Inhibition of GR-mediated transcription by p23 requires interaction with Hsp90

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Abstract p23 is a regulatory co-chaperone of heat shock protein (Hsp) 90, but can also act as a general molecular chaperone by itself. Using novel point mutations of p23 that disrupt its interaction with Hsp90 we found its co-chaperone function to be required for its inhibitory effect on glucocorticoid receptor (GR). The C-terminal region of p23, which is required for its chaperone activity, is dispensable for inhibition of GR. Importantly, similar results were obtained with a constitutively active GR. Thus, the action of p23 on the nuclear stage of GR regulation requires its Hsp90 co-chaperone function, but not its chaperone activity.

Key words: Glucocorticoid receptor; Heat shock protein 90; p23; Chaperone; Co-chaperone

1. Introduction

Molecular chaperones promote the correct folding of proteins to their native state [1,2]. Heat shock protein (Hsp) 90 is a highly conserved ubiquitous protein that functions as a central folding platform for diverse proteins including steroid receptors such as the glucocorticoid receptor (GR) [3–5]. Hsp90 acts together with Hsp70, p23 and various other protein cofactors to fold and stabilize the ligand binding domain of GR [4].

p23 interacts with Hsp90 as a regulatory cofactor and binds to the adenosine triphosphate (ATP)-bound form of Hsp90 [6–8]. Geldanamycin disrupts the Hsp90–p23 interaction [9,10]. It has been reported that human p23 reduces the ATPase activity of Hsp90 [11] and enhances the Hsp90-mediated folding of GR in vitro [12] and in vivo [13]. Upon hydrolysis of ATP by Hsp90, p23 also enhances dissociation of substrate from Hsp90 [14]. In mammalian and yeast cells, increased expression of p23 raised the ligand efficiency of GR by acting through its ligand binding domain [15].

In addition to its regulation of Hsp90, p23 acts as molecular chaperone itself and prevents protein aggregation [16,17]. The unstructured C-terminal region of p23 is necessary for its chaperone function [18], but this region is not required for interaction with Hsp90 or its regulatory co-chaperone function [14,19]. The crystal structure of a p23 lacking the C-terminal chaperone domain revealed a cluster of conserved residues on one face of the protein, possibly providing a binding face for Hsp90 [19].

More recently, p23 was found to act not only in the activation of GR [12], but also in its inhibition by disassembling transcriptional regulatory complexes of the receptor [20]. The transcriptional activity of GR in vivo was reduced by chromatin-targeted or intact p23. Moreover, p23 reduced the occupancy of glucocorticoid response elements by GR. Chromatin-targeted Hsp90 displayed similar effects on transcription complexes [20]. These observations give rise to the crucial question of whether the effects of p23 on transcriptional activity are due to its intrinsic chaperone activity, or its role as an Hsp90 co-chaperone.

We addressed this question by examining the in vivo inhibition of GR activity by p23. Using novel point mutants of p23 unable to bind Hsp90, we demonstrate that the co-chaperone function of p23 is required for its inhibition of GR. In contrast, the C-terminal region of p23 necessary for its intrinsic chaperone activity is not involved in GR inhibition. Importantly, p23 also inhibited the transcriptional activity of a GR devoid of the ligand binding domain in an Hsp90-dependent manner.

2. Materials and methods

2.1. Cell culture, transfection, luciferase and β-galactosidase assay

Cell cultivation and transfection were as described [21]. Unless otherwise indicated, amounts of transfected plasmids per $\sim 10^7$ cells were 1.5 μg steroid-responsive luciferase reporter plasmid MTVLuc, 2 μg β-galactosidase vector pCMVβ-Gal (Stratagene) as control, p23 expression plasmid as indicated and 0.75 μg pRK7GR that expresses human GR, or a FLAG-tagged version. The total amount of plasmids was kept constant for each condition by supplementing expression vector.

Luciferase and β-galactosidase assays were as described [21]. Unless described otherwise, luciferase data are presented as percent of stimulation. That is, after normalization of the data to galactosidase activities, fold stimulation of luciferase expression was calculated by comparing cells without to cells with hormone. The stimulation in the absence of cotransfected p23 was set to 100%. For the hormone-independent GR stimulation was calculated by comparing cells in the presence versus absence of cotransfected GR.

