The Co-repressor Hairless Protects RORα Orphan Nuclear Receptor from Proteasome-mediated Degradation*

Anna N. Moraitis and Vincent Giguère‡

From the Molecular Oncology Group, McGill University Health Center and the Departments of Biochemistry, Medicine, and Oncology, McGill University, Montréal, Quebec H3A 1A1, Canada

RORα is a constitutively active orphan nuclear receptor essential for cerebellar development and is previously shown to regulate genes involved in both myogenesis and adipogenesis. The transcriptional activity of RORα is dependent on the presence of a ubiquitous ligand and can be abolished by interaction with Hairless (Hr), a ligand-oblivious nuclear receptor co-repressor. In this study, we first demonstrate that RORα is a short-lived protein and that treatment with the MG-132 proteasome inhibitor results in the accumulation of ubiquitin-conjugated receptor and inhibition of transcription. These data show that RORα transcriptional activity and degradation are intrinsically linked. In addition, the introduction of inactivation mutations in the ligand-binding pocket and co-regulator-binding surface of RORα significantly increases protein stability, indicating that ligand and/or co-regulator binding perpetuates RORα degradation. Strikingly, expression of the co-repressor Hr results in the stabilization of RORα because of an inhibition of proteasome-mediated degradation of the receptor. Stabilization of RORα by Hr requires intact nuclear receptor recognition LXXLL motifs within Hr. Interestingly, the co-repressor nuclear receptor co-repressor (NCoR) has no effect on RORα protein turnover. This study shows that stabilization of RORα is an essential component of Hr-mediated repression and suggests a molecular mechanism to achieve transcriptional repression by a liganded receptor-co-repressor complex.

The ubiquitin-proteasome pathway is the major system employed by eukaryotes for the selective degradation of cellular proteins that play key roles in cellular processes such as cell cycle regulation, differentiation, signal transduction, transcription, and chromosomal stabilization (reviewed in Refs. 1 and 2). Proteolytic degradation by the ubiquitin-proteasome system involves ATP-dependent covalent attachment of a macromolecular chain of ubiquitin (Ub)1 molecules to the target protein, followed by degradation through the multicatalytic 26 S proteasome. The conjugation of Ub, a highly conserved 8.6-kDa protein, to its target protein is mediated by the sequential action of three enzymes: E1, the Ub-activating enzyme, activates Ub in an ATP-dependent manner; E2, the Ub-conjugating enzyme, catalyzes the attachment of Ub to the substrate protein; and E3, the Ub-ligases, serves as a scaffold between E2 and the substrate and provides recognition specificity of the substrate. Ubiquitinylation of a substrate is reversible, and Ub moieties can be cleaved from a target protein by deubiquitinating enzymes. These enzymes assure that the cell is not depleted of a Ub pool. A protein tagged with a polyubiquitin chain is recognized and degraded by the 26 S proteasome complex. This complex is composed of a 19 S regulatory subcomplex, consisting of a “lid” subunit and a “base” subunit, the latter containing the six ATPases required for the degradation executed by the 20 S catalytic subcomplex (2).

The Ub-proteasome pathway has recently emerged as a key regulator of transcription controlling the level, location, and activity of transcription factors and associated co-factors (3). Nuclear receptors are short-lived transcription factors whose turnover is mediated by the Ub-proteasome complex (reviewed in Ref. 4). A number of nuclear receptors, including the estrogen, progesterone, glucocorticoid, retinoic acid, and thyroid hormone receptors, as well as peroxisome proliferator-activated receptor γ, are degraded in a ligand-dependent fashion (5–11). Degradation of the vitamin D and pregnane X receptors reportedly occurs in a ligand-independent fashion, signaled by unstable interactions of the receptors with heat shock proteins (12, 13). In addition to ligand binding, phosphorylation of nuclear receptors by signal transduction pathways and co-regulator binding also serve as signals to the Ub-proteasome complex, targeting the receptor for degradation (9, 14). Co-repressors such as NCoR and co-activators such as members of the steroid receptor co-activator (SRC) family and CREB-binding protein are also substrates for proteasome-mediated degradation (5, 15, 16).

The ligand influences the stability of nuclear receptors by inducing a conformational change that permits co-factor docking. A number of these co-factors have been identified as Ub-proteasome or Ub-like pathway enzymes, with a role in both proteasomal degradation and transcriptional activation. The E3 Ub-ligases RSP5/RPF1 and the E6-associated protein and the ATPase subunit of the 26 S proteasome SUG1 (suppressor of Gal4) all participate in nuclear receptor transactivation while simultaneously mediating their degradation (17–22). Recently, p300/CREB-binding protein has been shown to mediate the polyubiquitination of the p53 transcription factor through its intrinsic Ub-ligase activity in addition to its function as a transcriptional co-activator (23). This dual action of p300/CREB-binding protein adds strong evidence that the Ub-proteasome pathway plays a direct regulatory role in transcription. Indication that transcriptional activation and protein

* This work was supported by the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Molecular Oncology Group, McGill University Health Centre, Rm. H5–21, 687 Pine Ave. West, Montréal, PQ H3A 1A1, Canada. Tel.: 514-843-1406; Fax: 514-843-1478; E-mail: vincent.giguere@mcgill.ca.

