analysis of the recombinant steroid-binding domain expressed in *E. coli* and affinity labelled with dexamethasone mesylate identified binding of the steroid to Cys-638 predominantly. However, all cysteine residues within the steroid-binding domain were affinity labelled to a certain degree indicating that the recombinant protein has a structure similar to the native receptor but more open and accessible.

INTRODUCTION

The glucocorticoid receptor (GR) is a signal transducer belonging to the steroid-receptor superfamily (for review see Ref. [1]). The domain structure of GR, consisting of the immunodominant, DNA-binding and steroid-binding domains, was first proposed following studies using limited proteolysis [2–4] and the domain borders determined by protein sequence analysis [5]. Sequencing of cDNAs for human [6], rat [7] and mouse [8] GRs and subsequent mutational analysis has given more information about the domains. The N-terminal, immunodominant domain has been shown to contain a transcriptional modulatory region called τ_1 [9, 10]. The central, DNA-binding domain recognises and binds to a specific DNA sequence, and is considered to form a zinc-finger structure [11–13]. A second transcriptional modulatory region, τ_2 [10, 14], has been identified within the N-terminal limit of the steroid-binding domain which is located at the C-terminal end of the receptor protein. The steroid-binding domain represses DNA-binding as well as the transactivation activity of the receptor in the absence of hormone [15]. Thus the role of the hormone-binding is now believed to primarily consist in turning off the repression by the steroid-binding domain [15].

The two ligand-binding domains bind their respective ligands, steroid and DNA, independently of other functional regions of the protein. Thus, the original proteolytic analysis showed that the steroid-binding domain could be isolated from the rest of the protein with bound hormone [16, 17]. Expression of truncated receptor protein lacking the steroid-binding domain resulted in constitutive DNA-binding and transcriptional activation, even following deletion of the N-terminal domain [9, 10, 18–20]. Expression of the isolated DNA-binding domain in *E. coli*, either as a fusion protein with protein A [21] or using the T7 polymerase system [12], resulted in a protein product with intact DNA-binding specificity.

*Present address: Department of Biochemistry, Iwate Medical University School of Dentistry, 19-1 Uchimaru, Morioka, Iwate 020, Japan.
†To whom correspondence should be addressed.

Several lines of evidence have shown that hsp90 is associated with the non-DNA-binding 9 S receptor (non-transformed or non-activated GR [22, 23]), and is lost on transformation from a 9 S form to a 4 S DNA-binding form [24, 25]. Dissociation of hsp90 is achieved under various conditions *in vitro*, and importantly, is accelerated by ligand-binding *in vitro* [25] and in cells [26]. The primary site of association of GR with hsp90 is located in the steroid-binding domain [27, 28]. Recently, Bresnick *et al.* [29] have reported that the association of hsp90 is essential for the steroid-binding ability of GR in mouse fibroblasts.

To investigate the fine structure of GR and the role of hsp90 for the steroid-binding ability, the steroid-binding domain of human GR (hGR) was expressed in *E. coli* and translated *in vitro*. Here we report the characteristics of the expressed proteins, and discuss the role of hsp90 in steroid-binding.

EXPERIMENTAL PROCEDURES

Materials

[6,7-^{3}H]Triamcinolone acetonide (TA; 29 Ci/mmol) and L-[^{35}S]methionine (1000 Ci/mmol) were purchased from Amersham Corp. [6,7-^{3}H]Dexamethasone mesylate (DM; 43.2 Ci/mmol) was from DuPont–New England Nuclear. IgG Sepharose 6 Fast Flow was from Pharmacia-LKB Biotechnology. Trypsin (sequencing grade), Asp-*N* endoproteinase and enzymes for the construction of plasmids were obtained from Boehringer Mannheim. T7 RNA polymerase and rabbit reticulocyte lysate were from Promega Biotech.

Construction of plasmids and bacterial strains

Fragments of the plasmid pRSVHGR carrying the full length cDNA for hGR (kindly provided by Dr Brian West, University of California, San Francisco, Calif.) were cloned into the pRIT vectors [30] to give the pEHGR series of plasmids. Proteins were expressed as C-terminal fusion-proteins with a part of protein A (32.5 kDa) which does not contain the signal sequence. The *Sph*I–*Xba*I fragment encoding amino acids 477–777 of hGR was cloned into pRIT32 cleaved with *Sma*I and *Xba*I (pEHGR4770). Plasmids pEHGR3700 [21] and pEHGR4160 (previously called pEHGR4150 [31]) have been described. To express the truncated GR fused with protein A, the *E. coli* strain MZ1, a strain containing the temperature-sensitive lambda repressor CI857, was transformed with the pEHGR plasmids.

Alternatively, to express the fragments of hGR without protein A in *E. coli* or to translate them *in vitro*, a plasmid pTHGR series was constructed. The *Sac*I–*Sal*I fragment of pEHGR3700 was introduced into the pT7-7 plasmid [32] cleaved with *Sma*I and *Sal*I, producing pTHGR3700, and the *Sac*I–*Sal*I fragment of pEHGR4770 introduced into pT7-7 cleaved with *Eco*RI and *Sal*I (pTHGR4770). *E. coli* K38[pGP1-2], expressing CI857 under the control of the lac UV5 promoter and T7 RNA polymerase under the control of the lambda P_L promoter, were transformed with the pTHGR plasmids. Proteins were expressed under the control of the T7 RNA polymerase promoter.

Expression of human GRs in E. coli

E. coli MZ1[pEHGR] were grown at 30°C in 250 ml of LB medium supplemented with 100 μg/ml of ampicillin, 1% (w/v) glucose and 1% (w/v) casamino acids. During the logarithmic growth phase, an equal volume of medium heated to 55°C was added to raise the temperature to 42°C, and the culture was maintained at 42°C for a further 2 h. *E. coli* K38[pGP1–2, pTHGR] were grown under the same conditions as the MZ1 strain with the addition of 50 μg/ml kanamycin. The production of the recombinant protein was also induced in the same way as for the MZ1 strain except that after 30 min, the temperature was lowered to 30°C and the cells incubated for a further 2 h. The cells were harvested, washed with ice-cold phosphate-buffered saline (pH 7.5), frozen in a dry ice–ethanol bath and stored at −80°C until used.