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Abbreviations: GFP, green fluorescence protein; GR, glucocorticoid receptor; Hsp, heat shock protein

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2.2. Plasmids

The plasmids MTVLuc have been described [21], pRK7GR1-515, pRK7GR were recloned by A. Hoffmann with an N-terminal HA-tag from cDNAs originally obtained from R. Evans. The green fluorescence protein (GFP)-tagged truncated GR was cloned into pEGFPC1 (Clontech).

For expression of p23 variants, the respective cDNAs were inserted in the vector pRK5MCS, and clones were verified by sequencing. Details are available on request.

2.3. Immunoprecipitation and immunoblots

For immunoprecipitation, a FLAG-tagged form of the protein of interest was expressed in human embryonic kidney (HEK) cells. Cells were solubilized in 1 ml lysis buffer (20 mM Tris/HCl pH 7.5; 130 mM NaCl; 20 mM Na_2MoO_4; 1 mM ethylenediamine tetraacetic acid (EDTA); 10% glycerol; 0.5% Triton X-100; 1:100 protease inhibitor cocktail, Sigma). The extract was incubated for 1 h on ice, centrifuged for 4 min at 13 000 rpm and protein concentration was determined (BCA kit; Pierce). Lysates were incubated with an anti-FLAG M2 affinity gel overnight at 4°C. Beads were washed three times with 1×Tris-buffered saline and samples were eluted with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer without reducing agent.

For immunoblot detection either 15 μg of total protein from the cell extracts used to determine luciferase activity or immunoprecipitates were separated by SDS–PAGE and transferred to nitrocellulose membrane (Schleicher&Schüll GmbH, Germany). Hsp90 was detected with rabbit polyclonal antibody (H-114, Santa Cruz), p23 with a mouse monoclonal antibody (ABR), FLAG-tagged proteins with a monoclonal anti-FLAG antibody (M2, Sigma), and HA-tagged GRs with a monoclonal anti-HA antibody (Roche, Germany). When two proteins were identified consecutively on the same blot, stripping buffer (Pierce) was used after detection of the first protein. Signals were visualized with appropriate secondary antibodies and the enhanced chemiluminescence (ECL) system and autoradiography.

2.4. Fluorescence detection

HeLa cells were seeded on gelatin-coated glass plates in a six-well plate and transfected with ExGen (Fermentas) as recommended by the manufacturer. 0.25 μg GFP-truncated receptor plasmid were used per well. After 24 h, cells were fixed with 4% paraformaldehyde (PFA) for 20 min, washed two times (1×TTBS, 5% fetal calf serum (FCS), 1% bovine serum albumin (BSA), 1% Triton X-100) and then embedded in 'ProTaqs Mount Fluor' (Biocyc GmbH and CoKG, Germany). The microscope was 'Axioplan 2 imaging' (Zeiss, Jena, Germany).

3. Results

3.1. p23 inhibits GR activity in a dose-dependent manner

To investigate the effect of p23 on GR activity, we developed an assay with a GR-dependent luciferase reporter gene in neuroblastoma cells. p23 decreased the transcriptional activity of GR in a dose-dependent manner in SK-N-MC cells (Fig. 1A). This decrease was not due to degradation of the receptor (Fig. 1B). Similar results were obtained in HEK, COS1 and HeLa cells (unpublished), strongly suggesting that the observed inhibition is not cell type-specific. FLAG-tagged and untagged p23 produced identical results (unpublished). The inhibition of GR by p23 was at least partially overcome by increasing concentrations of hormone (inset Fig. 1A). Western blotting revealed a moderate increase of total p23 levels under our assay conditions (Fig. 1C). We also tested whether overexpression of p23 leads to disruption of the Hsp90–GR complex in the cytosol, since this could explain the inhibitory activity of p23. We precipitated FLAG-tagged GR in the presence or absence of cotransfected p23 and found no difference in the amount of coprecipitated Hsp90 (Fig. 1D). Cells not expressing FLAG-tagged GR produced no background (unpublished).