1 The abbreviations used are: Ub, ubiquitin; SRC, steroid receptor co-activator; SUG, suppressor of Gal4; LBD, ligand-binding domain; Hr, Hairless; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; NCoR, nuclear receptor co-repressor; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin.

This paper is available on line at http://www.jbc.org

52511
Stabilization of RORα by the Co-repressor Hr

degradation occur concomitantly is further supported by the loss of nuclear receptor-mediated transcriptional activation observed upon inhibition of the 26 S proteasome function (5, 24). This observation suggests that the Ub-proteasomal complex is integral to nuclear receptor-mediated transcription. Down-regulation of an activation complex may be required for the exchange of co-activator complexes leading to disruption of the preinitiation complex, thereby allowing transcriptional elongation to proceed. The cell can then recycle components of the activation complex necessary for the initiation of a new round of transcription. This pathway provides a means of preventing the overstimulation by hormone (25).

RORα (genetic acid-related orphan receptor α; NR1F1) is a member of the ROR subfamily, which also includes RORβ (NR1F2) and RORγ (NR1F3), each regulating diverse physiological processes (26). Genetic ablation of the rora gene mimics the staggerer phenotype that is caused by massive neurodegeneration of Purkinje cells in the cerebellum (27–29). RORα knock-out and staggerer mice also serve as a model for age-related degenerative pathologies because they exhibit greater susceptibility to atherosclerosis, immunodeficiencies linked to an overexpression of inflammatory cytokines, abnormal formation and maintenance of bone, and changes in muscle differentiation (reviewed in Ref. 30). RORα is a potent transcriptional activator even in the absence of exogenously added ligand. However, the recent resolution of the crystal structures of RORα and RORβ ligand-binding domains (LBDs), in combination with mutagenesis assays of the RORα LBD, suggest that members of the ROR family require that their LBD be occupied by a ligand for transcriptional activation to occur (31–33). Regulation of RORα transcriptional activity is also mediated through co-repressor recruitment. RORα has been shown to interact with members of the SRC family and the p300/CREB-binding protein co-activators (32, 34, 35). Repression of RORα activity can be achieved by displacement from its binding site by the transcriptionally inactive orphan nuclear receptors RevErBα and RVR (36–38) and possibly by an active mechanism through interaction with the co-repressors NCoR and SMRT, although the effect of NCoR expression on RORα activity is negligible (39). However, more recently, RORα transcriptional activity was shown to be completely abrogated by the corepressor N12, RORα, SRC-1, NCoR, or actin when using anti-RORα antibody (C-18; Santa Cruz Biotechnology), anti-c-Myc antibody (Roche Applied Science), anti-SRC-1 antibody, anti-NCoR antibody (H-25; Santa Cruz Biotechnology), and anti-actin antibody (I-19; Santa Cruz Biotechnology), respectively. The lysates were prepared from transiently transfected Cos-1 cells harvested in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5) supplemented with protease and phosphatase inhibitors. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and the oligo(dT) primer, and the PCR amplification was performed using the Platinum Taq DNA polymerase (Invitrogen) in the presence of the following primers: RORA, 5′-AGG CTC CCA CAC TCG TTA CCA-3′ and 5′-AGA GCT CCA CGG TGG TCA-3′. Each PCR product was separated on a 2% agarose gel and the size of the PCR product was confirmed by sequencing.

**Experimental Procedures**

Plasmids—pCMX-hRORα wild type and ligand-binding domain mutants L381F, V364G, K357A, and E509K as well as pCMX-FLAG-hRORα have been previously described (32). N-terminal deletion mutants of hRORα, RORαΔN12, RORαΔN25, and RORαΔN35 have been described elsewhere (42). pCMV-HA-UB consists of a octameric Ub construct; each Ub is preceded at its N terminus by an HA tag as described in (43). PKR5-Myc-Hr and LXXLL mutants (HrΔp, HrΔαp, and HrNΔco) have been previously described (32). The pCMX-hNCoRα was a gift from G. Rosendal (La Jolla, CA) and was described in Ref. 44.

