Volume 322, number 2, 125–128 FEBS 12415 May 1993

Biochemical and immunological evidence that an acidic domain of hsp 90 is involved in the stabilization of untransformed glucocorticoid receptor complexes

N. Tbarka, C. Richard-Méreau, P. Formstecher and M. Dautrevaux

Laboratoire de Biochimie structurale, Faculté de Médecine, 1 Place de Verdun, 59045 Lille Cedex, France

Received 17 March 1993

Polyclonal antibodies (AS 232–266) have been raised against the 232–266 amino acid sequence of the mouse hsp 84. This sequence posseses 54% acidic residues. AS 232–266 react with both the denatured and the free native murine hsp 84, but not with the bound hsp 84 present in the untransformed glucocorticoid receptor complexes (GR). Both AS 232–266 and peptide 232–266 were shown to decrease [^{3}H]dexamethasone binding by GR. Moreover synthetic peptide 232–266, when added to 7 nm untransformed GR, convert them into 5 nm hsp 84-free GR. Taken together these data suggest that the acidic 232–266 sequence of hsp 84 is involved in the stabilization of the hsp 84–GR interaction, which is known to result in 7 nm complex formation and in GR ligand binding activity improvement. Both peptide 232–266 and AS 232–266 destabilize this interaction.

Glucocorticoid receptor; Heat shock protein, 90 kDa

1. INTRODUCTION

When prepared in buffers of low ionic strength glucocorticoid receptors (GR) exist as heteromeric complexes that do not bind DNA [1]. The core of these untransformed complexes consists of one steroid subunit and a dimer of hsp 90 [2,3], to which other heat shock proteins, including hsp 70 and hsp 56 are less tightly associated [4,5]. Hormone binding induces a temperature-dependent association of GR from hsp 90 and their conversion to the DNA-binding form [6]. Reassociation of transformed GR with hsp 90 is accompanied by functional reconstitution of the untransformed state of the receptor [7]. The recent demonstration that hsp 90 is necessary for the high affinity steroid binding activity of GR [8] and that hsp 90–GR complexes exist in intact cells [9] still reinforce interest for the hsp 90–GR interaction. Whereas several regions involved in the formation of stable complexes with hsp 90 have been localized in the sequence of GR [10–15] and other steroid hormone receptors [16–18], the corresponding regions of hsp 90 still remain unidentified. One possible candidate is a highly charged sequence present in all eukaryotic hsp 90 [19].

We demonstrate here that both a synthetic peptide chosen in this sequence and a polyclonal antibody raised against this peptide, are able to stabilize hsp 90–GR complexes.

Correspondence address: P. Formstecher, Laboratoire de Biochimie structurale, Faculté de Médecine, 1 Place de Verdun, 59045 Lille Cedex, France. Fax: (33) (20) 53 55 77.

2. MATERIALS AND METHODS

2.1. *Synthesis of peptide and preparation of antibodies*

A 36-mer peptide (corresponding to the mouse and rat hsp 90 sequences in Fig. 1 plus an additional N-terminal cysteine for coupling) was synthesized by the Merrifield solid-phase method [20] and purified by gel filtration on Biogel P4 (Bio-Rad, France) and reversed-phase HPLC on 5 μm Nucleosil C8 (Machery Nagel, Germany). Covalent coupling to bovine serum albumin (BSA) was performed using succinimidyl 4-*N*-maleimidomethyl cyclohexane carboxylate [21]. The peptide to BSA ratio in the conjugate was 18:1. Two New Zealand rabbits were immunized [22] and anti hsp 90 antisera were tested by Western blotting. Positive antisera were fractionated by 33% (w/v) ammonium sulfate precipitation, redissolved in phosphate-buffered saline (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) and submitted to gel filtration on an Ultrogel ACA 202 column (IBF, France) equilibrated in the same buffer.

2.2. *Cytosol preparation and steroid binding assay*

Cytosol of rat and mouse liver was prepared as previously described [23] in buffer A: 20 mM TES (*N*-tris-hydroxymethyl-methyl-2-aminoethane sulfonic acid) pH 7.4, 1 mM dithiothreitol, 1 mM PMSF (phenylmethane sulfonic fluoride), 10 mM sodium molybdate and 10% glycerol. Steroid binding was performed with [6,7-^{3}H]dexamethasone (40 Ci/mmol, NEN, Boston, MA) using a dextran-coated charcoal assay [23]. Non-specific binding was determined in parallel samples in the presence of a 1,000-fold molar excess of unlabelled dexamethasone.