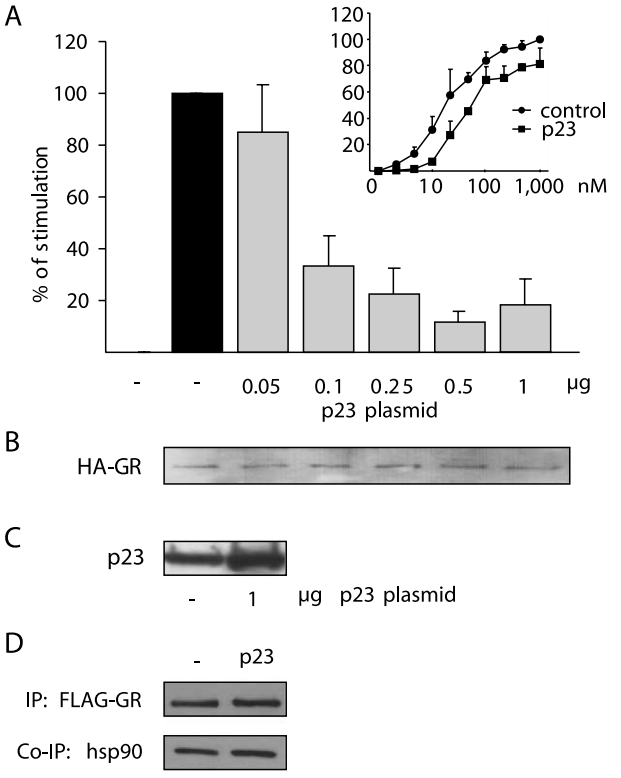

Fig. 1. p23 inhibits GR-dependent transcription. SK-N-MC were transfected with a reporter plasmid, a GR expressing plasmid, a β-galactosidase plasmid, increasing amounts of p23 expression plasmid as indicated, and cultivated for 16 h in the presence of 10 nM cortisol. A: Luciferase activities, calculated as percent of stimulation. Data represent four independent experiments performed in duplicate. Inset: Effect of p23 (1 μg plasmid) at different concentrations of cortisol. B: Representative Western blot of the HA-tagged GR. C: Western blot comparing endogenous levels of p23 with total levels of p23 after transfection. D: HEK cells were transfected with FLAG-tagged GR and p23 expressing plasmids, as indicated, FLAG-tagged GR was precipitated from lysates (upper panel) and was determined on the same blot after detecting coprecipitated Hsp90 (lower panel).

3.2. Two hydrophobic residues on the surface of p23 are required for binding to Hsp90

The goal of this study was to determine whether the Hsp90 co-chaperone function of p23 or its intrinsic chaperone function is required for GR inhibition. To target its chaperone function, we deleted the C-terminus of p23, which removes its chaperone activity [18], but leaves Hsp90 binding and co-chaperone function intact [14,19]. We verified that p23 lacking the C-terminal 35 amino acids indeed interacted with Hsp90 (C35, Fig. 2B). To target the Hsp90 co-chaperone function, we established mutants that could not bind Hsp90. Important clues were provided by the crystal structure [19] and the identification of conserved amino acids in p23 (Fig. 2A). There are conserved residues mainly within the first 108 amino acids with stronger conservation between residues 86 and 108. We mutated the hydrophobic amino acids phenylalanine (position 103) and tryptophan (106) to alanines (F103A/W106A, 'ANNA' Fig. 2A). These two conserved bulky aromatic amino acids are exposed on the surface of the protein [19]. Therefore, they are not contributing to maintain p23 structure, but likely to be involved in recognizing Hsp90. Mutation of these two amino acids indeed abolished binding to Hsp90 (Fig. 2B).

