UbcH7 interacts with the glucocorticoid receptor and mediates receptor autoregulation

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Abstract

Unlike other nuclear receptors, transactivation by the glucocorticoid receptor (GR) is increased by the inhibition of the ubiquitin/proteasome pathway. Here, we demonstrate that the ubiquitin-conjugating enzyme (E2), UbcH7, physically interacts with the GR and, when overexpressed, reduces the ability of the receptor to upregulate gene expression. Chemical inhibition of the 26S proteasome abolished the downregulation effect of overexpressed UbcH7, suggesting a role for the 26S proteasome, and GR protein stability in mediating the UbcH7 effect. Furthermore, a UbcH7 dominant negative mutant (C89S), unable to transfer ubiquitin, failed to repress GR

transactivation. Indeed, overexpression of the mutant UbcH7 was sufficient to augment GR transactivation to levels achieved using the proteasome inhibitor MG132, but there was no further induction when MG132 and the UbcH7 mutant were used together. Expression of the dominant negative UbcH7 abolished ligand-dependent downregulation of GR protein, suggesting that the UbcH7 effect was mediated by regulation of GR protein concentration. Taken together, these data show that UbcH7 is a key regulator of GR turnover and glucocorticoid sensitivity.

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Introduction

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily. It is a key regulator of many homeostatic mechanisms and is also the target of therapeutic glucocorticoids used to treat inflammatory diseases. Much is now understood about the structure and function of the GR. The activated receptor is capable of both up- and down-regulating target gene expressions. It acts through interaction with many other transcription factors and co-modulator proteins. Cellular glucocorticoid sensitivity is not only related to receptor expression; in most cell types GR expression is also negatively regulated by exposure to glucocorticoid hormones.

Upon ligand binding, the GR translocates from the cytoplasm to the nucleus where it binds to specific DNA sequences termed glucocorticoid-response elements (GREs) to regulate target gene transcription. A number of studies have shown that cellular sensitivity to glucocorticoids is influenced by glucocorticoid receptor density (Cidlowski & Cidlowski 1981, Vanderbilt *et al.* 1987, Hoeck *et al.* 1989, Silva *et al.* 1994) with ligand-binding initiating a process of glucocorticoid receptor downregulation. This ligand-dependent receptor downregulation is mediated both at the level of

gene transcription as well as protein stability (Svec & Rudis 1981, McIntyre & Samuels 1985, Dong *et al.* 1988, Vedeckis *et al.* 1989, Oakley & Cidlowski 1993).

The GR is subject to a number of post-translational modifications, which include phosphorylation and ubiquitylation. Activation of the receptor by ligand binding initiates N-terminal phosphorylation (Weigel 1996, Bodwell *et al.* 1998). The phosphorylation state of proteins can regulate their stability (Webster *et al.* 1997), by targeting the protein for polyubiquitylation and subsequent degradation via the 26S proteasome (Fuchs *et al.* 1998, Kornitzer & Ciechanover 2000, Wallace & Cidlowski 2001, Deroo *et al.* 2002). The recognition of a phosphorylated substrate relies on its interaction with a specific ubiquitin-protein ligase (E3). Protein ubiquitylation is an energy-dependent process, which requires sequential transfer of ubiquitin from an ubiquitin-activating enzyme (E1), to an ubiquitin-conjugating enzyme (E2) to target protein generally facilitated by an E3. Ubiquitylation of the GR is regulated by at least three different E3s: CHIP (Connell *et al.* 2001, Wang & DeFranco 2005), E6-AP (Nawaz & O'Malley 2004), and hmdm2 (human homolog of mdm2) via the formation of a trimeric complex with p53 (Sengupta & Wasylyk 2001).