**Cell Culture and Transient Transfection—**Cos-1 cells obtained from the American Type Culture Collection were cultured in Dulbecco’s minimal essential medium containing penicillin (25 units/ml), streptomycin (25 units/ml), and 10% fetal calf serum at 37 °C with 5% CO2. Twenty-four hours prior to transfection, the cells were split and seeded in 12-well plates. The cells were transfected with FuGENE 6 Transfection Reagent (Roche Applied Science), following the protocol supplied by the manufacturer. A total of 1 µg of DNA/well was transfected including 0.05 µg of pCMX-hRORα1 or mutant derivatives, 0.5 µg of pCMX-hNCoRα or pRK5-Myc-rHR, 0.5 µg of reporter plasmid, and 0.25 µg of internal control pCMV/SVGal. The cells were treated with ethanol vehicle (0.1% DMSO) or 0.1, 0.5, or 1.0 µM MG-132 for 6–24 h, as specified in the figure legends. The cells were harvested and assayed for luciferase and β-galactosidase activities. The normalized values are expressed in terms of relative luciferase units. The error bars represent the standard deviation between duplicate samples. Each graph is one representative experiment of a total of three independent experiments.

**Co-immunoprecipitation and Immunoblotting Assays—**Cos-1 cells in 10-cm dishes were transiently transfected as described above with 10 µg of FLAG-RORα and HA-UB and treated with ethanol (vehicle) or 1 µM MG-132 for 24 h. The cells were lysed in IP buffer (1% Nonidet P-40, 10% glycerol, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) supplemented with protease and phosphatase inhibitors. Complete Mini EDTA-free (Roche Applied Science). The lysates (containing a total of 250 µg of protein) were incubated with 5 µg of FLAG antibody (Sigma) overnight at 4 °C with gentle rotation. The proteins were collected on protein G-Sepharose for 2 h at 4 °C with mild rotation and then washed three times with ice-cold low salt buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0). The immunoprecipitates were resolved by SDS-PAGE, transferred to a hydrophobic polyvinylidene difluoride membrane (Amersham Biosciences), and immunoblotted with FLAG antibody or HA antibody (HA.11; Berkeley Antibody Company). The proteins were visualized with the POD chemiluminescence kit following the manufacturer’s instructions (Roche Applied Science). Immunoblotting for detection of RORα wild type and mutant derivatives, 0.5 µg of Myc-Hr, SRC-1, NCoR, or actin was done using anti-RORα antibody (C-18; Santa Cruz Biotechnology), anti-c-Myc antibody (Roche Applied Science), anti-SRC-1 antibody (M-314; Santa Cruz Biotechnology), anti-NCoR antibody (H-303; Santa Cruz Biotechnology), and anti-actin antibody (I-19; Santa Cruz Biotechnology), respectively. The lysates were prepared from transiently transfected Cos-1 cells harvested in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 mM Na3VO4, 1 mM NaF), resolved by SDS-PAGE, and transferred, and immunoblotted as described above.

**Protein Degradation Assay—**The cell extract was prepared from Cos-1 cells harvested in modified RIPA buffer. 5 µl of in vitro translated [35S]methionine-labeled RORα, using Tnt rabbit reticulocyte lysate (Promega, Madison, WI), was incubated with 50 µg of cell extract, ethanol (vehicle), or 50 µM MG-132, 20 µM lactacystin, 50 µg/ml expressed sequence tag, 200 mM phenylmethylsulfonyl fluoride in a final volume of 50 µl of degradation buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.2 mM diithiothreitol) for 2 h at 37 °C. The samples were resolved by SDS-PAGE. The gels were fixed, treated with the fluorographic reagent Amplify (Amersham Biosciences), dried, and exposed.

**Pulse-Chase Assay—**Cos-1 cells in 10-cm dishes were transiently transfected as described above, with 5 µg of pCMX-hRORα1 and pRK5-Myc-rHR or pCMX-hSRC-1 for 24 h as specified in figure legends. The cells were washed carefully with 1× phosphate-buffered saline, and the medium was replaced with Dulbecco’s modified Eagle’s medium (100 µCi/ml) for an additional 1 h. The cells were washed with 1× phosphate-buffered saline and chased with Dulbecco’s modified Eagle’s medium for the times indicated in the figure legends. The cells were treated with 400 µg of lysozyme and 10 µl of protein G-agarose slurry of protein G-Sepharose for an additional 30 min. The beads were then washed with high salt buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0), followed by a wash in high salt buffer (500 mM NaCl, 1% Nonidet P-40, 10% glycerol, and 50 mM Tris-HCl, pH 8.0), and then washed in 1× phosphate-buffered saline. The samples were boiled for 3 min and resolved by SDS-PAGE. The gels were fixed and treated with fluorographic reagent, dried, and exposed. Quantification was performed using the Typhoon 8600 PhosphorImager (Amersham Biosciences).
RESULTS

