Regulation of the Dynamics of hsp90 Action on the Glucocorticoid Receptor by Acetylation/Deacetylation of the Chaperone*

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It is known that inhibition of histone deacetylases (HDACs) leads to acetylation of the abundant protein chaperone hsp90. In a recent study, we have shown that knockdown of HDAC6 by a specific small interfering RNA leads to hyperacetylation of hsp90 and that the glucocorticoid receptor (GR), an established hsp90 “client” protein, is defective in ligand binding, nuclear translocation, and gene activation in HDAC6-deficient cells (Kovacs, J. J., Murphy, P. J. M., Gaillard, S., Zhao, X., Wu, J.-T., Nicchitta, C. V., Yoshida, M., Toft, D. O., Pratt, W. B., and Yao, T.-P. (2005) Mol. Cell 18, 601–607). Using human embryonic kidney wild-type and HDAC6 (small interfering RNA) knockdown cells transiently expressing the mouse GR, we show here that the intrinsic properties of the receptor protein itself are not affected by HDAC6 knockdown, but the knockdown cytосol has a markedly decreased ability to assemble stable GR-hsp90 heterocomplexes and generate stable steroid binding activity under cell-free conditions. HDAC6 knockdown cytосol has the same ability to carry out dynamic GR-hsp90 heterocomplex assembly as wild-type cytосol. Addition of purified hsp90 to HDAC6 knockdown cytосol restores stable GR-hsp90 heterocomplex assembly to the level of wild-type cytосol. hsp90 from HDAC6 knockdown cytосol has decreased ATP-binding affinity, and it does not assemble stable GR-hsp90 heterocomplexes when it is a component of a purified five-protein assembly system. Incubation of knockdown cell hsp90 with purified HDAC6 converts the hsp90 to wild-type behavior. Thus, acetylation of hsp90 results in dynamic GR-hsp90 heterocomplex assembly/disassembly, and this is manifest in the cell as a ~100-fold shift to the right in the steroid dose response for gene activation.

The hsp90/hsp70-based chaperone machinery regulates a wide variety of proteins involved in cellular signaling through the assembly of client protein-hsp90 heterocomplexes (reviewed in Ref. 1). The chaperones hsp90 and hsp70 play a key role in the balance between maintenance of protein integrity and degradation by the ubiquitin-proteasomal pathway, and inhibition of hsp90 function leads to degradation of hsp90 client proteins (2, 3). Yu et al. (4) have reported that treatment of cells with an inhibitor of histone deacetylases (see Refs. 5 and 6 for a review of deacetylase inhibitors) leads to acetylation of hsp90 and depletion of several hsp90 client proteins, including Raf-1, ErbB2, and mutant p53. This suggests that hsp90 function is affected by acetylation/deacetylation.

Histone deacetylase (HDAC)6 is a cytoplasmic HDAC that is associated with microtubules (7) and has been shown to regulate aggresome formation in response to misfolded protein stress (8). The cystic fibrosis transmembrane conducting regulator (CFTR) is a client protein of hsp90 (9), and a mutant form, CFTR-Δ508, is prone to misfolding and aggresome formation (reviewed in Ref. 10). Misfolded CFTR-Δ508 moves in a dynein-dependent fashion along microtubules to form the perinuclear aggresome (10). HDAC6 is a component of these aggresomes and cells deficient in HDAC6 cannot form aggresomes properly, apparently because of a failure to load polyubiquitinated misfolded protein onto the dynein motor for transport to aggresomes (8). Because ubiquitylated CFTR-Δ508 is coimmunoadsorbed from cytосol with HDAC6 and HDAC6 is coadsorbed with dynein, it was proposed that HDAC6 directly links ubiquitylated CFTR-Δ508 to the motor protein (8).

Other hsp90 client proteins, such as the glucocorticoid receptor (GR) (11) and the tumor suppressor protein p53 (12, 13), have been shown to utilize an hsp90-dependent mechanism for dynein-dependent retrograde movement along microtubules. In this movement system, client protein-hsp90 heterocomplexes formed by the hsp90/hsp70-based chaperone machinery are linked via hsp90-binding immunophilins to the dynein/dynactin motor protein complex (Refs. 11 and 13 and reviewed in Ref. 14). Because HDAC6 could be required for proper hsp90 function in forming these movement complexes rather than acting itself to directly link the client protein to the dynein motor, we have examined the effect of HDAC6 on hsp90 acetylation and function (15).

We showed that HDAC6 binds hsp90 in vivo and that purified HDAC6 deacetylates hsp90 in vitro (15). Inhibition of histone deacetylases by trichostatin A or knockdown (KD) of HDAC6 by specific small interfering RNA leads to hyperacetylation of hsp90, and hsp90 in cytосol of HDAC6 knockdown cells has much less of the hsp90 cochaperone p23 bound to it than the hsp90 in wild-type cytосol (15). The GR in HDAC6-deficient cells is defective in ligand binding, nuclear translocation, and gene activation, suggesting that HDAC6 is required for hsp90 function in vivo (15). Although we proposed that HDAC6 activity is critical for normal GR function, it was not determined that hsp90, rather than the GR itself, p23, or another component of the hsp90/hsp70-based chaperone machinery, was the critical target of HDAC6 action.