Interestingly, inhibition of proteasomal activity decreases the ligand-induced transcriptional activities of the estrogen and thyroid hormone receptors (Dace *et al.* 2000, Lonard *et al.* 2004). This observation suggests that protein ubiquitylation of these receptors, and/or regulatory co-factors, is required for continued transcriptional activity. By contrast, chemical inhibition of the proteasome increased the ligand-induced transactivation potential of the GR, while blocking the ligand-dependent downregulation of GR levels (Wallace & Cidlowski 2001, Wang & DeFranco 2005). These data point to a GR-specific mode of regulation by the ubiquitin–proteasome pathway. Importantly, the E3 ligase, CHIP, may determine the GR response to ubiquitylation by regulating the coupling to the ubiquitin/proteasome-dependent protein degradation pathway (Wang & DeFranco 2005).

Recently, Perissi *et al.* (2004) demonstrated that the E2, UbcH7, was recruited to DNA by activated nuclear receptors. This interaction enhanced their transcriptional activities. Moreover, UbcH7 modulated nuclear receptor transactivation, including GR function, by interaction with the co-activator SRC-1 (Verma *et al.* 2004). Independently, we identified UbcH7 as the physical interacting partner of the GR in a yeast two-hybrid screen. Here, we demonstrate that UbcH7 interacts directly with the GR *in vitro* and inhibits its transactivation function via targeting the protein for proteasome degradation. Therefore, UbcH7 interacts with multiple members of the nuclear receptor superfamily, and with their co-modulator proteins, but results in differential effects on their transcriptional activity depending on the model used. This may indicate the underlying differences in the mechanisms of transcriptional regulation used by these closely related proteins.

Methods

Plasmids

Full-length UbcH7 was cloned into pcDNA3-GFP to create UbcH7.pcDNA3 (Ardley *et al.* 2001). The UbcH7 C89S mutant was generated from UbcH7.pcDNA3 to create C89SUBcH7.pcDNA3 using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's protocol (Ardley *et al.* 2003). Mouse mammary tumor virus long terminal repeat luciferase reporter gene (MMTV-luc) and pcDNA3-GR have been previously described (Stevens *et al.* 2003a, Waters *et al.* 2004). Cytomegalovirus (CMV)-renilla vector was obtained from Promega, and used to control the transfection efficiency as previously described (Stevens *et al.* 2003a,b).

Yeast two-hybrid screen

Total RNA was extracted from 1×10^9 untreated CORL103 cells using TRIzol reagent (GIBCO BRL). An mRNA was isolated from 3 mg total RNA using GenElute

mRNA isolation (SIGMA), according to the manufacturer's guidelines. A cDNA library was constructed in B42 AD vector of the MATCHMAKER LexA hybrid screen system (Clontech) using the restriction endonucleases EcoRI and XhoI to ensure the correct orientation of the insert cDNA using the cDNA synthesis kit as per the manufacturer's instruction (Stratagene). This library formed the 'prey' for the yeast two-hybrid screen. Construction of the GR 'bait' construct (LexA-GR_{525–777}) was previously described (Stevens *et al.* 2003a).

Saccharomyces cerevisiae (EGY48) were sequentially transformed with reporter, bait, and then prey DNA constructs. Transformations were performed using a standard lithium acetate procedure (Stevens *et al.* 2003a). The transfected yeast cells were replica-plated onto synthetic dropout/Gal/Raf plates with X-gal, containing the appropriate treatment (50 μ M RU486 (mifepristone) or dimethylsulfoxide vehicle as the solvent control). The cDNA library plasmid DNA was isolated from positive colonies and re-introduced into fresh *S. cerevisiae* to confirm a positive interaction between the C-terminus of the GR (GR_{525–777}) and prey. Positive prey DNA were isolated, sequenced, and identified using BLAST.

Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris–HCl buffer (pH 7.4) containing 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and complete protease-inhibitor cocktail). Protein concentration was determined using the Bio-Rad Bradford assay reagent. Total cellular lysate protein, 500 μ g, was incubated with 2.0 μ g antibodies and 50 μ l resuspended protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, USA) for 16 h at 4 °C on a tube rotator. The beads were captured by centrifugation, and washed thrice in RIPA buffer. The bound proteins were released by boiling in SDS-PAGE loading buffer for 5 min.