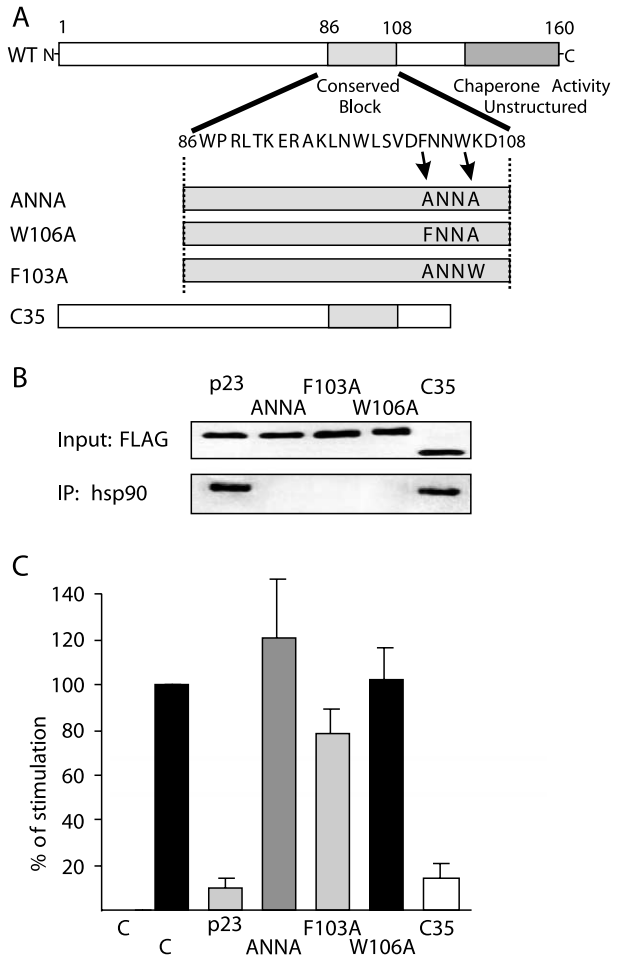

Fig. 2. Hsp90 interaction of p23 is required for its inhibitory effect on GR, but not its C-terminal region. A: Structure of wild-type p23 and mutants. Light gray, region of highest conservation; bold letters, conserved amino acids [19]; dark gray, unstructured C-terminus required for chaperone activity [18,19]. B: Immunoprecipitation of FLAG-tagged wild-type p23 and mutants. Upper panel: Western blot with anti-FLAG antibody to control input of precipitation. Lower panel: Western blot of the immunoprecipitation with an Hsp90 antibody. C: SK-N-MC cells were transfected with the reporter plasmid, the GR plasmid, β-galactosidase plasmid, and 3 μg of either control vector (c), wild-type, or mutated p23, respectively. Data represent four independent experiments performed in duplicate.

Also, the single point mutations F103A and W106A disrupted interaction with Hsp90 (Fig. 2B).

3.3. Hsp90 binding of p23, but not its C-terminal chaperone domain, is required for its inhibitory effect on GR

The mutants described above were tested in our reporter assay. The ANNA (F103A/W106A), F103A and W106A p23 mutants that were unable to bind Hsp90 were also unable to inhibit transcriptional activity of GR (Fig. 2C). To evaluate the contribution of the intrinsic p23 chaperone activity, we tested the effect of the C-terminal deletion mutant. Deletion of the 30 or 35 C-terminal amino acids of p23 severely impairs the intrinsic chaperone activity of the protein [18,19]. The C35 deletion mutant was as effective as wild-type p23 in the inhibition of GR activity (Fig. 2C). Therefore, the Hsp90-dependent co-chaperone function of p23 is required for its effect on GR, but not its intrinsic chaperone activity.

3.4. p23 also affects a constitutively nuclear GR in an Hsp90-dependent manner

p23 has been implicated in the folding reactions of steroid receptors [22], which take place in the cytosolic compartment of the cell, and in late nuclear steps of receptor activation [15], i.e. in disassembly of transcriptional regulatory complexes [20]. Using GFP-tagged p23 we found that p23 is evenly distributed in both the cytosol and the nucleus (unpublished). Therefore, the inhibition of GR by p23 could be due to an action in either one or both subcellular compartments.

To determine if p23 acts on transcriptionally active GR in the nucleus, we tested a truncated, constitutively active GR (amino acids 1–515), which activates transcription independently of hormone [23]. This GR was also inhibited by p23 in an Hsp90-dependent manner (Fig. 3A), because the ANNA (F103A/W106A) mutant was inactive. In contrast, the C-terminal truncation of p23 did not abolish the inhibitory effect on this GR (Fig. 3A). There was no degradation of the truncated GR (Fig. 3B). We also verified that the truncated GR is exclusively nuclear, as predicted (Fig. 3C). The relative inhibition of the hormone-independent GR seems lower when compared to full-length GR. This may be explained by the already reduced transcriptional activity of the truncated GR. Alternatively, the higher expression levels of the truncated GR may require also higher levels of p23 for comparable inhibition.