2.3. *Hsp 90 purification*

Hsp 90 from rat or mouse liver cytosol was purified using protamine sulfate precipitation followed by ion-exchange chromatography on a Mono Q HR 5/5 column (Pharmacia Biotechnology) and high-performance size-exclusion chromatography on a TSK G4000 SW column (Tbarka, in preparation). The purified protein was labelled with ^{125}I using the Bolton and Hunter procedure [24].

2.4. *Gel electrophoresis and immunoblotting*

SDS-PAGE was performed on 7.5% slab gels under reducing condi-

tions [25]. After electrotransfer to a nitrocellulose sheet, immunological revelation was performed [26] using anti-hsp 90 antibodies (monoclonal antibody Ac 88 was a kind gift of Dr. O. Toft) at a 1:1000 to 1:5000 final dilution. Peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were used for detection.

3. RESULTS

3.1. *An antibody specific of the free hsp 90*

A very charged peptide, mapping from residue 232 to residue 266 of mouse hsp 90β sequence [27,28], was chosen using prediction of antigenic regions of proteins and synthesized (Fig. 1). This peptide belongs to an acidic domain of eucaryotic hsp 90 in which charges are roughly conserved among species, despite unperfect sequence homology [19,27–30]. In human and mice two hsp 90 isoforms, hsp 90α and hsp 90β, encoded by separate genes have been described [28,29]. Both exist as homodimers [31] and are present in the untransformed GR complexes [32]. Peptide 232–266 was used to raise antibodies in rabbits and a positive antiserum, AS 232–266, was obtained. AS 232–266 recognized murine cytosolic hsp 90 in Western blots at a dilution greater than 1:8,000 (Fig. 2). A control using Ac 88, a monoclonal antibody recognizing all eukaryotic hsp 90 [33], is also shown. AS 232–266 binding appeared specific, since it was abolished by preincubation with peptide 232–266 and not with an unrelated peptide. Moreover AS 232–266 crossreacts with both hsp 90 isoforms and with hsp 90 from other species, including chick, rat and human (data not shown). Interestingly AS 232–266 recognizes hsp 90 in its native free form (Fig. 3A): ^{125}I-labelled highly purified rat hsp 90 was characterized by high-performance size-exclusion chromatography as a peak with a 6.8 nm Stokes radius, which was shifted to more than 8.5 nm after preincubation in the presence of AS 232–266. However no shift was observed with untransformed GR complexes (Fig. 3B), demonstrating that the epitope recognized by AS 232–266 in free hsp 90 was not accessible in these complexes, which do contain hsp 90 as demonstrated by Western blot experiments [26]. This result is consistent with a possible involvement of this epitope in the binding of hsp 90 to GR.

3.2. *Both AS 232–266 and peptide 232–266 interfere with [^{3}H]dexamethasone–GR complex formation*

When added to rat liver cytosol, AS 232–266, but not preimmune or unrelated sera, was able to lower [^{3}H]dexamethasone binding to GR in a dose-dependent manner (Fig. 4A). This phenomenon could be explained by the binding of AS 232–266 to ligand-free hsp 90–Gr complexes, resulting in the masking of the GR ligand binding domain. The hsp 90 epitope recognized by AS 232–266 and the GR binding site could be close enough to each other to result in mutually exclusive binding of AS 232–266 and [^{3}H]dexamethasone. This hypothesis is reinforced by the observation that in the presence of AS 232–266, performed [^{3}H]dexamethasone–GR complexes dissociate with the same kinetics as in the presence of an excess of unlabelled dexamethasone (data not shown). Interestingly peptide 232–266–SAB conjugates, but not free SAB or conjugates with unrelated peptides, also appeared able to compete for [^{3}H]dexamethasone binding to GR (Fig. 4B). This observation promted the hypothesis that peptide 232–266 could destabilize ligand-free hsp 90–GR complexes, yielding a GR form with lowered ligand-binding ability.