Preparation of cell lysate

The frozen cells from 100 ml bacterial culture were thawed at 0°C and suspended in 4 ml TEDG buffer (20 mM Tris–HCl, pH 7.5 at 0°C, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) or TEDGM buffer (TEDG buffer with 20 mM sodium molybdate) with 0.5 mM leupeptin, 1 μM pepstatin, 0.5 mM phenylmethanesulfonyl fluoride and 10 μg/ml soybean trypsin inhibitor. For [^{3}H]DM labelling, dithiothreitol was omitted from the lysis buffer (TEG buffer). Lysozyme, 1 mg/ml, was added. After 30 min at 0°C, the cells were freeze-thawed twice, sonicated briefly, and centrifuged at 150,000 *g* for 30 min. The resulting supernatant was used as bacterial lysate.

Transcription and translation in vitro

Transcription and translation *in vitro* were performed as described previously [33] with slight modifications. Briefly, plasmids of the pTHGR series were linearised with *Sal*I. 5 μg of the plasmid was used as a template and transcribed with T7 RNA polymerase at 37°C for 2 h, followed by phenol/chloroform extraction and ethanol precipitation. Transcripts were translated at 30°C in a final volume of 130 μl containing 90 μl of rabbit reticulocyte lysate, 20 μM each of 19 amino acids (methionine depleted), and 10 μM unlabelled methionine or 1 mCi/ml [^{35}S]methionine. After 1 h, the samples were cooled to 0°C and diluted 5-fold with TEDG or TEDGM buffer containing 1 mM leupeptin.

Steroid binding assay

Bacterial lysates or the *in vitro* synthesised products were incubated at 0°C with [^{3}H]TA in the presence or absence of a 200-fold molar excess of radioinert TA in a final volume of 50 μl. After 16–26 h for bacterial lysates or 2 h for the *in vitro* translated materials, 50 μl of hydroxylapatite suspension [50% (v/v) in TEDGM buffer] was added and incubated for 15 min at 0–4°C. After centrifugation, an aliquot of the supernatant was counted to quantitate free [^{3}H]TA concentration. The precipitate was washed four times with 1 ml TEDGM buffer. Bound [^{3}H]TA–receptor complexes were extracted with 0.2 ml 0.4 M potassium phosphate buffer (pH 7.3) and counted for radioactivity. The specific binding was determined by subtracting the binding in the presence of radioinert TA from that in the absence of radioinert TA.

Glycerol gradient centrifugation

Samples labelled with [^{3}H]TA were treated with dextran-coated charcoal and 200 μl aliquots were applied on linear 12.5–27.5% (v/v) glycerol gradients prepared in 20 mM Tris–HCl, (pH 7.5 at 0°C), 1 mM EDTA, 1 mM dithiothreitol, 0.15 M NaCl with or without 20 mM sodium molybdate. Gradients were centrifuged at 57,000 rpm for 16 h at 2°C in a Beckman SW60 rotor. Four-drop fractions were collected and counted.

SDS–PAGE and immunoblotting

SDS–PAGE was performed using a discontinuous system according to Laemmli [34] (4%(w/v) stacking gel and 12.5% (w/v) separating gel). Immunoblotting was performed to detect the protein A portion of fusion proteins as described previously [21].

Radiosequence analysis of affinity labelled PAHGR

The bacterial lysate (4 ml) was incubated with 200 nM [^{3}H]DM in TEG buffer at 0°C for 16 h. After charcoal adsorption of unbound ligand, the sample was filtrated through a 0.45 μm membrane filter and loaded onto an IgG Sepharose column (15 × 40 mm) equilibrated with 50 mM Tris–HCl (pH 7.5 at 0°C), 1 mM EDTA and 0.15 M NaCl. After washing with the same buffer, bound proteins were eluted with 0.5 M acetic acid and precipitated with 10% (w/v) trichloroacetic acid at 0°C. Cleavage with trypsin and chymotrypsin was performed as previously described [35]. Cleavage with Asp-*N* endoproteinase was carried out at a protein : enzyme ratio of 20 : 1 at 37°C for 18–20 h in 10% (v/v) acetonitrile and 10 mM ammonium bicarbonate. β-Lactoglobulin A was added as a carrier and internal standard for sequencing. The samples were analysed as previously described [35].

RESULTS

Characteristics of hGR expressed in bacteria

N-terminal truncated fragments of hGR comprising amino acids 370–777 (denoted as HGR370), 416–777 (HGR416) and 477–777 (HGR477) (Fig. 1) were expressed in *E. coli* as C-terminal fusion proteins with a part of protein A from *Staphylococcus aureus* (PAHGR). The amount of expressed proteins in the lysate

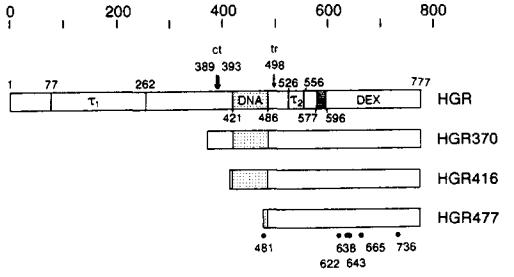

Fig. 1. Schematic illustration of human GR and its fragments expressed in *E. coli* and synthesised *in vitro*. The hGR and truncated fragments are schematically illustrated with putative domains including transactivation regions (τ_1 and τ_2), the steroid-binding domain (DEX) and the DNA-binding domain (DNA) [5, 10]. A region within the steroid-binding domain that is highly conserved among steroid receptors is represented by the striped box [8, 27]. Chymotrypsin (ct) amd trypsin (tr) cleavage sites are indicated by arrows [5], and cysteine residues within HGR477 by filled circles.