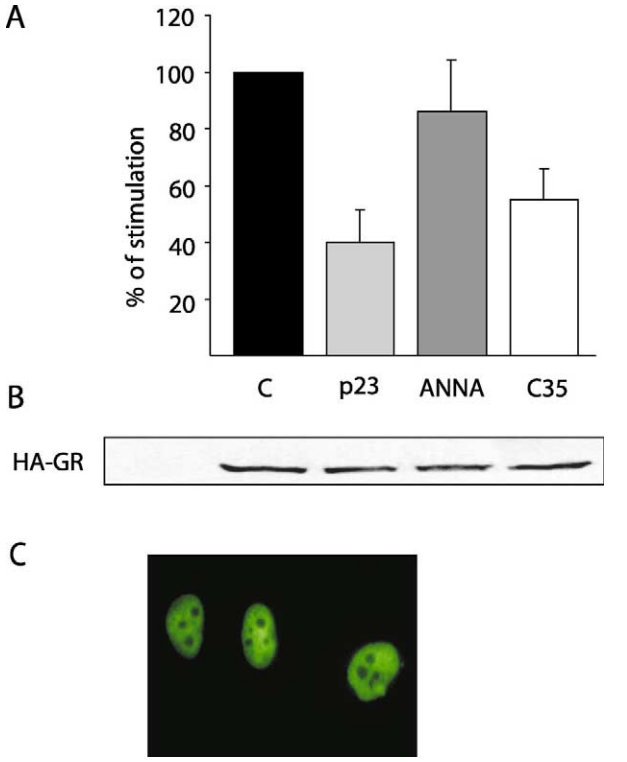

Fig. 3. p23 inhibits a constitutively active GR in an Hsp90-dependent manner. A: SK-N-MC cells were transfected with the reporter plasmid, 1 μg of a plasmid expressing a truncated GR, β-galactosidase plasmid and 3 μg of control vector (c) or respective p23 plasmid, and cultivated for 16 h. Data represent 15 independent experiments for wild-type p23, 14 for ANNA and eight for C35 performed in duplicate. B: Representative Western blot of the HA-tagged truncated GR. C: Representative fluorescence picture of GFP-tagged truncated GR in HeLa cells.

4. Discussion

p23 can act biochemically as co-chaperone by interacting with Hsp90, and as chaperone by binding to unfolded polypeptides. Here, we elucidate for the first time the contributions of these two biochemical activities to the function of p23 in vivo. In the negative regulation of GR transcriptional activation, p23 apparently functions through its interaction with Hsp90 rather than through its chaperone activity.

Although the p23–Hsp90 interaction was indispensable for inhibiting GR (Figs. 2 and 3), a C-terminal deletion mutant impairing the chaperone activity of p23 [18,19] was as effective as wild-type p23. Similar C-terminal deletions in p23 also did not affect the activity of p23 in the ATP-dependent substrate release from Hsp90 [14]. Together, these data suggest that the Hsp90 regulatory and chaperone activities of p23 operate independently of each other. The chaperone function of p23 has not been linked to a specific process in vivo, but may be relevant in stress conditions.

As part of our study we established point mutants of p23 that disrupt binding to Hsp90. Mutation of conserved bulky hydrophobic residues exposed on the surface of p23 is sufficient to disrupt its Hsp90 binding (Fig. 2). Very recently, other contact points of p23 with Hsp90 have also been reported [24], and the combined results outline an interaction surface that spans the conserved core of p23.

p23 inhibited the activity of both full-length GR and GR lacking the ligand binding domain, apparently through similar Hsp90-dependent mechanisms. Hsp90 together with p23 may act either on co-regulators recruited by GR, or directly on GR itself. For the truncated GR, the latter explanation seems unlikely, as the ligand binding domain of GR is thought to be the Hsp90 interaction site [25]. Nevertheless, a second, possibly weaker or transient interaction site of GR with Hsp90 cannot be excluded. Many cofactors are recruited by the N-terminus of GR [26], which may be targets of Hsp90.

An earlier study reported enhancement of rat GR activity by p23 while other steroid receptors like rat mineralocorticoid receptor or human estrogen receptor were inhibited [15]. The reason for the seeming discrepancy to our study is unknown, but may lay in differences of experimental settings, e.g. rat GR versus human GR. We observed inhibition of GR by p23 in four different cell lines and already at relatively low doses of p23 (Fig. 1). Importantly, our p23 expression conditions did not lead to disruption of the Hsp90 heterocomplex. Our results support and extend a more recent report that describes disassembly of transcriptional complexes and inhibition of GR activity by both chromatin-targeted and intact p23 [20], as well as chromatin-targeted Hsp90. Based on their model, our results suggest that Hsp90 and p23 work together in disassembling transcriptionally active GR from chromatin.