Immunoblotting

GR (M20; Santa Cruz; Waters *et al.* 2004), UbcH7 (Ardley *et al.* 2003), and β -actin (Sigma; anti-actin) antibodies were employed. Samples were resolved on SDS-PAGE gels, and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 6 h with 5% (w/v) non-fat dry milk in Tris-buffered saline with 0.05% (v/v) Tween 20 and incubated for 16 h with either M20 (1:2000), UbcH7 (1:3000) or β -actin (1:2000) primary antibodies diluted in blocking buffer. The membranes were washed thrice, and blotted with secondary antibody conjugated with horseradish peroxidase. After three more washes, the membrane was developed with Pierce Supersignal West Pico reagent. Membranes were subsequently stripped and blotted for β -actin to confirm equal loading of samples and transfer of protein.

Transfection

COS7 cells, and HeLa cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) with Glutamax-1 (Invitrogen Life Technologies), and 10% (v/v) fetal calf serum before being seeded at 5×10^5 cells/10 cm tissue culture dish. All transfections were performed using Eugene 6 according to the manufacturers' instructions (Roche). For glucocorticoid reporter gene studies, cells were transfected with 2.0 µg MMTV-luc, 0.5 µg pcDNA3-GR, 0.3 or 1.0 µg pcDNA3-UbcH7-GFP or empty vector control, and 0.2 µg CMV-Renilla. Post-transfection cells were transferred to DMEM with Glutamax-1 and 10% (v/v) charcoal/dextran-stripped calf serum, divided into 24-well plates and treated in triplicate with steroid for 18 h before harvest. For chemical inhibition of the proteasome, cells were transfected with 2.0 µg MMTV-luc, 0.5 µg pcDNA3-GR, 1.0 µg pcDNA3-UbcH7-GFP, the C89S.UbcH7 mutant or the empty vector control, and 0.2 µg CMV-Renilla. Cells were then transferred into 10% (v/v) charcoal/dextran-stripped fetal calf serum containing media and split into 12-well plates. They were then pre-treated for 1 h with 1.0 µM MG-132 (Calbiochem) or vehicle then with 100 nM dexamethasone as appropriate. Cell lysates were subjected to dual luciferase assay as per the manufacturer's instructions (Promega). Firefly luciferase results were normalized using renilla luciferase as control for differences in transfection efficiency. All transfections were performed on at least three occasions with similar results.

UbcH7 localization

COS7 cells were sedimented, resuspended in medium supplemented with 5% (v/v) charcoal/dextran-stripped serum (Hyclone, UK) and seeded onto 22 mm glass coverslips at a density of 3×10^5 cells/slide. After 24 h, cells were transfected with 0.5 µg pcDNA3-GR and either 0.5 µg UbcH7-green fluorescent protein (GFP) or 0.5 µg C89S-GFP using Eugene 6 (Roche). Eighteen hours post-transfection, cells were treated with vehicle (dimethylsulphoxide; DMSO) or dexamethasone (100 nM) for 1 h before fixing with 4% (w/v) paraformaldehyde. To visualize the GR, cells were then washed thrice with tris buffered saline (TD) buffer (10 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl) and treated with blocking buffer (TD plus 1% (w/v) BSA, 0.2% (v/v) Triton X-100) for 1 h at 20 °C. A 1:200 dilution of primary antibody, P20 sc-1002 (Santa Cruz) in washing buffer (TD plus 1% (w/v) BSA, 0.05% (v/v) Triton X-100), was added for 2 h at 20 °C. Cells were then washed thrice with wash buffer. A 1:200 dilution of the appropriate secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG; Molecular Probes, Eugene, OR, USA) in washing buffer was added for 1 h at 20 °C. The cells were then washed thrice and mounted on slides using Citifluor (glycerol/PBS; Citifluor Ltd, UK). The coverslips were sealed and stored at 4 °C. Images were taken using a Leica TCS-4D confocal microscope (Leica Microsystems, Heidelberg, Germany) using a 63× water

immersion objective. To visualize Alexa 568, cells were viewed using an excitation filter of 568 nm and the emission was collected using a 590 nm long pass filter. To visualise GFP, cells were excited with an argon laser at 488 nm and emission was collected using a band-pass filter of 525 ± 25 nm.