In this work, we have examined the role of HDAC6 on GR-hsp90

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3 The abbreviations used are: HDAC, histone deacetylase; GR, glucocorticoid receptor; hsp, heat shock protein; CFTR, cystic fibrosis transmembrane conducting regulator; KD, knockdown; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.
heterocomplex assembly and steroid binding activity in vitro. We show that cytosol from HDAC6 knockdown cells has a decreased ability to assemble stable GR-hsp90 heterocomplexes and that the assembly activity can be restored to the level of wild-type cytosol by addition of purified deacetylated hsp90. The stable heterocomplex assembly activity of hsp90 purified from HDAC6 knockdown cytosol can be functionally restored by incubating it with purified HDAC6. Interestingly, despite its deficiency in stable heterocomplex assembly, the acetylated hsp90 of HDAC6 knockdown cells is still able to carry out dynamic assembly of GR-hsp90 heterocomplexes. The acetylated hsp90 has a lower affinity for ATP than the wild-type hsp90, and the dynamic GR-hsp90 heterocomplex assembly/disassembly that occurs in HDAC6 knockdown cells is manifest as a ~100-fold shift to the right versus wild-type cells in the dose response of dexamethasone for transcriptional activation. Together with our previous report (15), our observations prove that hsp90 function is regulated through acetylation/deacetylation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Untreated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, WI). [6,7-3H]Dexamethasone (40 Ci/ml), [ring-3,5-3H]chloramphenicol (38 Ci/ml), and [125I]-conjugated goat anti-mouse and goat anti-rabbit IgGs were obtained from PerkinElmer Life Sciences. Protein A-Sepharose, non-radioactive dexamethasone, trichostatin A, goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies, and M2 monoclonal anti-FLAG IgG were from Sigma. Dubbecco’s modified Eagle’s medium was from Bio-Whittaker (Walkersville, MD). The BuGR2 monoclonal IgG used to immunoplot the mouse GR and the rabbit polyclonal antibody used to immunoplot human GR were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG used to immunoplot hsp90 was from StressGen Biotechnologies (Victoria, BC, Canada). The IF3 monoclonal IgG used to immunoplot p23 was a gift from Dr. David Toft (Mayo Clinic, Rochester, MN). The FiGR monoclonal IgG used to immunoadsorb the mouse GR was generously provided by Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH), and the 8D3 monoclonal IgM used to immunoadsorb hsp90 was kindly provided by Dr. Gary Perdew (Pennsylvania State University, University Park, PA). The pSV2Wrec plasmid encoding full-length mouse GR and the mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter plasmid were kindly provided by Dr. Edwin Sanchez (Medical College of Ohio, Toledo, OH). 293T human embryonic kidney cells stably expressing a pSuper control small interfering RNA (293T-wt) or HDAC6 small interfering RNA (293T-HDAC6KD) were described previously (8). The expression plasmid pcDNA3-FLAG-tagged HDAC6 and rabbit antiserum pSuper control small interfering RNA (293T-wt) or HDAC6 small interfering RNA (293T-HDAC6KD) were described previously (8). The experiment plasmid pcDNA3-FLAG-tagged HDAC6 and rabbit antiserum pSuper control small interfering RNA (293T-wt) or HDAC6 small interfering RNA (293T-HDAC6KD) were described previously (8).

**Methods**

**Cell Culture and Cytosol Preparation**—293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum. Cells were harvested by scraping into Hanks’ buffered saline solution and centrifugation. Cell pellets were washed in Hanks’ buffered saline solution, resuspended in 1.5 volumes of HERM buffer (10 mM NaOH–Hepes, 1 mM EDTA, and 20 mM sodium molybdate, pH 7.4) with 1 μM trichostatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of Complete–Mini protease inhibitor mixture (Roche Applied Science) per 3 ml buffer, and ruptured by Dounce homogenization. The lysate was then centrifuged at 100,000 × g for 30 min, and the supernatant, referred to as “cytosol,” was collected, aliquotted, flash-frozen, and stored at −70 °C. Mouse GR was expressed in Sf9 cells, and cytosol was prepared as described previously (16).