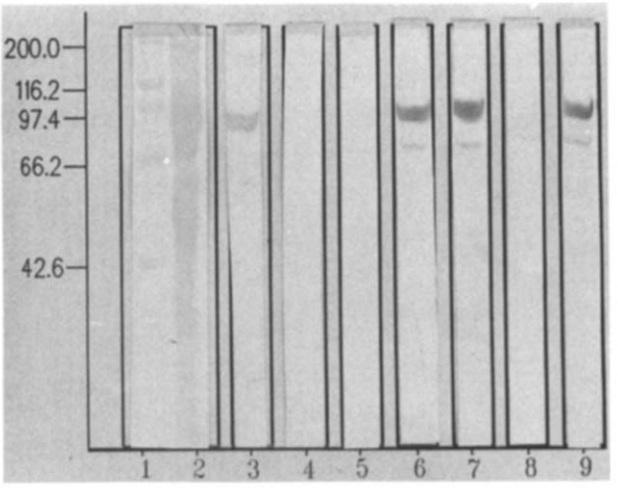

Fig. 2. Western blot analysis of cytosolic mouse hsp 90. Following SDS-PAGE and electrotransfer to nitrocellulose filters, blots were revealed with monoclonal antibody Ac 88 (lane 3), the pre-immune antiserum 232–266 (lane 4), a non-relevant antiserum (lane 5) and the immune antiserum AS 232–266 either crude (lane 6) or after immunoglobulin fractionation (lane 7). Control strips were also revealed with AS 232–266 preincubated with peptide 232–266 (lane 8) or with an unrelated peptide (lane 9). The positions of proteins size markers (kDa) are shown (lane 1).

3.3. *Dissociation of 7 nm hsp 90–Gr complexes in the presence of peptide 232–266*

When cytosolic GR were incubated overnight in the presence of 10 nM [^{3}H]dexamethasone and 25 μM free peptide 232–266 only a 20–25% decrease in binding activity was observed, i.e. far less than with the corresponding peptide–BSA conjugate. However, when analyzed by high-performance size-exclusion chroma-

RAT/MOUSE 232 E E K G E K E E E D K E D E E K P K I E D Y G S D - E E D D S G K D K K 266
HUMAN 232 ★★★★★★★★★★★★ D ★★★★★★★★★★★★★★★★ - ★★★★★★★★★★ 266
CHICK 239 ★★★ E ★★★★ - - ★ - T ★ D ★★ E ★★★★★★★★ E ★★ E K K D G ★★★ 271

Fig. 1. Amino acid sequence 232–266 from mouse [27], rat [30] and human [29] hsp 90β and the corresponding sequence from chick hsp 90 [19]. Conserved residues are indicated by an asterisk. Dashes represent gaps resulting from alignment.

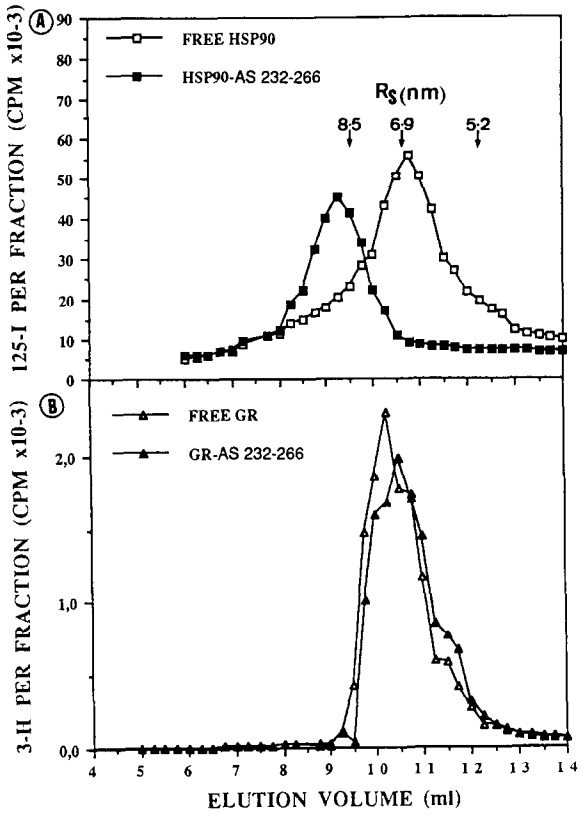

Fig. 3. Interaction of AS 232–266 with native hsp 90 and GR complexes. (A) Purified ^{125}I-labelled, rat liver hsp 90 samples (40 μg) were incubated overnight at 0°C in the presence or not of AS 232–266 (1:30 final dilution) and loaded on a TSK G4000 SW column (0.75 × 30 cm). Elution was performed in buffer C (20 mM TES, pH 7.0, 1 mM EDTA, 200 mM NaCl) at a 0.5 ml · min^{-1} flow rate, 0.25 ml fractions were collected and assayed for radioactivity. Arrows indicate elution volumes of Stokes radius markers: thyroglobuline (8.3 nm), β-galactosidase (6.9 nm) and aldolase (5.2 nm). (B) Rat liver cytosol samples were incubated with [^{3}H]dexamethasone 10 nM and in the presence or not of AS 232–266 (1:20 final dilution). Size-exclusion was performed in buffer C supplemented with 10 mM sodium molybdate.