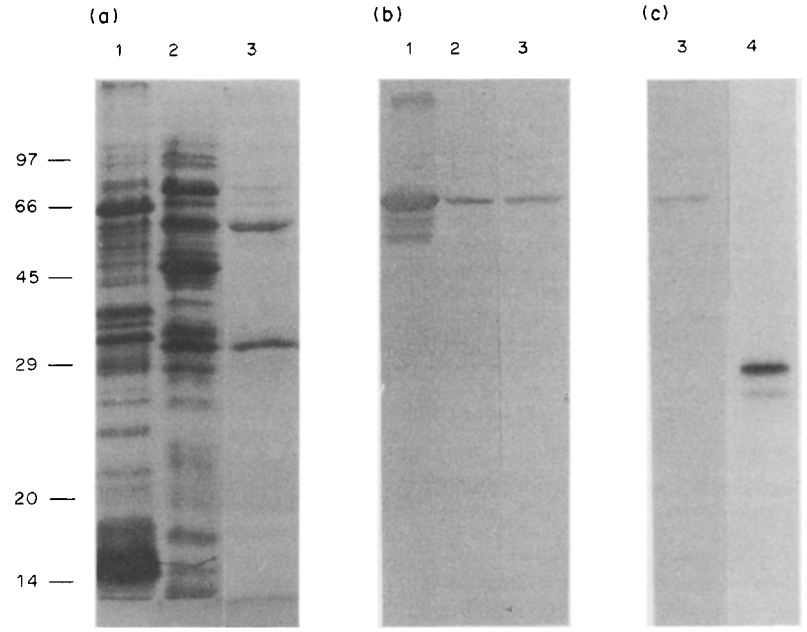

Fig. 2. Analysis of the fusion protein PAHGR477, expressed in *E. coli*. Aliquots of samples were analysed by SDS–PAGE on a 12.5% (w/v) polyacrylamide gel and (a) stained with Coomassie blue, or developed by (b) immunoblotting or (c) autoradiography. (1) Whole cells; (2) cell lysate; (3) PAHGR477 labelled with [^{3}H]DM chromatographed on IgG Sepharose and eluted with 0.5 M acetic acid; and (4) PAHGR477 labelled with [^{3}H]DM chromatographed on IgG Sepharose and eluted with the buffer after tryptic digestion. Molecular weight standards are phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and β-lactamase (14 kDa).

(150,000 *g* supernatant) estimated by ELISA (measuring the amount of protein A) was approximately 0.5 mg/l culture for PAHGR370, 2 mg/l for PAHGR416, and 5 mg/l for PAHGR477, in agreement with the previous observation that the expression levels and solubility of the fusion proteins were inversely related to the molecular weight of the expressed proteins [31]. Immunoblotting of a whole cell extract of *E. coli* MZ1[pEHGR4770] showed a predominant protein product of M_r 66,000, in accord with the expected size of PAHGR477 (68 kDa) (Fig. 2b). Minor bands at M_r 65,000–50,000 detected by immunoblotting may be proteolytic products. When the lysate and particulate fraction of the whole cell homogenate were analysed separately by immunoblotting after SDS–PAGE, less than half of the M_r 66,000 protein expressed was recovered in the lysate (data not shown).

Specific [^{3}H]TA-binding was observed with the lysates containing PAHGR416 and PAHGR477, while the binding to PAHGR370 was extremely low. No hormone binding was detected with the lysates from *E. coli* MZ1 cells without plasmid and with the parent vector (data not shown). An apparent equilibrium of [^{3}H]TA-binding was achieved after 16 h incubation at 0–4°C. Since the lysate containing PAHGR477 had a higher binding capacity due to the elevated expression level and solubility of the fusion protein, characterisation of the bacterially-expressed GR was carried out predominantly with PAHGR477.

Ligand binding specificity was examined at 100 nM [^{3}H]TA with various concentrations of radioinert steroids. As shown in Fig. 3, TA was the most potent competitor, followed by

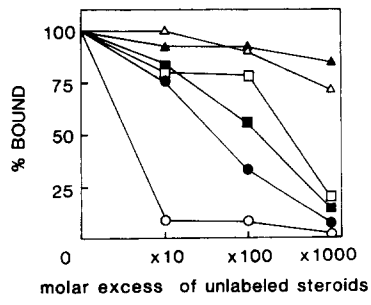

Fig. 3. Competition analysis of PAHGR477 steroid-binding. The lysate of *E. coli* expressing PAHGR477 was incubated with 100 nM [^{3}H]TA in the presence of various competitors. The results is expressed as a percentage of the [^{3}H]TA bound in the sample without addition of unlabelled steroid (9320 dpm). The same result was obtained in two separate experiments. Unlabelled steroids were TA(○), corticosterone (●), progesterone (■), aldosterone, (□), estradiol-17β (▲), and testosterone (△).

corticosterone. Among other steroids tested, progesterone and aldosterone competed to some extent, while testosterone and estradiol-17β did not. Therefore, the binding of PAHGR477 is specific for glucocorticoids and its specificity is identical to that of the native GR[36].

Scatchard analysis revealed that PAHGR477 had a single binding site for [^{3}H]TA (Fig. 4). The apparent dissociation constant (K_d) for PAHGR477 was 75.3 $\pm$ 2.1 nM (n = 3), and the number of binding sites was 6 nM. Compared with the expression level estimated by immunoassay of protein A, approximately 0.4% of the fusion protein in the soluble fraction (150,000 g supernatant) was functional (bound steroid). PAHGR416 also had a single binding site and the K_d was 68.5 nM (n = 2), virtually identical to that of PAHGR477. Notably, these K_d values were significantly lower than that of the native GR(K_d = 3–10 nM) [36].

To investigate the effect of the protein A moiety on the steroid-binding affinity, HGRs were expressed without protein A using the T7 polymerase system. The proteins expressed had a leader sequence at the N-terminus derived from the polylinker of the vector consisting of MARIRAPVPGDR (HGR370) and MARIRYP (HGR477). However, since the binding did not saturate at [^{3}H]TA concentrations between 0.5 and 200 nM, K_d values for these truncated GRs were not determined precisely, but seemed larger than 100 nM (data not shown).