Statistical analysis

Comparison of group data was done by ANOVA followed by Bonferroni *t*-test. Significance was taken as <0.05 , and all significant differences found are marked on the graphs. The package SPSS for windows 11.5 was used for analysis.

Results

Identification of UbcH7 as a GR-interacting protein

We used the C-terminal domain of the GR (amino acid residues 525–777) to screen a yeast two-hybrid library generated from the human small cell lung cancer cell line CORL103. We were interested in finding proteins that interacted in the presence of the GR antagonist RU486. Fifteen clones were identified as interacting with the GR bait in the presence of RU486. Interactions were confirmed in re-transformed yeast cells. Clones were sequenced, and revealed that one, XP15, had 100% identity with residues 41–154 of the coding sequence of UbcH7. The interaction between XP15 and the GR was strongest in the presence of RU486. These results identify UbcH7 as interacting with the C-terminus of the GR.

In vivo interaction between the GR and UbcH7

We studied the interaction between UbcH7 and the GR in COS7 cells in order to ensure that binding also occurred under physiological conditions in mammalian cells. These cells were chosen for two reasons. First, we demonstrated that transfected GR levels were reduced when the cells were cultured in the presence of glucocorticoid (Fig. 1a), and second that they expressed endogenous UbcH7 (Fig. 1b).

The addition of glucocorticoid produced no change in the levels of either endogenous UbcH7, or UbcH7-GFP or dominant negative (C89S).UbcH7-GFP in transfected COS7 cells (Fig. 1b). Therefore, the mechanism of action of glucocorticoid is not through a modulation of UbcH7 levels.

Immunoprecipitation with GR antibodies of COS7 cell lysates previously transfected with either UbcH7-GFP or C89S.UbcH7-GFP was followed by immunoblotting for UbcH7. Initial studies performed in the absence of MG-132, the 26S proteasome inhibitor, or dominant negative UbcH7, did not show co-immunoprecipitated UbcH7 with the GR. UbcH7 was only detected in lysates prepared from those cells transfected with the C89S.UbcH7-GFP construct, and co-incubated with MG-132. These levels were enhanced in the presence of dexamethasone (Fig. 1c). Cells were

Figure 1 *In vivo* interactions between glucocorticoid receptor (GR) and Ubch7. (a) COS7 cells were transfected with the GR pcDNA3 expression vector, or Ubch7-GFP, or C89S-GFP as indicated, and cultured in the presence of 1.0 μM dexamethasone for 18 h before harvest. Western blot analysis was then performed on cell lysates for GR protein expression. The immunoblot of whole cell lysate was subsequently stripped and re-probed for β-actin to ensure approximately equal loading of protein. (b) COS7 were transfected with the GR pcDNA3 expression vector and either wild-type (Ubch7), or dominant negative Ubch7 (C89S), and then incubated with (+) or without (–) 1 μM dexamethasone (Dex) for 18 h before harvest. Cell lysates were analysed for Ubch7 protein expression by western blot analysis. The presence of higher molecular weight bands indicates the expression of Ubch7-GFP or C89S.Ubch7-GFP (both marked Ubch7-GFP), whereas the lower bands represent endogenous Ubch7 (marked Ubch7). The immunoblot was subsequently stripped and re-probed for β-actin to confirm equal loading and transfer (not shown). (c) COS7 cells were co-transfected with the GR, and either wild-type Ubch7-GFP (Ubch7) or C89S.Ubch7-GFP (C89S), pcDNA expression constructs. The cells were then incubated with MG132 with (+) or without (–) 1.0 μM dexamethasone (Dex) as indicated for 18 h before harvest. The cell lysates were then immunoprecipitated with GR antibodies, before immunoblotting for Ubch7. Immunoblot analysis of the input lysates are shown in the left-hand four lanes (input), the immunoblot of the GR immunoprecipitate shown on the right-hand four lanes (GR IP). The IgG heavy chain band present in the immunoprecipitates is marked.