tography, [^{3}H]dexamethasone–GR complexes appeared completely dissociated into the 5 nm form (Fig. 5). This result was obtained at 0°C and in the presence of sodium molybdate, i.e. under conditions that stabilize the 7 nm hsp 90 containing GR complexes. Therefore peptide 232–266 appears able to destabilize and dissociate hsp 90–GR complexes.

4. DISCUSSION

Two modes of binding of hsp 90 to steroid hormone receptors are currently evoked. Hydrophobic interactions could involve the ligand binding domain (LBD) of the receptors. Certain critical regions of this domain have been localized, specially in the case of GR [10–13]. However it is now clear for the progesterone [16,18] and the estrogen receptor [17], and probably also for the GR [14,15], that hsp 90 bind to the LBD through interactions at multiple locations. The implication of putative leucine-zippers has been hypothesized on both the receptors and hsp 90 [34], which comprises highly hydrophobic regions [35]. On the other hand polar interactions could also contribute to the hsp 90–receptor complex stability. A very polar, mainly basic, region of the receptor, encompassing the terminal part of the DNA binding domain (DBD) and the hinge region between the DBD and the LBD could be recognized by specific antibodies in transformed, (hsp 90-free) but not in untransformed (hsp 90 containing) GR and PR complexes [36,37]. This region, which could be masked by hsp 90 in the untransformed complexes, appeared essential for hsp 90 binding to the estrogen receptor [17], but not to

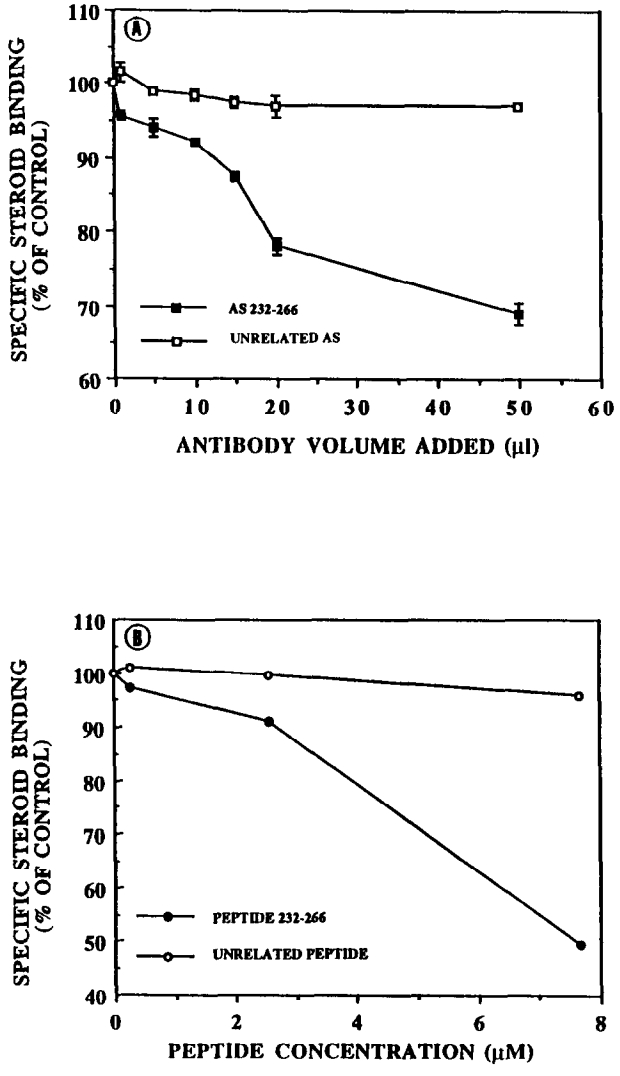

Fig. 4. Inhibition of GR steroid binding activity by AS 232–266 and peptide 232–266–SAB conjugates. Rat liver cytosol samples (0.5 ml) were incubated overnight in the presence of [^{3}H]dexamethasone and of (A) increasing amounts of fractionated AS 232–266 or unrelated antibodies and (B) increasing concentrations of peptide 232–266–BSA conjugates or unrelated peptide–BSA conjugates. Specific binding is expressed as percent of a control incubated in the presence of [^{3}H]dexamethasone alone.