As shown in Fig. 5, [^{3}H]TA–PAHGR477 sedimented as a 5 S complex irrespective of the presence or absence of molybdate (5.1 $\pm$ 0.2 S, n = 5, and 5.1 $\pm$ 0.2 S, n = 3, with or without molybdate, respectively). Glycerol gradient centrifugation followed by SDS–PAGE and immunoblotting revealed that most of the PAHGR477 molecules in the bacterial lysate did not bind [^{3}H]TA and formed large aggregates sedimenting to the bottom of the gradient (larger than 15 S) (data not shown).

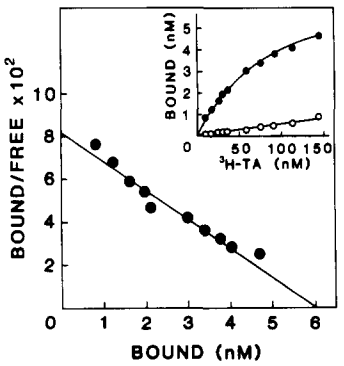

Fig. 4. Scatchard analysis of PAHGR477. The lysate of *E. coli* expressing PAHGR477 was incubated with 1–150 nM [^{3}H]TA in the presence or absence of a 200-fold molar excess of unlabelled TA. After 16 h, [^{3}H]TA binding was measured. The insert shows total binding (●) and non-specific binding (○).

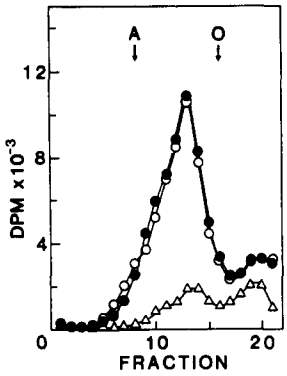

Fig. 5. Glycerol gradient centrifugation of PAHGR477. The lysate was incubated with 100 nM [^{3}H]TA in the absence (●, ○) or presence (△) of 20 μM unlabelled TA. After 20 h, free hormone was adsorbed onto dextran-coated charcoal and the samples (200 μl) were loaded on top of linear 12.5–27.5% (v/v) glycerol gradients prepared in TEDGM (●, △) or TEDG (○) buffer containing 0.15 M NaCl. Aldolase (A, 7.8 S) and ovalbumin (O, 3.0 S) were used as standards.

Purification of affinity-labelled PAHGR477

PAHGR477 was labelled with 200 nM [^{3}H]DM and purified by IgG Sepharose chromatography. Coomassie blue staining showed that there were at least three bands (M_r 74,000, 58,000 and 32,000) in the purified fraction in addition to the M_r 66,000 species corresponding to the PAHGR477 molecule (Fig. 2a, lane 3). Among them, only the band corresponding to PAHGR477 was detected by immunoblotting and autoradiography (Fig. 2b and c, lane 3). The M_r 74,000 and 58,000 bands probably represent endogenous bacterial proteins. The M_r 32,000 band is probably protein A cleaved from the fusion protein since it was visualised by immunoblotting only.

Following digestion of [^{3}H]DM–PAHGR477 immobilised on IgG Sepharose with 0.5 μg/ml trypsin at 4°C for 30 min, two labelled bands of M_r 30,000 and 27,000 were observed in the eluted fraction (Fig. 2c, lane 4). The size of these bands are equivalent to those obtained by tryptic digestion of the purified rat [2, 4] and hGR [37]. Thus the structure which is susceptible to trypsin cleavage between the DNA and steroid binding domains was maintained in the fusion protein.

Radiosequence analysis of [^{3}H]DM-labelled PAHGR477

Analysis of purified GR labelled with [^{3}H]DM has identified a unique site affinity labelled by the steroid, Cys-656 in rat GR [35, 38] and Cys-644 in mouse GR [39] (equivalent to Cys-638 in hGR). Radiosequence analysis is thus a potent analytical criterion of the conformation of the protein in relation to its function. The method was therefore applied to the steroid-binding domain expressed in *E. coli*.

The purified [^{3}H]DM-PAHGR477 was cleaved with trypsin, Asp-*N* endoproteinase or chymotrypsin, and the resultant mixture of peptides was analysed on a gas-phase sequenator. After cleavage with trypsin, two major peaks were obtained at cycles 5 and 10 (Fig. 6). In addition, there were three cycles (2, 8 and 16) with radioactivity higher than the background. Cleavage with Asp-*N* protease also resulted in the recovery of peaks of radioactivity at several cycles (3, 13, 18, 20 and 25; Fig. 6). Cleavage with chymotrypsin resulted in a broad peak at cycles 2–6 with maximal values at cycle 4 (data not shown). The α-mesyl group of DM is known to react only with cysteine residues [38, 40] and the protein A moiety in PAHGR477 does not contain any cysteine. Therefore, the labelled residues are restricted to six cysteines located in the GR moiety of the fusion protein (Fig. 1). By comparison with the amino acid sequence of hGR [3], the putative specific cleavage sites and labelled cysteines are summarised in Table 1.

Cys-638 (cycle 5 after cleavage with trypsin, cycle 13 with Asp-*N* protease) was identified as the most reactive residue with [^{3}H]DM. Both Cys-643 and Cys-665 would be expected to be recovered at cycle 10 following trypsin cleavage. Indeed, the result of Asp-*N* protease digestion indicated that both cysteines were labelled (cycles 3 and 18 for Cys-643, cycle 25 for Cys-655) but that Cys-643 was labelled with much higher efficiency. In addition, two cysteines, Cys-622 (cycle 8 after cleavage with trypsin) and Cys-736 (cycle 16 after cleavage with trypsin) were also identified. However, the efficiency of labelling of these two residues was very low and they could not be demonstrated following cleavage with Asp-*N* protease. These assignments were compatible with the results of chymotryptic cleavage (data not shown). No evidence was obtained indicating that Cys-481 was labelled.