**Transient Transfection of Mouse GR and MMTV-CAT Reporter**—293T cells were grown as monolayer cultures in 162-cm² culture flasks to ~50% confluency, washed, and incubated with 5 ml of serum-free medium containing 25 μg of plasmid DNA and 75 μl of TransFast transfection reagent (Promega). After 1 h, 10 ml of Dubeccco’s modified Eagle’s medium with 10% bovine calf serum was added, and the incubations were continued for 48 h. For transfection of MMTV-CAT reporter, wild-type and knockdown cells were grown as monolayer cultures in 35-mm culture wells to ~50% confluency, washed, and incubated for 1 h with 1 ml of serum-free medium containing 5 μg of plasmid DNA and 15 μl of TransFast transfection reagent. The transfection medium was replaced with regular medium, and the cells were incubated for 48 h. During the incubation, cells were treated for 20 h with various concentrations of dexamethasone.

**Immunoaodsoption of GR**—Receptors were immunoadsorbed from aliquots of 50 μl (for measuring steroid binding) or 100 μl (for Western blotting) of Sf9 cell cytosol by rotation for 2 h at 4 °C with 18 μl of protein A-Sepharose precoupled to 10 μl of FIGR ascites suspended in 200 μl of TEG (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Immunoadsorbed GR was stripped of endogenously associated hsp90 by incubating the immunopellet for an additional 2 h at 4 °C with 350 μl of 0.5 M NaCl in TEG buffer. The pellets were then washed once with 1 ml of TEG buffer followed by a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4).

**GR-hsp90 Heterocomplex Reconstitution**—Immunopellets containing GR stripped of chaperones were incubated with 50 μl of reticulocyte lysate or 293T cell cytosol and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase). For heterocomplex reconstitution with purified proteins, immunopellets containing stripped GR were incubated with 15 μg of ATP-agarose-purified knockdown or wild-type HEK 293 hsp90, 15 μg of purified rabbit hsp70, 0.6 μg of purified human Hop, 6 μg of purified human p23, 0.125 μg of purified YDJ-1 adjusted to 55 μl with HKD buffer (10 mM Hepes, pH 7.4, 100 mM KCl, 5 mM dithiothreitol) containing 20 mM sodium molybdate and 5 μl of the ATP-regenerating system. The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity and for GR-associated hsp90. In the experiments shown in Fig. 7, the five-protein mixture containing purified knockdown hsp90 was incubated for 5 min at 30 °C with an α-FLAG immune pellet prepared from cytosols of control or FLAG-tagged HDAC6-expressing cells prior to addition of the mixture to stripped GR immune pellets for heterocomplex reconstitution for 20 min at 30 °C.

**Assay of Steroid Binding Capacity**—For cytosols to be assayed for steroid binding, a 50-μl aliquot of cytosol was incubated overnight at 4 °C in 50 μl of HEM buffer plus 50 nM [3H]dexamethasone ± 1.000-fold excess of non-radioactive dexamethasone. Samples were mixed with dextran-coated charcoal, centrifuged, and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts/min of [3H]dexamethasone bound/100 μl of cytosol.

Washed immune pellets to be assayed for steroid binding to stable GR-hsp90 heterocomplexes were incubated overnight at 4 °C in 50 μl of HEM buffer plus 50 nM [3H]dexamethasone. Samples were then washed three times with 1 ml of TEGM buffer and counted by liquid scintillation spectrometry. For assay of steroid binding under dynamic
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GR-hsp90 assembly conditions, 50 nM [3H]dexamethasone was present during the assembly incubation at 30 °C, and pellets were then washed and counted. In both cases, the steroid binding is expressed as counts/min of [3H]dexamethasone bound/FIGR immunopellet prepared from 100 μl of S99 cell cytosol.

Gel Electrophoresis and Western Blotting—Immune pellets were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.25 μg/ml BuGR2 for GR, 1 μg/ml AC88 for hsp90, 1 μg/ml J3 for p23, 0.1% α-AcK, or 0.1% α-HDAC6. The immunoblots were then incubated a second time with the appropriate 125I-conjugated or horseradish peroxidase-conjugated counter-antibody to visualize the immunoreactive bands.

Protein Purification—hsp70, Hop, YDJ-1 (the yeast homolog of hsp40), and p23 were purified as described by Kanelakis and Pratt (17). When hsp90 was purified from HDAC6 knockdown cells by our usual three-step procedure (17), it was deacetylated and functionally identical to purified wild-type hsp90 in supporting stable GR-hsp90 heterocomplex assembly in the five-protein assembly system. Because hsp90 binds to ATP-agarose when the salt concentration of the application buffer is low and can then be eluted with a salt gradient, we used a single-step procedure of ATP-agarose chromatography both to partially purify hsp90 and to compare the relative ATP-binding properties of hsp90 from knockdown and wild-type cells. This procedure rapidly separates hsp90 from deacetylating activity and yields acetylated hsp90 from knockdown cytosol that does not support stable GR-hsp90 heterocomplex assembly (Fig. 6). For ATP affinity chromatography, 2.0 ml of cytosol prepared in HEM buffer was applied to a 50-ml column of ATP-agarose, the column was washed with 100 ml of HE buffer (10 mM Hepes, pH 7.4, 2 mM EDTA), and the column was then eluted with a 125-ml gradient of (0–500 mM) KCl in HE buffer. hsp90 was eluted with the KCl gradient, and the matrix was subsequently cleared of hsp70 and other high affinity ATP-binding proteins by elution with 5 mM ATP. The hsp90-containing fractions were identified by Western blotting, pooled, and contracted to 200–250 μl of S99 cell cytosol.