In principle, increased p23 expression could act on both the cytosolic and the nuclear Hsp90-regulated processes. By using the hormone-independent GR (Fig. 3), we have now analyzed specifically the nuclear function of p23, and shown that this involves Hsp90 interaction.

p23-mediated inhibition of a steroid receptor-independent promoter has been reported [20]. However, Hsp90 and p23 are not general transcription inhibitors: we found that the receptor-independent transcription from the MMTV promoter and the CMV promoter-driven transcription were unaffected (unpublished). It will be interesting to learn which transcription factors and co-regulators are specifically targeted by p23 in concert with Hsp90.

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References

[1] Hartl, F.U. and Hayer-Hartl, M. (2002) Science 295, 1852–1858.
[2] Bukau, B. and Horwich, A.L. (1998) Cell 92, 351–366.
[3] Buchner, J. (1999) Trends Biochem. Sci. 24, 136–141.
[4] Pratt, W.B. and Toft, D.O. (1997) Endocr. Rev. 18, 306–360.
[5] Young, J.C., Moarefi, I. and Hartl, F.U. (2001) J. Cell Biol. 154, 267–273.
[6] Sullivan, W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E.S., Litwack, G. and Toft, D. (1997) J. Biol. Chem. 272, 8007–8012.
[7] Fang, Y., Fliss, A.E., Rao, J. and Caplan, A.J. (1998) Mol. Cell. Biol. 18, 3727–3734.
[8] Obermann, W.M., Sondermann, H., Russo, A.A., Pavletich, N.P. and Hartl, F.U. (1998) J. Cell Biol. 143, 901–910.
[9] Bohen, S.P. (1998) Mol. Cell. Biol. 18, 3330–3339.
[10] Schulte, T.W., Akinaga, S., Soga, S., Sullivan, W., Stensgard, B., Toft, D. and Neckers, L.M. (1998) Cell. Stress Chaperones 3, 100–108.
[11] McLaughlin, S.H., Smith, H.W. and Jackson, S.E. (2002) J. Mol. Biol. 315, 787–798.
[12] Hutchison, K.A., Stancato, L.F., Owens-Grillo, J.K., Johnson, J.L., Krishna, P., Toft, D.O. and Pratt, W.B. (1995) J. Biol. Chem. 270, 18841–18847.
[13] Morishima, Y., Kanelakis, K.C., Murphy, P.J., Lowe, E.R., Jenkins, G.J., Osawa, Y., Sunahara, R.K. and Pratt, W.B. (2003) J. Biol. Chem. 278, 48754–48763.
[14] Young, J.C. and Hartl, F.U. (2000) EMBO J. 19, 5930–5940.
[15] Freeman, B.C., Felts, S.J., Toft, D.O. and Yamamoto, K.R. (2000) Genes Dev. 14, 422–434.
[16] Bose, S., Weikl, T., Bugl, H. and Buchner, J. (1996) Science 274, 1715–1717.
[17] Freeman, B.C., Toft, D.O. and Morimoto, R.I. (1996) Science 274, 1718–1720.
[18] Weikl, T., Abelmann, K. and Buchner, J. (1999) J. Mol. Biol. 293, 685–691.
[19] Weaver, A.J., Sullivan, W.P., Felts, S.J., Owen, B.A. and Toft, D.O. (2000) J. Biol. Chem. 275, 23045–23052.
[20] Freeman, B.C. and Yamamoto, K.R. (2002) Science 296, 2232–2235.
[21] Schmidt, U., Wochnik, G.M., Rosenhagen, M.C., Hartl, F.U., Holsboer, F. and Rein, T. (2003) J. Biol. Chem. 278, 4926–4931.
[22] Dittmar, K.D., Demady, D.R., Stancato, L.F., Krishna, P. and Pratt, W.B. (1997) J. Biol. Chem. 272, 21213–21220.
[23] Hollenberg, S.M., Giguere, V., Segui, P. and Evans, R.M. (1987) Cell 49, 39–46.
[24] Oxelmark, E., Knoblauch, R., Arnal, S., Su, L.F., Schapira, M. and Garabedian, M.J. (2003) J. Biol. Chem. 278, 36547–36555.
[25] Dalman, F.C., Scherrer, L.C., Taylor, L.P., Akil, H. and Pratt, W.B. (1991) J. Biol. Chem. 266, 3482–3490.
[26] Wallberg, A.E., Flinn, E.M., Gustafsson, J.A. and Wright, A.P. (2000) Biochem. Soc. Trans. 28, 410–414.