RORα, a Target of the Ub-Proteasome Complex—Nuclear receptors are short-lived proteins that are rapidly turned over by the Ub-proteasome complex. Using pulse-chase analysis, we first determined that the half-life of RORα in transiently transfected Cos-1 cells is ~1.3 h (Fig. 1A). This rapid turnover is reminiscent of liganded nuclear receptors, which have a shorter half-life than their unliganded counterparts. We next investigated whether RORα degradation is mediated by the Ub-proteasome complex using pharmacological inhibitors. Peptide aldehydes (MG-132) or natural products (lactacycin) act as pseudosubstrates that become covalently linked to the 20 S proteasome and inactivate its chymotryptic and tryptic-like activities (45). As shown in Fig. 1B, RORα is not expressed endogenously in Cos-1 cells, although RORα expressed through transient transfection is detected by immunoblotting with anti-RORα antibody. Blocking of the 26 S proteasome with MG-132, lactacycin, expressed sequence tag (EST), or phenylmethylsulfonyl fluoride (PMSF) inhibitors as specified under “Experimental Procedures.” C, Cos-1 cells transiently transfected with HA-tagged Ub (HA-Ub) and FLAG-tagged RORα (Flag-RORα) treated with ethanol (−) or 1 μM MG-132 (+) (Fig. 2C). The lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibody and immunoblotted (Blot) with anti-FLAG or anti-HA antibodies as specified under “Experimental Procedures.”

Ub-conjugated RORα complexes in cells treated with MG-132 (Fig. 2C). Given the absence of Ub-RORα complexes in untreated cells, Ub-tagged RORα is likely rapidly degraded by the 26 S proteasome under normal conditions (Fig. 2C).

A Putative PEST Motif Is Not Involved in Degradation—
Proteins targeted for degradation by the Ub-proteasome complex often contain a short hydrophilic stretch of at least 12 amino acids termed a PEST motif. A PEST region serves as a proteolytic signal leading to rapid destruction of the protein (46, 47). We first used a PESTfind program (at.embnet.org/embnet/tools/biol/PESTfindabout.htm) to identify putative PEST sequences in RORα. This algorithmic program scores the hydrophilicity in a range of −50 to +50, and scores above +5.0 are considered more probable PEST motif candidates. A putative PEST motif with a score of +6.88 was located in the N-terminal region of the protein (Fig. 3A). To determine the involvement of this putative PEST motif in signaling RORα degradation, we tested three N-terminal deletion mutants referred to as RORαΔ12, RORαΔ25, and RORαΔ35 (Fig. 3B). These constructs were transiently transfected in Cos-1 cells, and their transactivation potential was assessed on a RORE-driven reporter. All deletion proteins displayed potent transcriptional activity, which was inhibited by treatment of the cells with the MG-132 (Fig. 3B). Given that protein expression of RORαΔ25 is stabilized upon treatment with MG-132 (Fig.

Fig. 1. RORα protein is rapidly turned over. A, a pulse-chase analysis was used to determine the half-life of RORα in transiently transfected Cos-1 cells labeled with [35S]methionine/cysteine and chased for 0, 1, 2, 3, 4, 5, and 20 h, followed by immunoprecipitation with anti-RORα antibody as specified under “Experimental Procedures.” One of three experiments is shown in the inset. B, Cos-1 cells were transiently transfected with empty vector (control) or RORα expression plasmid, treated with ethanol (vehicle) or 1 μM MG-132 proteasome inhibitor for 16 h. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-RORα or anti-actin antibody. WB, Western blot.

Fig. 2. RORα is ubiquitinated and degraded by the 26 S proteasome. A, in vitro degradation assay of in vitro translated and labeled RORα incubated with Cos-1 cell extracts treated with ethanol (−) or 50 μM MG-132 (+). The input (i) represents labeled RORα not subjected to the 37 °C incubation required for the degradation reaction. B, in vitro degradation assay of in vitro translated and labeled RORα incubated with Cos-1 extract in the presence of vehicle (ethanol), MG-132, lactacycin, expressed sequence tag (EST), or phenylmethylsulfonyl fluoride (PMSF) inhibitors as specified under “Experimental Procedures.” C, Cos-1 cells transiently transfected with HA-tagged Ub (HA-Ub) and FLAG-tagged RORα (Flag-RORα) treated with ethanol (−) or 1 μM MG-132 (+) for 16 h. The lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibody and immunoblotted (Blot) with anti-FLAG or anti-HA antibodies as specified under “Experimental Procedures.”
transactivation potential of a RORE.

A potential PEST motif located in the N-terminal region of RORα and identified using a PESTfinder program with a score of +6.88 is shown (filled box). B, transcriptional activity of RORα N-terminal deletion mutants transfected in Cos-1 cells treated with ethanol (vehicle, open columns) or 1 μM MG-132 (closed columns) for 16 h and assayed for transactivation potential on a RORe2-TkLuc reporter. C, expression of RORα and RORαΔ25 in cells treated with ethanol (+) or 0.5 μM MG-132 (+) analyzed by immunoblotting using the anti-RORα antibody. The actin was immunoblotted as a loading control. wt, wild type; RLU, relative luciferase units.