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GR-mediated Transcriptional Activation—Dexamethasone-induced CAT gene expression was assayed by measuring CAT enzymatic activity in wild-type and knockdown cell cytosol, using a modified version of the CAT assay described in Kwok et al. (18). Cells transfected with MMTV-CAT reporter and treated for 20 h with various concentrations of dexamethasone were washed, harvested, resuspended in potassium phosphate buffer (100 mM potassium phosphate, 1 mM dithiothreitol, pH 7.8), and ruptured by exposing the cell suspensions to three freeze-thaw cycles. Cell suspensions were centrifuged at 18,000 × g for 10 min, and the protein concentration of the supernatants was measured by Bradford assay. Aliquots of the supernatants containing 10 μg of total protein were incubated for 15 min at 70 °C in 150 mM Tris-HCl buffer, pH 7.4. The aliquots were added to a CAT reaction mixture (50 mM purified [3H]chloramphenicol, 150 mM Tris-HCl, pH 7.4, 0.25 mM butyryl-CoA) and incubated for 2 h at 37 °C. An organic phase mixture consisting of 2 parts pristane and 1 part mixed xylenes was added, and the samples were thoroughly vortexed. The reaction mixture was centrifuged at 20,000 × g for 10 min, and 150 μl of the organic phase was counted by liquid scintillation spectrometry.

RESULTS

hsp90 Binding to GR and Steroid Binding Activity Are Decreased in HDAC6 Knockdown Cells—FK228, an inhibitor of multiple histone deacetylases, has been reported to deplete cells of several hsp90 client proteins (e.g. p53, ErbB2, Raf-1) (4). However, selective knockdown of HDAC6 in HEK 293T cells results in decreased glucocorticoid binding activity without any decrease in the level of endogenous human GR (Fig. 1A). To further examine the effect of HDAC6 knockdown, we wanted to immunoadsorb the GR and directly examine its activity in formation of GR-hsp90 heterocomplexes. Because we were unable to find an immunoadsorbing antibody against the human GR, we transiently expressed the mouse (m)GR in HEK cells. Again, we found decreased steroid binding activity in HDAC6 knockdown cells without any decrease in the level of expressed mGR (Fig. 1B). mGR was immunoadsorbed from 293T cell cytosol prepared as in B with anti-GR (8) or a nonimmune IgG (NI), and the immune pellets were washed and Western blotted for mGR, hsp90, and p23.

![FIGURE 1. Both steroid binding activity and GR-hsp90 heterocomplex assembly are deficient in HDAC6 knockdown cells. A, steroid binding to human GR in HEK cells. Cytosol prepared from 293T wild-type (wt) and 293T-HDAC6KD (KD) cells was Western blotted for human GR (hGR) expression or assayed for steroid binding activity. B, steroid binding to mouse GR in HEK cells. Cytosol prepared from 293T cells transiently transfected with mouse GR (mGR) was Western blotted for mGR expression or assayed for steroid binding activity. The steroid binding values represent the mean ± S.E. from three experiments. C, effect of HDAC6 knockdown on GR-hsp90 heterocomplex assembly. mGR was immunoadsorbed from 293T cell cytosol prepared as in B with anti-GR (8) or a nonimmune IgG (NI), and the immune pellets were washed and Western blotted for mGR, hsp90, and p23.](image-url)
activity as mGR from wild-type cells. Thus, the mGR from HDAC6 knockdown cells appears to be intrinsically normal and competent to become a functional receptor in the presence of wild-type hsp90 chaperone machinery.

HDAC6 Knockdown Cytosol Is Deficient at Stable GR-hsp90 Heterocomplex Assembly—To determine whether HDAC6 knockdown cells were deficient at GR-hsp90 heterocomplex assembly, baculovirus-expressed mGR was immunoadsorbed from Sf9 cytosol, stripped of insect chaperones, and incubated with cytosols prepared from wild-type and knockdown cells. It can be seen in Fig. 3 that cytosol from HDAC6 knockdown cells has a reduced ability to form GR-hsp90 heterocomplexes and generate steroid binding activity.

In all cases where client proteins form heterocomplexes with hsp90 that are stable enough to survive immunoadsorption and washing, inhibition of hsp90 function (e.g. by geldanamycin) leads to degradation via the ubiquitylation/proteasome pathway (3). This is the case for the GR (21), and it is surprising that the level of GR in HDAC6 knockdown cells is the same as that in wild-type cells (Fig. 1), despite the reduced ability of knockdown cells to form GR-hsp90 heterocomplexes. However, in some cases, hsp90 client proteins engage in a very dynamic cycle of heterocomplex assembly/disassembly, with disassembly being so rapid that no, or only trace amounts of, client protein-hsp90 heterocomplexes are observed with biochemical techniques. This is the case with neuronal nitric-oxide synthase, for example, which associates with hsp90 in a very dynamic manner that is sort of a "hit-and-run" mode of hsp90 regulation (22). However, such a dynamic cycle of heterocomplex assembly/disassembly nevertheless stabilizes neuronal nitric-oxide synthase to proteasomal degradation (23).