Table 1. Radiosequence analysis of PAHGR477 labelled with [^{3}H]DM

Cycle	Cleavage site and labelled amino acid
	Cleavage with trypsin
2[a]	×
5	..R_{633}▼MTLPC$_{638}$..
8	..R_{614}▼QSSANLLC$_{622}$..
10	..R_{633}▼MTLPCMYDQC$_{643}$..
	..R_{655}▼LQVSYEEYLC$_{665}$..
16	..K_{720}▼LLDSMHEVVENLLNYC$_{736}$..
	Cleavage with Asp-*N*endoproteinase
1[a]	×
2[a]	×
3	..▼D_{641}QC$_{643}$..
13	..▼D_{626}LIINEQRMTLPC$_{638}$..
18[b]	..▼D_{626}LIINEQRMTLPCMY▽D_{641}QC$_{643}$..
20[c]	..▼N(D)$_{619}$LLCFAP▽D_{626}LIINEQRMTLPC$_{638}$..
25	..▼D_{641}QCKHMLYVSSELHRLQVSYEEYLC$_{665}$..

Identification of radiolabelled residues following cleavage with trypsin or Asp-*N* endoproteinase (Fig. 6). The amino acid sequences shown, derived from the nucleotide sequence of human cDNA [6], show the specific cleavage site and end at the affinity-labelled residues. Putative cleavage and partial cleavage sites are indicated with closed and open wedges respectively.

[a]Not identified.

[b]Assuming incomplete cleavage at Asp-641.

[c]Assuming deamidation of Asn-619 and incomplete cleavage at Asp-626.

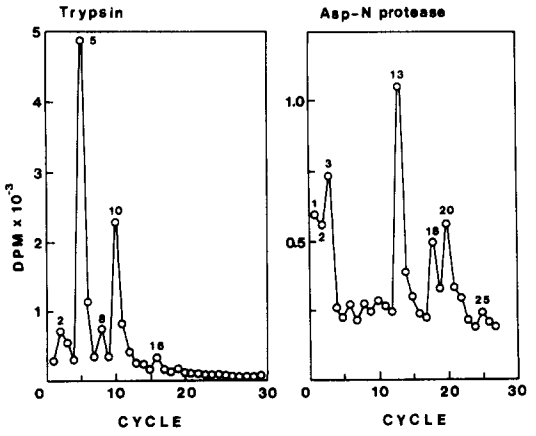

Fig. 6. Radiosequence analysis of affinity-labelled PAHGR477. Purified PAHGR477 labelled with [^{3}H]DM was cleaved with trypsin or Asp-*N* protease. The resulting mixture of peptides was applied on a protein sequenator, and 90% of the extract recovered in each cycle was analysed for radioactivity.

Although it was previously shown that [^{3}H]DM binds to a unique cysteine residue in both the rat and mouse GR [35, 38, 39], it could not be excluded that the heterogeneity of labelling described above represented a species difference. However, GR purified from human leukemia cells and labelled with [^{3}H]DM gave rise to one single peak on radiosequence analysis, corresponding to Cys-638. The same material photo-affinity labelled with [^{3}H]TA gave rise to two peaks of radioactivity on radiosequence analysis, corresponding to Met-604 and Cys-736 as described for the rat GR [35] (P.-E. Strömstedt and J. Carlstedt-Duke, unpublished results).

Steroid-binding affinity of GR translated in vitro

When translation of pTHGR mRNA was carried out *in vitro* in the presence of [^{35}S]methionine, the main translation products were polypeptides of M_r 50,000 (HGR370) and M_r 37,000 (HGR477) (Fig. 7). Two kinds of binding sites were observed on Scatchard analyses and the K_d value of the higher affinity binding site was 2 nM in both cases. This is in marked contrast to that of bacterially expressed GRs which have a single low affinity [^{3}H]TA-binding site.

When the *in vitro* translated products were analysed by glycerol gradient centrifugation, [^{3}H]TA–HGR370 sedimented at 8.1 S ($n = 2$) in the presence of molybdate (Fig. 8a). This sedimentation rate is similar to that of the molybdate-stabilised 9 S form of GR expressed in COS-7 cells [27] and the trypsinised M_r 27,000 fragment of the rat GR [28], both of which were shown to be associated with hsp90. [^{3}H]TA–HGR370 complexes were found to convert to a 4 S form (4.5 ± 0.1 S, $n = 3$) in the absence of molybdate. These results indicated that the truncated GR synthesised under cell-free conditions is associated with hsp90 in the presence of molybdate, as is the *in vitro* synthesised full-length GR [33]. [^{3}H]TA–HGR477 complexes sedimented at about 7 S (6.8 S, $n = 2$, with molybdate; 7.7 S, $n = 2$, without molybdate) (Fig. 8b). Incubation of [^{3}H]TA–HGR477 complexes at 25°C in the absence of molybdate resulted in a partial 4 S shift (data not shown). This suggests that HGR477 is also associated with hsp90.

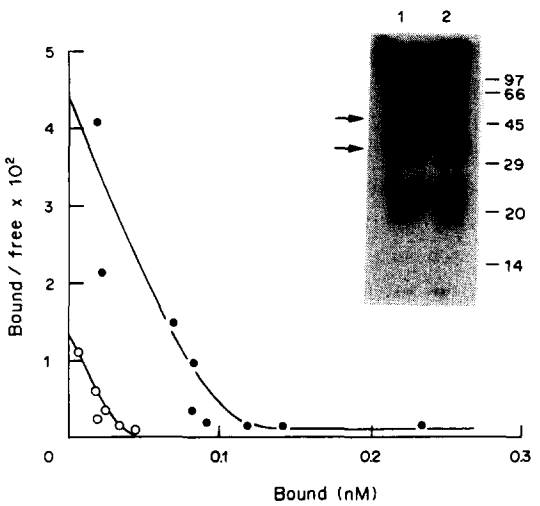

Fig. 7. Scatchard analysis of *in vitro* synthesised HGR370 and HGR477. Scatchard analysis was performed with HGR370 (○) and HGR477 (●) synthesised by *in vitro* translation. The inserted figure shows the autoradiography of the product of rabbit reticulocyte lysates programmed with *in vitro* synthesised mRNA from pTHGR3700 (lane 1) and pTHGR4770 (lane 2). Arrows indicate the peptides corresponding to HGR370 (M_r 50,000) and HGR477 (M_r 37,000). Molecular weight standards are the same as in Fig. 2.