Fig. 3. A putative PEST motif is not involved in signaling to the Ub-proteasome complex. A, schematic representation of RORα. A potential PEST motif located in the N-terminal region of RORα and identified using a PESTfinder program with a score of +6.88 is shown (filled box). B, transcriptional activity of RORα N-terminal deletion mutants transfected in Cos-1 cells treated with ethanol (vehicle, open columns) or 1 μM MG-132 (closed columns) for 16 h and assayed for transactivation potential on a RORe2-TkLuc reporter. C, expression of RORα and RORαΔ25 in cells treated with ethanol (+) or 0.5 μM MG-132 (+) analyzed by immunoblotting using the anti-RORα antibody. The actin was immunoblotted as a loading control. wt, wild type; RLU, relative luciferase units.

3C), we have to conclude that deletion of the N-terminal PEST motif does not affect RORα degradation.

Proteasomal Degradation Is an Integral Part of RORα Transactivation Potential—Ub-mediated degradation of nuclear receptors and other transcription factors is tightly coupled to their transactivation potential, providing the cell with a mechanism that protects it against possible deleterious prolonged periods of transcription at specific genes. In particular, it has been demonstrated that this pathway is imperative for a functional hormone-mediated transcriptional response of the estrogen receptor (5, 24). However, it is not known whether this finding could be extended to orphan nuclear receptors such as RORα that display potent and constitutive transcriptional activity. As shown in Fig. 4A, treatment of Cos-1 cells transiently transfected with RORα and a RORE-driven reporter with increasing concentrations of MG-132 results in a progressive inhibition of RORα-mediated transactivation. Blocking of the 26 S proteasome most likely leads to the accumulation of transcriptionally inactive Ub-conjugated RORα. These data show that a functional Ub-proteasome pathway is critical for efficient transcriptional activation by RORα. We next investigated whether the presence of a ligand could influence RORα degradation. As mentioned above, recent crystallographic and mutational studies have shown that the presence of a ligand, possibly cholesterol or a close derivative trapped within the RORα LBD, is essential for its transcriptional activity (32, 33). However, the harsh methodology used to manipulate cholesterol levels in the cells necessary to observe a cholesterol-mediated regulation of RORα activity could indirectly affect the Ub-proteasome pathway (33). We have therefore used the LBD mutants RORαV364G and RORαL361F to simulate unliganded and liganded receptor conditions, respectively (32). The valine residue at position 364 lines the ligand-binding pocket and is in close proximity to the bound cholesterol molecule, whereas the leucine residue at position 361 is not within the vicinity of the putative ligand. Transiently transfected Cos-1 cells were treated with the proteasome inhibitor MG-132, and the transactivation potential as well as protein expression levels of both wild type and mutant RORα were assayed. As expected, RORαV364G displays considerably reduced transcriptional activity, whereas RORαL361F is functional and activates transcription from the reporter gene (Fig. 4B). Interestingly, RORαV364G expression is greater than that of wild type RORα in the absence of the proteasomal inhibitor, demonstrating that the transcriptional inactivity is independent of protein expression levels of this mutant (Fig. 4C). RORαL361F expression is similar to that of wild type RORα, indicating that this mutation does not affect the function of the receptor (Fig. 4C). Treatment of cells with MG-132 decreases the expression levels of both wild type RORα and RORαL361F, whereas no significant effect can be observed on RORαV364G expression (Fig. 4C). These data suggest that transcriptionally inactive RORαV364G is not degraded by the Ub-proteasome complex and indicates that ligand binding is required for rapid degradation of the receptor.
We and others have demonstrated that RORα transcriptional activity is dependent on the integrity of a functional co-activator binding surface (32, 34). To assess the importance of this interface in proteasomal-mediated RORα degradation, we tested the expression levels of an AF-2-deficient mutant (RORαE509K), as well as a functional hydrophobic clef mutant (RORαK357A) as a positive control. As shown in Fig. 4B, the RORαK357A mutant exhibits constitutive transcriptional activity, whereas RORαE509K is transcriptionally inactive. We have previously shown that their transactivation potentials correlate to their ability to interact with SRC co-activators (32). In a manner analogous to wild type RORα, RORαK357A degradation is blocked by MG-132, and its transcriptional activity is inhibited (Fig. 4B). The transcriptionally inactive AF-2-deficient mutant RORαE509K exhibits a higher protein expression level than wild type RORα, whereby treatment with MG-132 has no effect. This suggests that the proteasomal-mediated degradation of RORα requires an intact AF-2 domain and the concomitant recruitment of co-factor proteins.