The GR undergoes a similar dynamic cycle of hsp90 heterocomplex assembly/disassembly in vitro when p23 is omitted from the purified assembly system (19). Such a dynamic assembly cycle can be detected by having radiolabeled dexamethasone present during the assembly incubation (24). The [3H]dexamethasone binds to the receptor as GR-hsp90 complexes are formed, thus steroid binding constitutes evidence that the chaperone machinery has carried out hsp90-dependent opening of the steroid binding cleft. In Fig. 4, replicate GR immune pellets were incubated with cytosols from wild-type and HDAC6 knockdown 293T cells in the absence of dexamethasone and then washed and incubated with [3H]dexamethasone to detect stable heterocomplex assembly (Fig. 4A), or they were incubated with cytosols in the presence of [3H]dexamethasone to detect dynamic heterocomplex assembly (Fig. 4B). Although the HDAC6 knockdown cytosol is deficient at stable GR-hsp90 heterocomplex assembly, it has the same activity as wild-type cytosol at dynamic heterocomplex assembly.

Purified Rabbit hsp90 Restores Stable Heterocomplex Assembly of HDAC6 Knockdown Cytosol to the Level of Wild-Type Cytosol—To determine whether the decreased stable heterocomplex assembly activity of HDAC6 knockdown cytosol was because of altered function of hsp90, purified rabbit hsp90 was added to knockdown cytosol and GR-hsp90 heterocomplex assembly activity and steroid binding activity were assayed. As shown in Fig. 5A, addition of purified hsp90 brings stable heterocomplex assembly activity and steroid binding activity up to the levels of wild-type cytosol. This suggests that components of the assembly machinery other than hsp90 are not affected by HDAC6 knockdown.

We have recently reported that hsp90 immunoadsorbed from knockdown cells has much less p23 bound to it (15). Thus, it is possible that acetylation of hsp90 reduces its p23-binding affinity and that increasing the concentration of p23 could overcome this deficiency. We have shown previously that the stoichiometry of p23 to hsp90 in reticulocyte lysate is ~1:9 and that p23 is the limiting component of the hsp90/hsp70-based chaperone system in lysate (20). When purified p23 is added to reticulocyte lysate to achieve approximate stoichiometric equivalence with hsp90, there is an increase in stable GR-hsp90 heterocomplex recovery and steroid binding activity (20). A similar increase in
stable assembly is seen when purified p23 is added to wild-type 293T cytosol (Fig. 5B). Addition of purified p23 to HDAC6 knockdown cytosol yields the same percentage increase in stable assembly, but it does not alter the deficiency in assembly with respect to wild-type cytosol (Fig. 5B). Thus, it seems unlikely that acetylation of hsp90 just reduces its affinity for p23, and it is likely that acetylation makes hsp90 unable to respond to p23 at all.

**Purified hsp90 from HDAC6 Knockdown Cells Has Decreased ATP-binding Affinity**—Cytosols prepared from HDAC6 knockdown 293T cells are deficient at stable GR/hsp90 heterocomplex assembly, but they nevertheless have 20–50% of the stable assembly activity of cytosols from wild-type cells (Figs. 3–5). The ability to form some stable heterocomplexes suggests that there is a mixture of acetylated and deacetylated hsp90 in knockdown cytosol. We purified hsp90 from knockdown cytosol using our usual three-step protocol involving sequential chromatography on DEAE-cellulose, hydroxypatite, and ATP-agarose (17). The purified hsp90 was then assayed for GR/hsp90 heterocomplex assembly activity in a five-protein mixture containing purified rabbit hsp70, purified human Hop, purified human p23, and purified YDJ-1, the yeast homolog of hsp40 (17). Unexpectedly, the purified HDAC6 knockdown cell hsp90 had the same activity at stable GR/hsp90 heterocomplex assembly as hsp90 purified from wild-type 293T cells (data not shown). This suggested that the knockdown cell hsp90 was deacetylated during its purification, and partial deacetylation probably also occurred when knockdown cytosol was incubated at 30 °C during GR/hsp90 heterocomplex assembly.