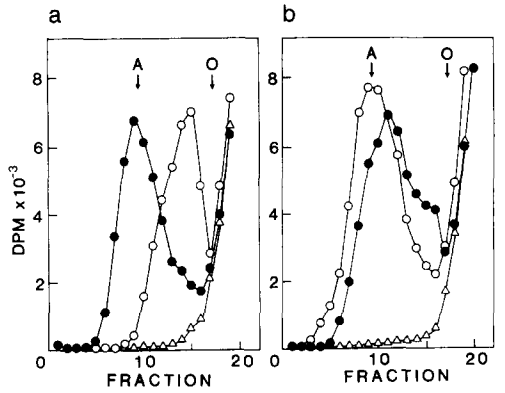

Fig. 8. Glycerol gradient centrifugation of *in vitro* synthesised HGR370 and HGR477. Rabbit reticulocyte lysate programmed with (a) pTHGR3700 mRNA or (b) pTHGR4770 mRNA were incubated with 50 nM [^{3}H]TA in the absence (●, ○) or presence (△) of 1 μM of unlabelled TA. After 4 h, free hormone was adsorbed onto dextran-coated charcoal and the samples (200 μl) were analysed by glycerol gradient centrifugation in TEDGM (●, △) or TEDG (○) buffer containing 0.15 M NaCl as described in Fig. 5.

DISCUSSION

In this study the steroid-binding domain of GR was expressed in *E. coli* using two different systems: as a fusion protein with protein A and expression under control of the T7 RNA polymerase promoter. The former approach was employed for the characterisation of the bacterially expressed steroid-binding domain because the fusion protein with protein A could be readily monitored and purified. These two systems have been successfully used to express the DNA-binding domain of GR in a functional form [12, 21]. However, in the case of the steroid-binding domain, most of the fusion proteins expressed were insoluble in either system presumably due to the more hydrophobic nature of this domain compared to the DNA-binding domain. In addition, expression of a larger eukaryotic polypeptide in *E. coli* tends to be less soluble [31, 41].

The bacterially expressed steroid-binding domain reported here had a low steroid-binding affinity, whereas the *in vitro* synthesised polypeptides consisting of the identical regions of GR exhibited a high affinity for the steroid, similar to that of the native receptor. Thus, the possibility of a post-translational event(s)

affecting the steroid binding ability of GR must be considered.

Detection of only a few amino acids at the C-terminus of GR is known to reduce steroid binding affinity [20]. However, although proteolytic products were found in the whole cell extract, immunoblotting analysis showed that the M_r 66,000 species was predominant in the bacterial lysate (Fig. 2b). In addition, limited trypsin cleavage of [^{3}H]DM–PAHGR477 resulted in two labelled species, M_r 27,000 and 30,000 respectively (Fig. 2c), corresponding exactly to the tryptic fragments of the purified rat and human GR. Therefore, we conclude that the low steroid-binding affinity of the expressed GRs was not due to proteolysis of the fusion protein.

Interestingly, we found that the non-liganded transformed GR from rat liver binds to [^{3}H]TA with a lower affinity ($K_d = 64$ nM) than the nontransformed GR($K_d = 0.5$ nM) [42]. This finding seems closely related to the difference in the steroid-binding affinities of bacterially expressed GR fragments and those synthesised *in vitro*. Comparing the physicochemical characteristics of the high affinity species with those of the low affinity entities, it is evident that GR fragments associated with hsp90 have a high affinity for steroid.

The data in Fig. 5 showed that the bacterially expressed GR did not associate with hsp83, the *E. coli.* protein homologous to eukaryotic hsp90. Comparison of the primary structures of hsp83 and hsp90 show only 58% homology with the absence of a highly charged stretch of 50 amino acids in hsp83 [43]. This very region has been proposed as a region associating with the steroid receptors [44]. If this is the case, it is not surprising that hsp83 fails to interact with the hGR expressed in *E. coli.*

Scatchard analysis of the *in vitro* translated HGR477 revealed the existence of a low affinity binding site as well as a high affinity site (Fig. 7). It is likely that the low affinity site corresponds to the 4 S form not associated with hsp90, while the high affinity site corresponds to the 9 S form. A 4 S form with no steroid-binding ability has been observed for the *in vitro* synthesised full-length rat GR [33].

The primary associating site of GR with hsp90 is located in the steroid-binding domain [27, 28]. It is therefore not surprising that association with hsp90 influences the fine structure of the steroid-binding domain. Bresnick *et al.* [29] also reported close correlation between the association of hsp90 with the mouse GR and its steroid binding capacity and proposed that the hsp90-free receptor had no steroid binding capacity.

In contrast to this study, the bacterially expressed progestin receptor has the same affinity for progestins as the native receptor [45]. However, since the native progestin receptor free from hsp90 still retains a relatively high steroid binding ability [46], association with hsp90 may have no or little effect on the progestin-binding affinity.

To prove the possible role of hsp90 for the steroid-binding ability of GR, reconstruction of the complex between hsp90 and GR would be necessary. So far, to our knowledge, reconstruction of the non-DNA binding form of GR has not been successful. Denis and Gustafsson [33] showed that the association with hsp90 probably occurs during translation of the receptor. In fact, addition of hsp90 purified from rat liver to the bacterial lysate had no effect on either the steroid-binding ability or the sedimentation pattern of PAHGR477 (data not shown).

Radiosequence analysis showed that Cys-638 in the bacterially expressed steroid-binding domain of hGR was the major residue covalently labelled with [^{3}H]DM. This residue is equivalent to Cys-656 in rat GR and Cys-644 in mouse GR which is uniquely labelled with [^{3}H]DM [35, 38, 39]. Radiosequence analysis of hGR affinity labelled with [^{3}H]DM or [^{3}H]TA showed that exactly the same three residues were labelled as in rat GR (cf. above). All other cysteines (Cys-622, -643, -665 and -736; cf. Fig. 1) within the steroid-binding domain of the bacterially expressed proteins were labelled to some extent, suggesting that the bacterially expressed steroid-binding domain has a more open structure than in the native GR. This conformation presumably confers the reduced steroid-binding affinity, but still maintains normal specificity. In addition, radiosequence analysis indicates that none of the cysteines in PAHGR477 expressed in bacteria appear to form intra-domain disulfide bridges. Inter-domain disulfide bridges have previously been excluded by limited proteolysis [4]. Thus, all the cysteine residues in the steroid-binding domain appear to occur in the reduced form, and may play a role in steroid binding.