The Co-repressor Hr Protects RORα from Proteasome-mediated Degradation—The results presented above show that proteasomal degradation of RORα is closely linked to its activation state and therefore provides a mechanism regulating RORα-mediated transcription. Evidence that the mechanisms for destroying active nuclear receptors involved direct participation of co-activator proteins is also accumulating. However, there is little evidence for the involvement of co-repressor proteins in this process. Recently, we have identified Hr as a potent repressor of RORα transcriptional activity (32). We therefore investigated whether co-expression of Hr and mutant derivatives (Fig. 5A) affects RORα protein stability. As shown in Fig. 5B, co-expression of increasing amounts of Hr together with RORα in Cos-1 cells leads to a 2-fold increase in RORα stability. We next tested whether direct interaction between Hr and RORα was required for protein stabilization. We had previously demonstrated that Hr possesses two functional nuclear receptor recognition LXXLL motifs and that ablation of both motifs was necessary to abrogate Hr/RORα interaction (32). As shown in Fig. 5C, mutation of an individual Hr LXXLL motif (Hrmut and Hrmut) does not significantly hinder stabilization of RORα by Hr (Fig. 5C, compare lanes 3 and 4 with lane 1), whereas mutation of both motifs (Hrmut) leads to a complete loss of stabilization, even in the presence of increasing amount of the mutant protein (Fig. 5D). These data indicate that Hr protects RORα from degradation as a result of direct interaction. As further controls for these experiments, we tested the effects of co-expression of NCoR and SRC-1 on RORα stability. NCoR expression had no effect on RORα protein stability (Fig. 5C, lane 6), whereas expression of SRC-1 led to a decrease of RORα protein detected (Fig. 5C, compare lanes 1 and 7 in top panel). The effect or lack thereof of the expression of both repressor proteins on RORα stability correlates with their respective effect on transcriptional activity (Fig. 5E). Interestingly, the SRC-1-mediated destabilization of RORα is blocked by MG-132 (Fig. 5C, lane 7, second panel). Finally, using pulse-chase analyses, we demonstrate that Hr stabilizes RORα and increases the receptor half-life from ~1.3 to ~2.8 h (Fig. 5F), whereas SRC-1 does not significantly affect RORα half-life (Fig. 5G). These data suggest that the potent Hr repression of RORα-mediated transcriptional activity may result as a consequence of stabilized RORα-Hr complex.

**DISCUSSION**

The Ub-proteasome system is involved in regulating the turnover of many transcription factors, including members of the nuclear receptor superfamily (4). Transcription factor activation and destruction are closely linked, providing the cell with an efficient mechanism for attenuating transcription (48). The more potently a given transcription factor activates transcription, the more rapidly it is Ub-tagged and degraded. An inverse correlation has been established between the strength of an activation domain and the protein half-life (49–51). Given that the RORα orphan nuclear receptor is a strong transcriptional activator, we investigated whether its potent activation domain is involved in the down-regulation of the receptor by signaling to the Ub-proteasome complex. In this study, we show that RORα is Ub-conjugated and rapidly degraded by the Ub-proteasome pathway with a half-life of only 1.3 h. Treatment of cells with MG-132, a pseudosubstrate that inhibits the catalytic function of the 26 S proteasome, results in a marked increase of RORα protein levels both in vivo and in vitro. Interestingly, blocking the Ub-proteasome pathway also impairs RORα transcriptional activity, indicating that degradation is an integral part of RORα-mediated transcription.

Recognition of target proteins by the Ub-proteasome complex is mediated through specific motifs that signal to E3 Ub ligase enzymes that a given protein is to be tagged with a polyubiquitin chain. Many rapidly degraded regulatory proteins contain PEST motifs, regions rich in proline, glutamic acid, serine, and threonine residues. The glucocorticoid receptor encodes a PEST motif, essential for ligand-mediated degradation, because point mutation of this motif abrogates down-regulation (9). RORα encodes a putative PEST sequence in its N-terminal domain. We have demonstrated that this motif is not required for proteasomal degradation of this protein. Similarly, RXR encodes putative PEST motifs in the N-terminal and hinge domains, although mutation of these motifs does not affect proteolytic degradation of this receptor (6). The absence of a functional PEST motif is not uncommon given that a number of receptors, including estrogen receptor α and thyroid hormone receptor, are down-regulated by the Ub-proteasome complex despite the lack of this consensus signaling motif (5, 7). Proteolysis may also be triggered by phosphorylation and/or recruitment of co-factors. For example, ligand-dependent degradation of retinoic acid receptor γ is dependent on its phosphorylation and dimerization states (14). Ligand-dependent glucocorticoid receptor degradation is also signaled by phosphorylation, because a phosphorylation-deficient mutant does not undergo proteolysis (9). It is currently unknown whether RORα serves as a substrate for specific kinases and whether such post-translational modification plays a role in RORα stabilization.