With the goal of minimizing deacetylation, we wanted a rapid, single-step isolation of hsp90 from the bulk of 293T cytosol proteins. Our experience with purification of rabbit hsp90 is that the chaperone is retained by ATP-agarose under low salt conditions but appears in the dropthrough fraction if it is applied to the column in buffer containing 500 mM KCl (17). Thus, we applied HDAC6 knockdown or wild-type 293T cytosol prepared in low salt buffer to a column of ATP-agarose and eluted with a gradient of 0–500 mM KCl. The hsp90-containing
fractions identified by immunoblotting were pooled, contracted, and tested for both stable and dynamic GR/hsp90 assembly in the purified five-protein system. As shown in Fig. 6A, the HDAC6 knockdown cell hsp90 elutes from ATP-agarose at a low salt concentration and hsp90 from the wild-type cell elutes at high salt. This suggests that the acetylated hsp90 in knockdown cells has a lower ATP-binding affinity than the deacetylated hsp90 in wild-type cells. The hsp90 purified from HDAC6 knockdown cells has no stable GR/hsp90 heterocomplex assembly activity in the purified five-protein system (Fig. 6B, solid bar), but it retains dynamic assembly activity (Fig. 6B, open bar).

Incubation with HDAC6 Restores Stable GR/hsp90 Heterocomplex Assembly Activity to Knockdown hsp90—We have shown previously that immunopurified FLAG-HDAC6 deacetylates hsp90 whereas a catalytically dead HDAC6 mutant does not (15). In the experiments in Fig. 7, the five-protein assembly mixture containing purified HDAC6 knockdown cell hsp90 was incubated with immunopurified FLAG-HDAC6, and stable GR-hsp90 heterocomplex assembly was assayed. It can be seen that the stable heterocomplex assembly activity of purified knockdown cell hsp90 (Fig. 7, lane 3) is restored to the level of purified wild-type hsp90 (lane 2) by incubation with immunopurified FLAG-HDAC6 (lane 4). Incubation of wild-type hsp90 with FLAG-HDAC6 does not affect its ability to generate steroid binding (data not shown).

The Dexamethasone Dose-response Curve Is Shifted to the Right ~100-Fold in HDAC6 Knockdown Cells—The GR contains a short 7-amino-acid segment at the N terminus of the ligand binding domain that is required for hsp90 binding and steroid binding activity (25). Mutations of three amino acids in this segment of the rat GR to alanine (P548A/V551A/S552A) yields a triple mutant GR that engages in dynamic GR-hsp90 heterocomplex assembly/disassembly in vivo (26). The dose-response curve for dexamethasone-dependent gene transcription is shifted ~300-fold to the right in cells expressing the triple mutant GR compared with the wild-type GR (26). Because GR-hsp90 heterocomplex assembly/disassembly is similarly dynamic in HDAC6 knockdown 293T cells, we asked whether they had the same phenotype.
with regard to the dose response for dexamethasone. Wild-type or HDAC6 knockdown 293T cells transiently transfected with an MMTV-CAT reporter plasmid as described under “Experimental Procedures” and treated for 20 h with various concentrations of dexamethasone. The data represent the mean ± S.E. for three experiments expressed as relative CAT activity. The curves were drawn using a nonlinear regression fit provided from Prism software.

**FIGURE 7.** Incubation of purified knockdown hsp90 with HDAC6 restores its stable GR/hsp90 heterocomplex assembly activity. The five-protein assembly mixture containing hsp90 purified from HDAC6 knockdown cytosol was incubated with immune pellets prepared with α-FLAG from cytosol of nonexpressing or FLAG-tagged HDAC6-expressing cells for 5 min at 30 °C prior to addition to stripped GR immune pellets for stable GR/hsp90 heterocomplex assembly. Conditions are: stripped GR immunopellet incubated with the four-protein mixture without hsp90 (lane 1), with mixture and wild-type hsp90 (lane 2), with mixture and knockdown hsp90 (lane 3), with mixture containing knockdown hsp90 incubated with α-FLAG immune pellet of nonexpressing cytosol (lane 4), with mixture containing knockdown hsp90 incubated with α-FLAG immune pellet of nonexpressing cytosol (lane 5). The steroid binding values represent the mean ± S.E. from three experiments.

**FIGURE 6.** The dexamethasone dose response is shifted to the right in HDAC6 knockdown cells versus wild-type cells. 293T wild-type cells (closed circles) and HDAC6 knockdown cells (open circles) were transiently transfected with MMTV-CAT reporter plasmid as described under “Experimental Procedures” and treated for 20 h with various concentrations of dexamethasone. The data represent the mean ± S.E. for three experiments expressed as relative CAT activity. The curves were drawn using a nonlinear regression fit provided from Prism software.