Figure 2 Co-localisation of glucocorticoid receptor (GR) and UbchH7. COS7 cells were transiently transfected with pcDNA3-GR and (a) either UbchH7-GFP or (b) C89S-GFP. Post-transfection cells were treated with 100 nM dexamethasone (Dex) for 30 min before fixation. The GR was visualized by immunostaining using a GR antibody and anti-rabbit Alexa 568-conjugated secondary antibody. Cells were then imaged with a Leica TCS-4D confocal microscope (Leica Microsystems, Heidelberg, Germany) using a 63× water immersion objective. To visualize Alexa 568 fluorescence, cells were viewed using an excitation filter of 568 nm and emission was collected using a 590 nm long pass filter. To visualise GFP fluorescence, cells were excited with an argon laser at 488 nm and emission was collected using a band-pass filter of 525 ± 25 nm.

co-incubated with MG-132 to inhibit proteasomal degradation of ubiquitinated proteins.

Analysis of the effect of glucocorticoid on the localization of Ubch7 and GR in COS7 cells

We employed a combined immunofluorescence, transfected fluorophore procedure to examine if Ubch7 and GR shared an overlapping intracellular distribution. This would not only provide further evidence of interaction, but may also evidence for an interaction that was restricted to specific subcellular compartments. It would be a prerequisite for functional interaction *in vivo*.

An intense nuclear and relatively weaker cytoplasmic Ubch7 expression was noted in COS7 cells transfected with the Ubch7-GFP (Fig. 2a). The localization of (C89S).Ubch7 was indistinguishable from that of Ubch7 (Fig. 2b). By immunofluorescence analysis, the GR is expressed principally in the cytoplasm in the absence of ligand (Fig. 2a and b; 0), but almost exclusively in the nucleus in the presence of it (Fig. 2a and b; Dex). The pattern of intranuclear distribution is similar to that we and other researchers, have reported before (Garside *et al.* 2004). It is clear from the overlapping staining patterns that the site of functional interaction could be either in the cytoplasm or in the nucleus, or both (Fig. 2a and b).

Overexpression of Ubch7 reduces GR transactivation

The data presented earlier indicate that Ubch7 and the GR physically interact within living cells. Consequently, a transient transfection system employing a glucocorticoid sensitive luciferase reporter gene construct was used to examine the functional effects of Ubch7 on glucocorticoid action. We observed that Ubch7 inhibited glucocorticoid-induced transactivation of the reporter in a concentration-dependent manner (Fig. 3). It caused both a significant reduction in the maximum activation and a significant increase in the EC50 (Fig. 3).

Inhibition of proteosomal enzymatic activity blocks Ubch7 repression of GR transactivation

We next determined whether the repressive effect of Ubch7 on dexamethasone-induced transactivation was dependent on the ubiquitin-26S proteasome pathway. As noted earlier (Fig. 3), transactivation was significantly lower when Ubch7 was overexpressed in COS7 cells (Fig. 4a, compare column 1 with column 2). The Ubch7-mediated inhibition of this transactivation was abolished when cells were cultured in the presence of the 26S proteasomal inhibitor, MG132 (Fig. 4a, compare columns 1 and 2 with columns 3 and 4 respectively). Indeed, MG132 enhanced dexamethasone-dependent transactivation in the absence of exogenous Ubch7 (Fig. 4a,

Figure 3 COS7 cells were transiently transfected with MMTV-Luc, pcDNA3-GR, CMV-renilla, Ubch7-GFP, or the empty vector control to keep a constant amount of transfected DNA. Post-transfection cells were treated for 18 h with a dose–response of dexamethasone (1–1000 nM) in triplicate then lysed and assayed for firefly and renilla luciferase activity. Firefly expression was corrected for renilla luciferase, and expressed as fold induction over a non-treated control. Results representative of three independent experiments are shown as mean ± s.d. Statistical significance is indicated compared with the empty control vector transfection.

compare column 1 with column 3). These data indicated that protein degradation via the 26S proteasome was necessary for repression of GR signaling by Ubch7.