To date, studies of nuclear receptor degradation by the Ub-proteasome complex have been limited to receptors that are regulated by a known ligand (5, 7, 9, 10, 12, 52). The ligand plays a key role in mediating substrate recognition by inducing the transconformation of the LBD that allows docking of proteins involved in the Ub-proteasome pathway. One caveat in the study of orphan nuclear receptors is the absence of a bona fide ligand. Although cholesterol or a cholesterol derivative has recently been suggested as a physiological RORα ligand (33), we found it difficult to manipulate endogenous cellular cholesterol levels without affecting the general state of the cells, making it impractical to use such ligands to study specific protein degradation. Mutagenesis assays have therefore been instrumental to the understanding of the mechanisms involved in RORα transcriptional activity. Mutations in the ligand-binding pocket render the receptor transcriptionally inactive, providing additional evidence that RORα activity is regulated by an endogenous ubiquitous ligand (32). The ligand-binding pocket mutant RORαV364G provides a means of mimicking unliganded receptor conditions. Strikingly, RORαV364G exhibits greater protein expression than wild type RORα and is unaffected by inhibition of the 26 S proteasome complex, showing...
that proteolytic degradation requires an intact ligand-binding pocket and is ligand-dependent. The AF-2-deficient mutant, RORα<sup>K509E</sup>, is highly expressed irrespective of treatment with the proteasome inhibitor MG-132, suggesting that only a transcriptionally active receptor undergoes rapid proteasomal degradation. Moreover, these results also demonstrated that an intact co-activator-binding surface is required for proteolytic degradation. Given that both activation and degradation are regulated by ligand, binding of a putative RORα ligand may not only recruit co-activator proteins necessary for transcriptional activation but may also recruit proteins of the Ub-proteasome complex. This is the first demonstration of an orphan nuclear receptor that is degraded by the Ub-proteasome pathway.

Recently, a number of Ub-proteasome and Ub-like pathway enzymes have been shown to be nuclear receptor co-activators. The E3 Ub ligases E6-associated protein and RNF105/RSP5 play a dual role as nuclear receptor co-activators and may be integral components of the RNA polymerase II machinery (17–19).
RNA polymerase II recruits E3 Ub ligases through phosphorylation of its C-terminal domain. In addition, the ATPase enzymes of the 19 S proteasome subcomplex, namely SUG1 and SUG2, have recently been shown to associate with actively transcribed genes (53, 54). SUG1 binds directly to the activation domains of GAL4 and other transcription factors and functions as a nuclear receptor co-activator (20–22, 55, 56). RORα has been shown to recruit SUG1 to its LBD in a yeast two-hybrid assay, although it is not yet known whether this putative co-activator can potentiate RORα transcriptional activity or recruit the Ub-proteasome complex for RORα degradation (34). The involvement of SUG1 in the regulation of RORα transcriptional activity and stability warrants further investigation. Recently, p300 was shown to contain intrinsic E3 Ub-ligase activity catalyzing the ubiquitination of the p53 transcription factor (23). Given that RORα recruits p300 via its LBD, p300 may, in addition to functioning as a co-activator, participate in the ubiquitinylation and subsequent degradation of this orphan receptor (35).

Interestingly, we have also demonstrated that expression of the AF-2-dependent Hr co-repressor leads to the stabilization of RORα and a prolonged half-life of the protein. Mutation of the LXXLL nuclear receptor recognition motifs that are involved in mediating Hr-RORα interaction leads not only to an inability of Hr to repress RORα transcriptional activity as previously demonstrated (32) but to the loss of the protective effect against RORα degradation. This suggests that to effectively repress transcription, the co-repressor-nuclear receptor must stably exist on the promoter for a prolonged period. Similarly, Sin3 protects the p53 transcription factor from proteasome-mediated degradation, thus increasing its efficacy as a repressor (57). Moreover, the E2F-1 and c-Myc transcription factors are also stabilized by their transcriptional repression partners, pRB and miz-1, respectively (58, 59). Co-repressor-mediated stabilization of a transcription factor is therefore a mechanism that extends beyond Hr-RORα and may be a general requirement for efficient transcriptional repression. However, despite previous demonstration of interaction between RORα and NCoR in vitro (39), the current study has shown that co-repressor-mediated stabilization of RORα does not extend to NCoR. It thus remains to be determined whether the co-repressor-NCoR complex participates in the stabilization of other classes of nuclear receptors or whether NCoR utilizes other mechanisms to achieve its repressive function.

In conclusion, we have shown that expression of the co-repressor Hr results in stabilization of RORα caused by an inhibition of proteasome-mediated degradation of the receptor. It thus appears that by protecting RORα from degradation, Hr is enhancing the efficacy of the Hr-RORα complex as a transrepressor, either by prolonging the association of histone deacetylases or preventing receptor turnover to co-activator-bound receptor. The effective interference of Hr on the interdependency of RORα-mediated transcription and Ub-proteasome degradation may explain its potent repressive action on RORα transcriptional functions. A mechanism rendered essential for shutting off a powerful and constitutively active nuclear receptor.

Acknowledgments—We thank Dr. Janelle Barry for helpful comments on this manuscript. HA-Ub was kindly provided by Dr. Morag Park.