**DISCUSSION**

Here, we have shown that specific depletion of HDAC6 renders glucocorticoid receptors in HEK 293T cells deficient in steroid binding activity (Fig. 1, A and B) and in stable heterocomplex assembly (Fig. 1C). Neither the level of the GR (Fig. 1) nor its intrinsic ability to be assembled into stable GR-hsp90 heterocomplexes with steroid binding activity are affected by HDAC6 knockdown (Fig. 2). Cytosols prepared from knockdown cells is deficient in its ability to assemble stable GR-hsp90 heterocomplexes (Fig. 3), but the assembly activity is restored to the level of wild-type cytosol by addition of purified rabbit hsp90 (Fig. 5A). This suggests that hsp90 is the only component of the multichaperone assembly machinery that is affected by depletion of HDAC6. Consistent with this, hsp90 purified from HDAC6 knockdown cells is deficient at stable GR-hsp90 heterocomplex assembly when it is the hsp90 component of a purified five-protein assembly system (Fig. 6B). The deficiency in stable assembly by hsp90 from knockdown cytosol is reversed by preincubating with HDAC6 (Fig. 7).

Taken together, three differences between HDAC6 knockdown and wild-type hsp90s suggest how acetylation affects hsp90 function. The acetylated hsp90 has decreased ATP-binding affinity (Fig. 6A), it has reduced p23 binding (15), and it is capable of dynamic, but not stable, GR/hsp90 heterocomplex assembly (Fig. 6B). A decreased affinity of hsp90 for ATP was also seen when cells were treated with the general HDAC inhibitor FK228 (4). The chaperone p23 (reviewed in Ref. 27) binds specifically to the ATP-dependent conformation of hsp90 (28, 29), and to have an open steroid binding cleft, the receptor-bound hsp90 must assume its ATP-dependent conformation (30). The acetylated hsp90 in HDAC6 knockdown cells can act as a component of the chaperone machinery to open the steroid binding cleft of the GR, as shown in Fig. 4B, but the opening is transient because p23 does not bind to stabilize the GR-hsp90 complex. p23 itself binds dynamically in stabilizing GR-hsp90 complexes in the steroid binding form (19). It is possible that the acetylated hsp90 achieves a p23 binding conformation, but it is so transient that p23 binding does not occur. Alternatively, acetylation may affect an ATP binding site on hsp90 such that ATP binds only weakly and the chaperone never achieves a p23 binding conformation. hsp90 contains a unique “Bergerat fold” ATP binding site at the N terminus that is the binding site for the hsp90 inhibitor geldanamycin (31–33), and it contains another nucleotide binding site near its C terminus (34–36), but it is not known whether acetylation affects one or both of these sites to yield decreased binding affinity for ATP-agarose. Regardless of the mechanism for the decreased p23 binding, the acetylation of hsp90 renders the chaperone incapable of stable GR-hsp90 assembly.

The dynamic assembly seen with acetylated hsp90 occurs with deacetylated hsp90 when p23 is not present in a purified assembly system (19, 24). In the normal cell with HDAC6 activity, very little of the hsp90 is acetylated and in the low ATP-binding affinity state. In the HDAC6 knockdown cell, the majority of hsp90 is acetylated, but cytosols prepared from knockdown cells still have some deacetylated hsp90 with high ATP-binding affinity that is capable of stable GR-hsp90 heterocomplex assembly (Fig. 4). The HDAC6 knockdown cells contain very little HDAC6 protein (15), and it is possible that activity of other deacetylating enzymes accounts for the presence of some hsp90 deacetylating activity in cytosol prepared from knockdown cells. Nevertheless, it is clear that HDAC6 activity is critical for normal response to hormone.

The wild-type GR in HDAC6 knockdown cells behaves like the 548/549/551 triple mutant GR (26) in that the dose response for dexamethasone-dependent transactivation is shifted about two orders of magnitude to the right (Fig. 8) with respect to wild-type cells expressing a normal level of HDAC6. This is the phenotype expected if the sole difference in both cases is a more rapid disassembly of the receptor-hsp90 complex. At any instant, only a few of the receptors in the cell would have an open steroid binding cleft and be accessible to
steroid. Because of rapid disassembly of the receptor-hsp90 heterocomplex, the proportion of time that the steroid binding cleft is open in the cell is very short, and a high concentration of steroid must be present to ensure entry before hsp90 dissociates and the ligand binding cleft closes. Thus, the GR in HDAC6-deficient cells is capable of ligand binding, nuclear translocation, and gene activation, and the HDAC6 deficiency with resulting hsp90 acetylation yields a phenotype in which much higher concentrations of steroid are required to achieve the same effect.