Further support for this conclusion came from the observation that overexpression of the C89S.Ubch7 dominant negative mutant also blocked the inhibition of GR transactivation induced by endogenous Ubch7 (Fig. 4b, compare column 1 with column 2). There was no additive effect of the MG132 and the dominant negative Ubch7 on glucocorticoid transactivation (Fig. 4b, compare column 2 with column 4).

Transfection of HeLa cells, which express endogenous GR, also showed that Ubch7 impaired and the dominant negative Ubch7 enhanced dexamethasone-dependent transactivation (Fig. 5).

Discussion

To identify novel proteins capable of interacting with the GR C-terminus, a yeast two-hybrid screen was established using RU486 as the ligand. RU486 was selected as it has been shown to recruit co-modulators for GR that are important to function both in the presence of agonists and antagonist, notably NCoR (Stevens *et al.* 2003a). In this study, we

Figure 4 COS7 cells were transiently transfected with MMTV-Luc, pcDNA3-GR, CMV-renilla, Ubch7-GFP, C89S-GFP or the empty vector control to keep a constant amount of transfected DNA. Post-transfection cells were treated for 18 h with dexamethasone (100 nM), with (+) or without (–) MG-132 in triplicate then lysed and assayed for firefly and renilla luciferase activity. Firefly expression was corrected for renilla luciferase, and expressed as fold induction over a non-treated control. Results representative of three independent experiments are shown as mean $\pm$ s.d. Statistical analysis was performed using ANOVA and Bonferroni *t*-test. * $P < 0.05$ compared with control (no MG132 or Ubch7).

explored the interactions between the GR and Ubch7, a direct interaction we identified in the yeast two-hybrid screen, and a functional interaction, which has been reported by other groups for the GR and other members of the nuclear receptor superfamily (Verma *et al.* 2004). Co-immunoprecipitation experiments also identified interaction between GR and endogenous Ubch7, but only in cells transfected with the dominant negative Ubch7. This may reflect altered stability of the GR or its interactions with other proteins in the absence of authentic Ubch7 activity. For example, Ubch7 has been shown to interact with GR co-modulator proteins, and may be important for stabilizing the GR, or its interactions with Ubch7 (Verma *et al.* 2004). By inhibiting the enzymatic activity of endogenous Ubch7 on these partner proteins, the dominant negative Ubch7 may stabilize interactions between the GR and the Ubch7.

Figure 5 HeLa cells were transiently transfected with MMTV-Luc, CMV-renilla, Ubch7-GFP, C89S-GFP or the empty vector control to keep a constant amount of transfected DNA. Post-transfection cells were treated for 18 h with dexamethasone (100 nM in triplicate then lysed and assayed for firefly and renilla luciferase activity). Firefly expression was corrected for renilla luciferase, and expressed as fold induction over a non-treated control. Results representative of three independent experiments are shown as mean $\pm$ s.d. * $P < 0.05$ compared with control.

Immunofluorescence studies showed co-localisation of GR and Ubch7 in cells, allowing such interactions to take place. It seems clear that the cytosolic Ubch7 does not translocate with the GR to the nucleus. The mechanism of interaction between the GR and Ubch7 remains to be determined, but the related protein, Ubch9, also interacts with the GR C-terminus through an unidentified mechanism (Kaul *et al.* 2002).

Inhibition of either Ubch7, with dominant negative expression, or of the proteasome, with MG132, potentiated glucocorticoid action. Interestingly, other nuclear receptors, notably estrogen receptor, show the opposite effect, with proteasomal inhibition reducing transactivation (Lonard *et al.* 2004). Hence, we examined directly the effect of Ubch7 on GR protein expression levels. These showed that overexpression of dominant negative Ubch7 resulted in higher GR levels. This further strengthens the conclusion that Ubch7 primarily modifies glucocorticoid sensitivity by regulating glucocorticoid receptor stability in the presence of its ligand. Disruption of this regulatory step, as for example, by overexpression of a dominant negative Ubch7 or inhibition of 26S proteasomal activity by MG132 leads to enhancement of glucocorticoid sensitivity. This mechanism is likely to be an important component in the autoregulation of GR expression as Ubch7 expression is ubiquitous and may, therefore, modulate glucocorticoid sensitivity throughout the body.