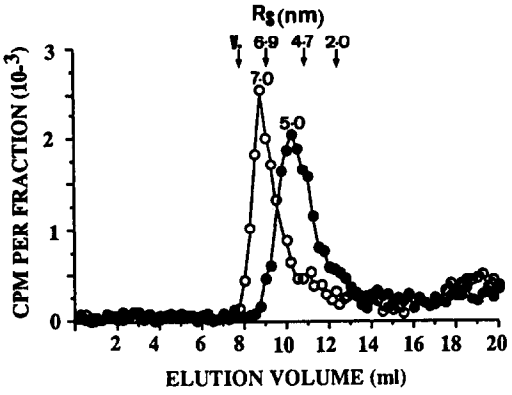

Fig. 5. Effect of peptide 232–266 on the Stokes radius of untransformed GR. Rat liver cytosol samples were incubated overnight in the presence of 10 nM [^{3}H]dexamethasone and in the presence or absence of 25 μM peptide 232–266, and analyzed by size-exclusion chromatography on a TSK G3000 column as described in Fig. 3.

GR [38]. However, even if it is not essential, this region could nevertheless contribute to GR binding. Its counterpart on hsp 90 could be the polar region including peptide 232–266 [19]. Indirect arguments for the implication of this very acidic region in hsp 90 binding to GR are the dissociation of hsp 90–GR complexes at pH 4.0, a value close to the pK of the Asp and Glu residues present in abundance in this region [39], and the fact that this region is absent in the bacterial hsp 90 equivalent, which does not bind steroid receptors [40]. Our data afford new and more direct evidence that the acidic domain of hsp 90 plays a role in the stabilization of hsp 90–GR complexes.

Acknowledgements: This work was supported by the University of Lille II and grants from INSERM (CJF 9203), the 'Association pour la Recherche contre le Cancer' and the 'Ligue contre le Cancer'. We are also indebted to Dr. D.O. Toft for providing the AC 88 antibody.