REFERENCES

1. Voges, D., Zwickl, P., and Baumeister, W. (1999) Annu. Rev. Biochem. 68, 1015–1068
2. Glickman, M. H., and Ciechanover, A. (2002) Physiol. Rev. 82, 373–429
3. Muratani, M., and Tansey, W. P. (2000) Nat. Rev. Mol. Cell Biol. 4, 192–201
4. Dennis, A. P., Haq, R. U., and Nawaz, Z. (2001) Prot. Sci. 10, D554–D559
5. Loundal, D. M., Nawaz, Z., Smith, C. L., and O’Malley, B. W. (2000) Mol. Cell 5, 939–948
6. Boudjelal, M., Wang, Z., Voorhees, J. J., and Fisher, G. J. (2000) Cancer Res. 60, 2247–2252
7. Dace, A., Zhao, L., Park, K. S., Furuno, T., Takamura, N., Nakanishi, M., West, B. L., Hanover, J. A., and Cheng, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8985–8990
8. Syvala, H., Pekki, A., Blauer, M., Pasanen, S., Makinen, E., Ylikomi, T., and Tsuchimori, P. (1996) J. Steroid Biochem. Mol. Biol. 58, 517–524
9. Wang, S. A., and Didie, W. A. (2001) J. Biol. Chem. 276, 42714–42721
10. Nomura, Y., Nagaya, T., Hayashi, Y., Kambe, F., and Seo, H. (1999) Biochem. Biophys. Res. Commun. 260, 729–733
11. Huber, S., Adelman, G., Sarraf, P., Wright, H. M., Mueller, E., and Song, J. (2000) J. Biol. Chem. 275, 18527–18533
12. Li, X. Y., Boudjelal, M., Xiao, J. H., Asuru, A., Kang, S., Fisher, G. J., and Voorhees, J. J. (1999) Mol. Endocrinol. 13, 1686–1694
13. Massaguay, H., Inouhita, H., Hiramatsu, Y., and Kudo, T. (2002) Endocrinology 143, 55–61
14. Kopf, E., Plassat, J. L., Vivat, V., De The, H., and Rochette-Egly, C. (2000) J. Biol. Chem. 275, 32820–32828
15. Zhang, J., Guenther, M. G., Carthew, R. W., and Lazar, M. A. (1998) Genes Dev. 12, 1775–1780
16. Baumann, C. T., Ma, H., Wolford, R., Reyes, J. C., Maruvada, P., Lim, C., Yen, J. T., Staal, C. H., and O’Malley, B. W. (1999) Mol. Cell Biol. 19, 1182–1189
17. Kanowsi, L. K., and O’Malley, B. W. (2000) Front. Biosci. 5, a8985–a8990
18. Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A., and Moore, D. D. (1995) Cell 81, 110–124
19. Glickman, M. H., and Ciechanover, A. (2002) Annu. Rev. Biochem. 71, 899–957
20. Lee, J. W., Ryan, F., Swaffield, J. C., Wefes, I., Sadis, S., Fu, H., Goldberg, A., Vierstra, R., and Finley, D. (1997) Mol. Biol. Rep. 24, 17–26
21. Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., and Becker-Andre, N., Daniel, H., Crepel, F., Mariani, J., Sotelo, C., and Becker-Andre, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3424–3429
22. Reid, G., Hubner, M. R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., and Ellenberger, P. (2001) Mol. Cell 11, 695–707
23. Reid, G., Denger, S., and Kanowsi, L. K., and O’Malley, B. F. (2002) Cell. Mol. Life Sci. 59, 821–831
24. Jett, A. M., and Ueda, E. (2002) Cell Death Differ. 9, 1167–1171
25. Hamilton, B. A., Frankel, W. N., Kerrebrock, A. W., Hawkins, T. L., FitzHugh, W. S., Kusumi, K., Russell, B. L., Mueller, K. L., van Berkel, V., Birren, B. W., Kruglyak, L., and Lane, G. L. (1997) EMBO J. 16, 275–284
26. Zhu, J., Gianni, M., Kopf, E., Honore, N., Chelbi-Alix, M., Koken, M., and Giguere, V. (2000) Mol. Cell Biol. 20, 3665–3674
52518

Stabilization of RORα by the Co-repressor Hr

53. Ottosen, S., Herrera, F. J., and Triezenberg, S. J. (2002) Science 296, 479–481
54. Gonzalez, F., Delahodde, A., Kodadek, T., and Johnston, S. A. (2002) Science 296, 548–550
55. Melcher, K., and Johnston, S. A. (1995) Mol. Cell Biol. 15, 2839–2848
56. Chang, C., Gonzalez, F., Rothermel, B., Sun, L., Johnston, S. A., and Kodadek, T. (2001) J. Biol. Chem. 276, 30956–30963
57. Zilfou, J. T., Hoffman, W. H., Sank, M., George, D. L., and Murphy, M. (2001) Mol. Cell Biol. 21, 3974–3985
58. Salghetti, S. E., Kim, S. Y., and Tansey, W. P. (1999) EMBO J. 18, 717–726
59. Hofmann, F., Martelli, F., Livingston, D. M., and Wang, Z. (1996) Genes Dev. 10, 2949–2959