Whether Ubch7 acts independent of other factors such as E3s in mediating the downregulation remains to be determined. There may be redundancy in the targeting process as E3s, CHIP, E6-AP, and hmdm2 also interact with the GR (Connell *et al.* 2001, Sengupta & Waslyk 2001, Wallace & Cidlowski 2001, Nawaz & O'Malley 2004). The choice of E3 may be dependent on cell type, and indeed, there is evidence that CHIP may contribute not only to the ligand-dependent degradation of GR via the proteasome, but

also to transactivation, with overexpression of CHIP reversing the potentiation of transactivation seen with MG132 (Wang & DeFranco 2005). As the GR dissociates from the heat-shock protein complex, it exposes new surfaces. Its conformation, particularly in the C-terminal ligand-binding domain, changes markedly to allow effective recruitment of co-modulator proteins (Stevens *et al.* 2003a). In addition, the N-terminal region becomes hyperphosphorylated. One or more of these changes could provide a signal for ubiquitylation.

Regulation of GR expression is a key determinant for cell and tissue glucocorticoid sensitivity. There is clearly a complex relationship between GR and its targeting by the ubiquitin system, as evidenced by our studies presented here, and those recently published (Verma *et al.* 2004, Wang & DeFranco 2005). As the dominant negative Ubch7 appears to enhance GR protein concentration, this suggests that Ubch7 acts to suppress such expression *in vivo*. Whether this modulation of GR protein expression is sufficient to completely explain the actions of Ubch7 is called into question by the findings of Verma *et al.* (2004). However, we have demonstrated direct interaction between GR and Ubch7, and showed that proteasomal activity is important for the Ubch7 effect, whereas earlier work showed interaction between Ubch7 and SRC-1 that resulted in Ubch7 potentiating GR transactivation, essentially the opposite result to those presented here (Verma *et al.* 2004). Therefore, it is possible that Ubch7 is acting both directly on the GR to mediate degradation, and also on SRC-1 to enhance transactivation. If so, the relative expression of GR, co-activator, or E3 (for example, CHIP; Wang & DeFranco 2005) may be critical for determining whether Ubch7 enhances or diminishes GR transactivation. It is also possible that the degree of ubiquitylation is important. For example, monoubiquitylation may be required for transactivation, but once transcription is triggered then polyubiquitylation and proteasomal degradation of the GR may follow. Indeed, there is evidence that the GR contains multiple ubiquitylation sites, but quantification of levels of ubiquitylation has proved difficult (Wallace & Cidlowski 2001). Extending our studies to HeLa cells, those used in the Verma study, showed similar effects of the Ubch7 overexpression to those found in COS cells. Taken together, our results and those of Verma *et al.* (2004) show that Ubch7 influences GR transactivation but other factors, as has previously been shown for the E3, CHIP, may determine the magnitude and direction of such change. Ubch7 acts on multiple proteins in the assembly of the GR-catalyzed transcription regulatory complex, and interactions between GR and Ubch7 are likely to be of low affinity, and transitory, as evidenced by co-immunoprecipitation studies. Therefore, the net result of altered Ubch7 on transactivation is likely to be dependent on the expression levels of GR, and multiple co-modulator proteins, including SRC-1 (Kaul *et al.* 2002, Verma *et al.* 2004).

Modulation of GR expression levels, particularly in response to agonist ligand, plays an important role in

determining glucocorticoid sensitivity. Aberrant regulation of these key enzymatic steps may explain pathological alterations in glucocorticoid sensitivity such as those found within sites of inflammation. In addition, the divergent effects of Ubch7 on different members of the nuclear receptor super family with considerable structural homology has implications for understanding underlying differences in the mode of action of these related ligand-activated transcription factors.

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