REFERENCES

[1] Pratt, W.B. (1987) J. Cell. Biochem. 35, 51–68.
[2] Rexin, M., Bush, W., Segnitz, B. and Gehring, U. (1988) FEBS Lett. 241, 234–238.
[3] Denis, M., Wikström, A.C. and Gustafsson, J.A. (1987) J. Biol. Chem. 262, 11803–11806.
[4] Sanchez, E.R., Hirst, M., Scherrer, L.C., Tang, H.Y., Welch, M.J., Harmon, J.M., Simons, S.S. Jr., Ringold, G.M. and Pratt, W.B. (1990) J. Biol. Chem. 265, 20123–20130.
[5] Sanchez, E.R. (1990) J. Biol. Chem. 265, 22067–22070.
[6] Mendel, D.B., Bodwell, J.E., Gametchu, B., Harrison, R.W. and Munck, A. (1986) J. Biol. Chem. 261, 3758–3763.
[7] Scherrer, L.C., Dalman, F.C., Marsa, E.M., Meschinchi, S. and Pratt, W.B. (1990) J. Biol. Chem. 265, 21397–21400.
[8] Bresnick, E.H., Dalman, F.C., Sanchez, E.R. and Pratt, W.B. (1989) J. Biol. Chem. 264, 4992–4997.
[9] Rexin, M., Busch, W., Segnitz, B. and Gehring, U. (1992) J. Biol. Chem. 267, 9619–9621.
[10] Dalman, F., Scherrer, L.C., Taylor, L.P., Hakil, H. and Pratt, W.B. (1991) J. Biol. Chem. 266, 3482–3490.
[11] Denis, M., Gustafsson, J.A. and Wikström, A.C. (1988) J. Biol. Chem. 263, 18520–18523.
[12] Howard, K.J., Holley, S.J., Yamamoto, K.R. and Diestelhorst, C.W. (1990) J. Biol. Chem. 265, 11928–11935.
[13] Chakraborti, P.K. and Simons, S.S. (1991) Biochem. Biophys. Res. Commun. (1991) 176, 1338–1344.
[14] Cadepond, F., Schweitzer-Groyer, G., Segard-Maurel, J., Jibard, N., Hollenberg, S.M., Giguere, V., Evans, R.M. and Baulieu, E.E. (1991) J. Biol. Chem. 266, 5383–5841.
[15] Schlatter, L.K., Howard, K.J., Parker, M.G. and Diestelhorst, C.W. (1992) Mol. Endocrinol. 6, 132–140.
[16] Carson-Jurica, M.A., Lee, A.T., Dobson, A.W., Conneely, O.M., Schrader, W.T. and O'Malley, B.W. (1989) J. Steroid Biochem. 34, 1–9.
[17] Chambrand, B., Berry, M., Redenilk, G., Chambon, P. and Baulieu, E.E. (1990) J. Biol. Chem. 265, 20686–20691.
[18] Schowalter, D.B., Sullivan, W.P., Maihle, N.J., Dobson, A.D.W., Conneely, O.M., O'Malley, B.W. and Toft, D.O. (1991) J. Biol. Chem. 266, 21165–21173.
[19] Binart, N., Chambraud, B., Dumas, B., Rowlands, D., Bigogne, C., Levin, J.M., Garnier, J., Baulieu, E.E. and Catelli, M.G. (1989) Biochem. Biophys. Res. Commun. 159, 140–147.
[20] Stewart, J.M. and Young, J.D. (1984) in: Solid-Phase Peptide Synthesis, 2nd Ed., pp. 1–176, Rockford, USA.
[21] Rothbard, J.B., Fernandez, R. and Schoolnik, G.K. (1984) J. Exp. Med. 160, 208–221.
[22] Vaitukatis, J., Robbins, J.B., Nieschlag, E. and Rose, G.T. (1971) J. Clin. Endocr. 33, 988–991.
[23] Idziorek, T., Formstecher, P., Danze, P.M., Sablonniere, B., Lustenberger, P., Richard, C., Dumur, V. and Dautrevaux, M. (1985) Eur. J. Biochem. 153, 65–74.
[24] Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539.
[25] Laemmli, U.K. (1970) Nature 277, 680–685.
[26] Lefebvre, P., Sablonniere, B., Tbarka, N., Formstecher, P. and Dautrevaux, M. (1989) Biochem. Biophys. Res. Commun. 159, 677–686.
[27] Moore, S.K., Kozak, K., Robinson, E.A., Ulrich, S.J. and Appella, E. (1987) Gene 56, 29–40.
[28] Moore, S.K., Kozak, K., Robinson, E.A., Ulrich, S.J. and Appella, E. (1990) J. Biol. Chem. 264, 5343–5351.
[29] Rebbe, N.F., Ware, J., Bertina, R.M., Mochich, P. and Stafford, D.W. (1987) Gene 53, 235–245.
[30] Mc Guire, J.A., Poellinger, L., Wikström, A.C. and Gustafsson, J.A. (1992) J. Steroid Biochem. Mol. Biol. 42, 813–822.
31] Minami, Y., Kawasaki, H., Miyata, Y., Susuki, K. and Yahara, I. (1991) J. Biol. Chem. 266, 10099–10103.
32] Mendel, D.B. and Orti, E. (1988) J. Biol. Chem. 263, 6695–6702.
33] Riehl, R.M., Sullivan, W.P., Vroman, B.T., Bauer, V.J., Pearson, G.R. and Toft, D.O. (1985) Biochemistry 24, 6586–6591.
34] Schwartz, J.A., Mizukami, H. and Skafar, D.F. (1993) FEBS Lett. 315, 109–113.
35] Yamamoto, M., Takahashi, Y., Inano, K., Horigome, T. and Sugano, H. (1991) J. Biochem. 110, 141–145.
36] Urda, L.A., Yen, P.M., Simons, S.S. and Hamon, J.M. (1989) Mol. Endocrinol. 3, 251–260.
37] Smith, D.F., Lubahn, D.B., McCormick, D.J., Wilson, E.M. and Toft, D.O. (1988) Endocrinology 122, 2816–2825.
38] Pratt, W.B., Jolly, D.J., Pratt, D.V., Hollenberg, S.M., Giguere, V., Cadepond, F.M., Schweizer-Groyer, G., Catelli, M.G., Evans, R.M. and Baulieu, E.E. (1988) J. Biol. Chem. 263, 267–273.
39] Nemoto, T., Ohara-Nemoto, Y. and Ota, M. (1988) J. Biochem. 103, 920–927.
40] Ohara-Nemoto, Y., Strömstedt, P.E., Dahlman-Wright, K., Nemoto, T., Gustaffon, J.A. and Carlstedt-Duke, J. (1990) J. Steroid Biochem. Mol. Biol. 37, 481–490.