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REFERENCES

1. Pratt, W. B., and Toft, D. O. (2003) Exp. Biol. Med. 228, 111–133
2. Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N., and Hartl, F. U. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14536–14541
3. Issacs, J. S., Xu, W., and Neckers, L. (2003) Cancer Cell 3, 213–217
4. Yu, X., Guo, Z. S., Marcu, M. G., Neckers, L., Nguyen, D. M., Chen, G. A., and Schrump, D. S. (2002) J. Natl. Cancer Inst. 94, 504–513
5. Yoshida, M., Furumai, R., Nishiyama, M., Komatsu, T., Nishino, N., and Horinouchi, S. (2001) Cancer Chemother. Pharmacol. 48, S20–S26
6. Marks, P. A., Richon, V. M., Breslow, R., and Rifkind, R. A. (2001) Curr. Opin. Oncol. 13, 477–483
7. Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., and Yao, T-P. (2002) Nature 417, 455–458
8. Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A., and Yao, T-P. (2003) Cell 115, 727–738
9. Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B., and Riordan, J. R. (1998) EMBO J. 17, 6879–6887
10. Kopito, R. R. (2000) Trends Cell Biol. 10, 524–530
11. Harrell, J. M., Murphy, P. J. M., Morishima, Y., Chen, H., Mansfield, J. F., Galigniana, M. D., and Pratt, W. B. (2004) J. Biol. Chem. 279, 45647–45654
12. Giannakakou, P., Sackett, D. L., Ward, Y., Webster, K. R., Blagosklonny, M. V., and Fojo, T. (2000) Nat. Cell Biol. 2, 709–717
13. Galigniana, M. D., Harrell, J. M., O’Hagen, H. M., Ljungman, M., and Pratt, W. B. (2004) J. Biol. Chem. 279, 22483–22489
14. Pratt, W. B., Galigniana, M. D., Harrell, J. M., and DeFranco, D. B. (2004) Cell. Signal. 16, 857–872
15. Kovacs, J. J., Murphy, P. J. M., Gailllard, S., Zhao, X., Wu, J-T., Nicchita, C. V., Yoshida, M., Toft, D. O., Pratt, W. B., and Yao, T. P. (2005) Mol. Cell 18, 601–607
16. Morishima, Y., Murphy, P. J. M., Li, D. P., Sanchez, E. R., and Pratt, W. B. (2000) J. Biol. Chem. 275, 18054–18060
17. Kanelakis, K. C., and Pratt, W. B. (2003) Methods Enzymol. 364, 159–173
18. Kwok, R. P. S., Lundblad, J. R., Chrvina, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. (1994) Nature 370, 223–226
19. Dittmar, K. D., Demady, D. R., Stancato, L. F., Krishna, P., and Pratt, W. B. (1997) J. Biol. Chem. 272, 21213–21220
20. Morishima, Y., Kanelakis, K. C., Murphy, P. J. M., Lowe, E. R., Jenkins, G. J., Osawa, Y., Sunahara, R. K., and Pratt, W. B. (2003) J. Biol. Chem. 278, 48754–48763
21. Whitesell, L., and Cook, P. (1996) Mol. Endocrinol. 10, 705–712
22. Billecke, S. S., Draganov, D. I., Morishima, Y., Murphy, P. J. M., Dunbar, A. Y., Pratt, W. B., and Osawa, Y. (2004) J. Biol. Chem. 279, 30252–30258
23. Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B., and Osawa, Y. (1999) J. Biol. Chem. 274, 1472–1478
24. Dittmar, K. D., and Pratt, W. B. (1997) J. Biol. Chem. 272, 13047–13054
25. Xu, M., Dittmar, K. D., Giannoukos, G., Pratt, W. B., and Simons, S. S., Jr. (1998) J. Biol. Chem. 273, 13918–13924
26. Kaul, S., Murphy, P. J. M., Chen, J., Brown, L., Pratt, W. B., and Simons, S. S., Jr. (2002) J. Biol. Chem. 277, 36223–36232
27. Felts, S. J., and Toft, D. O. (2003) Cell Stress Chaperones 8, 108–113
28. Sullivan, W., Stensgard, G., Cascutt, G., Bartha, B., McMahon, N., Alnemri, E. S., Litwack, G., and Toft, D. (1997) J. Biol. Chem. 272, 8007–8012
29. Sullivan, W. P., Owen, B. A. L., and Toft, D. O. (2002) J. Biol. Chem. 277, 45942–45948
30. Grenert, J. P., Johnson, B. D., and Toft, D. O. (1999) J. Biol. Chem. 274, 17525–17533
31. Grenert, J. P., Sullivan, W. P., Fadden, P., Haystead, T. A. J., Clark, J., Minnaugh, E., Krutzsch, H., Ochel, H. J., Schulte, T. W., Sausville, E., Neckers, L. M., and Toft, D. O. (1997) J. Biol. Chem. 272, 23843–23850
32. Stehbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Cell 89, 239–250
33. Prodrumou, C., Roe, S. M., O’Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (2000) Cell 99, 65–75
34. Marcu, M. G., Chadi, A., Bouhouche, I., Catelli, M., and Neckers, L. M. (2000) J. Biol. Chem. 275, 37181–37186
35. Soti, C., Racz, A., and Csermely, P. (2002) J. Biol. Chem. 277, 7066–7075
36. Garnier, C., Laffitte, D., Tsvetkov, P. O., Barbier, P., Leclerc-Devin, J., Millot, J. M., Briand, C., Makarov, A. A., Catelli, M. G., and Peyrot, V. (2002) J. Biol. Chem. 277, 12208–12214