In conclusion, expression of the isolated steroid-binding domain of GR in *E. coli* results in a protein which binds steroid specifically, but with reduced affinity. The structure of the

expressed protein shows some similarity to the native GR but is more open. Expression of the same fragment of GR *in vitro*, in the presence of hsp90, results in a product with normal affinity for steroid. Thus, the association of hsp90 appears to stabilise the receptor structure and steroid-binding properties and we propose that hsp90 is required for GR to maintain a high affinity ligand-binding state. A possible involvement of other component(s) or modifications of the non-transformed receptor cannot be ruled out.

Acknowledgements—This work was supported by grants from the Swedish Medical Research Council (2819), the Swedish National Board for Technical Development, Karo Bio AB, and by Keiryokai Research Foundation. Salary support was provided by the Swedish Medical Research Council to K. D.-W. and P.-E. S. (studentship) and to J. C.-D. (MRC scientist).

REFERENCES

1. Evans R. M.: The steroid and thyroid hormone receptor superfamily. *Science* **240** (1988) 889–895.
2. Carlstedt-Duke J., Okret S., Wrange Ö. and Gustafsson J.-Å.: Immunochemical analysis of the glucocorticoid receptor: identification of a third domain separate from the steroid-binding and DNA-binding domains. *Proc. Natn. Acad. Sci. U.S.A.* **79** (1982) 4260–4264.
3. Dellweg H.-G., Hotz A., Mugele K. and Gehring U.: Active domains in wild-type and mutant glucocorticoid receptors. *EMBO J.* **1** (1982) 285–289.
4. Wrange Ö., Okret S., Radojcic M., Carlstedt-Duke J. and Gustafsson J.-Å.: Characterization of the purified activated glucocorticoid receptor from rat liver cytosol. *J. Biol. Chem.* **259** (1984) 4534–4541.
5. Carlstedt-Duke J., Strömstedt P.-E., Wrange Ö., Bergman T., Gustafsson J.-Å. and Jörnvall H.: Domain structure of the glucocorticoid receptor protein. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 4437–4440.
6. Hollenberg S. M., Weinberger C., Ong E. S., Cerelli G., Oro A., Lebo R., Thompson E. B., Rosenfeld M. G. and Evans R. M.: Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318** (1985) 635–641.
7. Miesfeld R., Rusconi S., Godowski P. J., Maler B. A., Okret S., Wikström A.-C., Gustafsson J.-Å. and Yamamoto K. R.: Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. *Cell* **46** (1989) 389–399.
8. Danielsen M., Northrop J. P. and Ringold G. M.: The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. *EMBO J.* **5** (1986) 2513–2522.
9. Giguère V., Hollenberg S. M., Rosenfeld M. G. and Evans R. M.: Functional domains of the human glucocorticoid receptor. *Cell* **46** (1986) 645–652.
10. Hollenberg S. M., Giguère V., Segui P. and Evans R. M.: Co-localization of DNA-binding and transcriptional activation function in the human glucocorticoid receptor. *Cell* **49** (1987) 39–46.
11. Severne Y., Wieland S., Schaffner W. and Rusconi S.: Metal binding "finger" structures in the glucocorticoid receptor defined by site-directed mutagenesis. *EMBO J.* **7** (1988) 2503–2508.
12. Freedman L. P., Luisi B. F., Korszun Z. R., Basavappa R., Sigler P. B. and Yamamoto K. R.: The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* **334** (1988) 543–546.
13. Carlstedt-Duke J., Strömstedt P.-E., Dahlman K., Rae C., Berkenstam A., Hapgood J., Jörnvall H. and Gustafsson J.-Å.: Structural analysis of the glucocorticoid receptor protein. In *The Steroid/Thyroid Hormone Receptor Family and Gene Regulation* (Edited by J. Carlstedt-Duke, H. Eriksson and J.-Å. Gustafsson). Birkhäuser Verlag, Basel (1989) pp. 93–108.
14. Hollenberg S. M. and Evans R. M.: Multiple and cooperative transactivation domains of the human glucocorticoid receptor. *Cell* **55** (1988) 899–906.
15. Picard D. and Yamamoto K. R.: Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.* **6** (1987) 3333–3340.
16. Carlstedt-Duke J., Gustafsson J.-Å. and Wrange Ö.: Formation and characteristics of hepatic dexamethasone-receptor complexes of different molecular weight. *Biochim. Biophys. Acta* **497** (1977) 507–524.
17. Wrange Ö. and Gustafsson J.-Å.: Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. *J. Biol. Chem.* **253** (1978) 856–865.
18. Godowski P. J., Rusconi S., Miesfeld R. and Yamamoto K. R.: Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* **325** (1987) 365–368.
19. Miesfeld R., Godowski P. J., Maler B. A. and Yamamoto K. R.: Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. *Science* **236** (1987) 423–427.
20. Rusconi S. and Yamamoto K. R.: Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *EMBO J.* **6** (1987) 1309–1315.
21. Dahlman K., Strömstedt P.-E., Rae C., Jörnvall H., Flock J.-I., Carlstedt-Duke J. and Gustafsson J.-Å.: High level expression in *Escherichia coli* of the DNA-binding domain of the glucocorticoid receptor in a functional form utilizing domain-specific cleavage of a fusion protein. *J. Biol. Chem.* **246** (1989) 804–809.
22. Housley P. R., Sanchez E. R., Westphal H. M., Beato M. and Pratt W. B.: The molybdate stabilized L-cell glucocorticoid receptor isolated by affinity chromatography or with a monoclonal antibody is associated with a 90–92 kDa nonsteroid-binding phosphoprotein. *J. Biol. Chem.* **260** (1985) 13810–13817.
23. Sanchez E. R., Toft D. O., Schlesinger M. J. and Pratt W. B.: Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. *J. Biol. Chem.* **260** (1985) 12398–12401.
24. Mendel D. B., Bodwell J. E., Gametchu B., Harrison R. W. and Munck A.: Molybdate-stabilized non-activated glucocorticoid-receptor complexes contain a 90-kDa non-steroid binding phosphoprotein that is lost on activation. *J. Biol. Chem.* **261** (1986) 3758–3763.
25. Denis M., Poellinger L., Wikström A.-C. and Gustafsson J.-Å.: Requirement of hormone for thermal conversion of the glucocorticoid receptor to a DNA-binding state. *Nature* **333** (1988) 686–688.
26. Raaka B. M. and Samuels H. H.: The glucocorticoid receptor in GH_1 cells. Evidence from dense amino acid labeling and whole cell studies for an equilibrium explaining the influence of hormone on the intracellular distribution of the receptor. *J. Biol. Chem.* **258** (1983) 417–425.
27. Pratt W. B., Jolly D. J., Pratt D. V., Hollenberg S. M., Giguère V., Cadepond F. M., Schweizer-Groyer G., Catelli M.-G., Evans R. M. and Baulieu E.-E.: A region in the steroid binding domain determines formation of the non-DNA-binding, 9S glucocorticoid receptor complex. *J. Biol. Chem.* **263** (1988) 267–273.

28. Denis M., Gustafsson J.-Å. and Wikström A.-C.: Interaction of the M_r 90,000 heat shock protein with the steroid-binding domain of the glucocorticoid receptor. *J. Biol. Chem.* **263** (1988) 18520–18523.
29. Bresnick E. H., Dalman F. C., Sanchez E. R. and Pratt W. B.: Evidence that the 90-kDa heat shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. *J. Biol. Chem.* **264** (1989) 4992–4997.
30. Nilsson B., Abrahamsén L. and Uhlén M.: Immobilization and purification of enzymes with staphylococcal protein A gene fusion vectors. *EMBO J.* **4** (1985) 1075–1080.
31. Bonifer C., Dahlman K., Strömstedt P.-E., Flock J.-I. and Gustafsson J.-Å.: DNA binding of glucocorticoid receptor protein A fusion proteins expressed in *E. coli*. *J. Steroid Biochem.* **32** (1989) 5–11.
32. Tabor S. and Richardson C. C.: A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natn. Acad. Sci. U.S.A.* **82** (1985) 1074–1078.
33. Denis M. and Gustafsson J.-Å.: Translation of glucocorticoid receptor mRNA *in vitro* yields a non-activated protein. *J. Biol. Chem.* **264** (1989) 6005–6008.
34. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (1970) 680–685.
35. Carlstedt-Duke J., Strömstedt P.-E., Persson B., Cederlund E., Gustafsson J.-Å. and Jörnvall H.: Identification of hormone-interacting amino acid residues within the steroid-binding domain of the glucocorticoid receptor in relation to other steroid hormone receptors. *J. Biol. Chem.* **263** (1988) 6842–6846.
36. Rousseau G. G., Baxter J. D. and Tomkins G. M.: Glucocorticoid receptors: relations between steroid binding and biological effects. *J. Molec. Biol.* **67** (1972) 99–115.
37. Brönnegård M., Poellinger L., Okret S., Wikström A.-C., Bakke O. and Gustafsson J.-Å.: Characterization and sequence-specific binding to MTV DNA of the purified activated human glucocorticoid receptor. *Biochemistry* **26** (1987) 1697–1704.
38. Simons S. S., Pumphrey J. G., Rudikoff S. and Eisen H. J.: Identification of cysteine 656 as the amino acid of hepatoma tissue culture cell glucocorticoid receptors that is covalently labeled by dexamethasone 21-mesylate. *J. Biol. Chem.* **262** (1987) 9676–9680.
39. Smith L. I., Bodwell J. E., Mendel D. B., Ciardelli T., North W. G. and Munck A.: Identification of cysteine-644 as the covalent site of attachment of dexamethasone 21-mesylate to murine glucocorticoid receptor in WEHI-7 cells. *Biochemistry* **27** (1988) 3747–3753.
40. Simons S. S.: Selective covalent labeling of cysteines in bovine serum albumin and in hepatoma tissue culture cell glucocorticoid receptors by dexamethasone 21-mesylate. *J. Biol. Chem.* **262** (1987) 9669–9675.
41. Marston F. A. O.: The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240** (1986) 1–12.
42. Nemoto T., Ohara-Nemoto Y., Denis M. and Gustafsson J.-Å.: The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. *Biochemistry* **29** (1989) 1880–1886.
43. Bardwell J. C. A. and Craig E. A.: Eukaryotic M_r 83,000 heat shock protein has a homologue in *Escherichia coli*. *Proc. Natn. Acad. Sci U.S.A.* **84** (1987) 5177–5181.
44. Baulieu E.-E., Binart N., Cadepond F., Catelli M. G., Chambraud B., Garnier J., Gasc J. M., Groyer-Schweizer G., Oblin M. E., Radanyi C., Redeuilh G., Renoir J. M. and Sabbah M.: Do receptor-associated nuclear proteins explain the earliest steps of steroid hormone function? In *The Steroid/Thyroid Hormone Receptor Family and Gene Regulation* (Edited by J. Carlstedt-Duke, H. Eriksson and J.-Å. Gustafsson). Birkhäuser Verlag, Basel (1989) pp. 301–318.
45. Eul J., Meyer M. E., Tora L., Bocquel M. T., Quirin-Stricker C., Chambon P. and Gronemeyer H.: Expression of active hormone and DNA-binding domains of the chicken progesterone receptor in *E. coli*. *EMBO J.* **8** (1989) 83–90.
46. Moudgil V. K., Eessalu T. E., Buchou T., Renoir J.-M., Mester J. and Baulieu E.-E.: Transformation of chick oviduct progesterone receptor *in vitro*: effects of hormone, salt, heat, and adenosine triphosphate. *Endocrinology* **116** (1985) 1267–1274.