Degradation of the Basic Helix-Loop-Helix/Per-ARNT-Sim Homology Domain Dioxin Receptor Via the Ubiquitin/Proteasome Pathway*

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Ben J. Roberts‡§ and Murray L. Whitelaw

From the Department of Biochemistry, University of Adelaide, Adelaide, South Australia, Australia 5005

The basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor (DR) translocates to the nucleus upon binding of aromatic hydrocarbon ligands typified by dioxin, whereupon it partners the Ah receptor nuclear translocator and initiates transcription. Concurrently, ligand binding down-regulates receptor levels via an unknown mechanism. In this study we show that receptor levels are dependent upon cellular compartmentalization, with entry into the nucleus leading to the rapid destruction of the DR. Ligand-induced DR translocation was bypassed by adding a heterologous nuclear localization signal to the DR, creating a constitutively nuclear form of the dioxin receptor (DRNLS). The DRNLS protein was shown to be unstable with a half-life of \( \leq 1 \) h whether partnering ARNT or HSP90. Thus, the structural changes induced by ligand binding have no inherent effect on DR stability but are critical in transporting the receptor prior to degradation. The proteolytic pathway that degrades the nuclear receptor is suggested to involve ubiquitination as it was inhibited by the proteasome inhibitor MG132 or co-expression of DRNLS with the ubiquitin mutant UbK48R. Incubation of cells expressing DRNLS with the phosphatase inhibitor calyculin resulted in the rapid phosphorylation and ubiquitination of DRNLS, suggesting that a nuclear kinase is required to trigger receptor proteolysis. Overall, this study demonstrates a novel mechanism of proteolysis whereby the simple relocation of a transcription factor from cytoplasm to nucleus initiates its rapid destruction.

The bHLH/PAS transcription factor family is involved in a diverse array of physiological pathways including hypoxia, circadian rhythms, salivary gland development, neurogenesis, and chemical metabolism (for review see Ref. 1). In general, bHLH/PAS proteins function as heterodimers with an appropriate cellular stimulus providing the signal for dimerization and subsequent DNA binding. In many cases one component of the heterodimer is specialized to respond to individual cellular signals such as xenobiotic influx or hypoxia, whereas the other is a general partner factor, ARNT (Ah receptor nuclear translocator), which functions in the absence of exogenous stimuli in the nuclear compartment. In response to ligand binding, the DR translocates from the cytoplasm to the nucleus whereupon it dimerizes with ARNT and binds to its cognate DNA recognition sequence (2–5). In comparison with the rapidly turned over bHLH/PAS hypoxia-inducible factor 1a (HIF1a), the cytoplasmic DR is a stable protein with a half-life reported to be approximately 8 h (6). However, both proteins contain NLS regions that confer nuclear translocation in response to their respective physiological stimuli (7, 8). When these sequences are mutated or deleted, DR (7) and HIF1a (8) fail to enter the nuclear compartment and initiate transcription. Given these similarities it is intriguing that the DR has been shown to be rapidly down-regulated upon ligand binding (9, 10) whereas HIF1a is rapidly up-regulated during hypoxia (11). Such opposing forces in regulating transcription factor levels have several precedents in the literature (for example; the cytokine-induced phosphorylation and degradation of signal transducers and activators of transcription 1a (12) or the mitogen-activated protein kinase-mediated stabilization of c-Jun (13)). In the case of signal transducers and activators of transcription 1a it has been suggested (12) that rapid down-regulation is a mechanism of attenuating potentially deleterious extended periods of transcription. Likewise, DR-responsive genes may also require pulsatile rather than continuous expression; however, it is unknown at present why overexpression of the DR-responsive genes so far identified, which are primarily involved in xenobiotic metabolism, would be harmful to the cell. In addition to down-regulation of the DR protein, it has recently been suggested that a DR homologue exerts a repressive effect on DR-mediated transcription via a negative feedback loop (14). Thus, there are at least two separate pathways by which DR signaling, once initiated, can be quickly attenuated. Such mechanisms may reflect developmental cues rather than those associated with DR-mediated xenobiotic metabolism.

In the cytoplasm the DR is complexed with 2 molecules of HSP90 and other proteins of uncertain function; the immunophilin-related AIP/XAP/ARA9 (15–18) and p23 (19). This protein complex is suggested to allow the ligand-binding site of DR to remain correctly folded. Ligand binding is thought to expose an amino-terminal bipartite NLS on the DR such that importin binding and nuclear transport rapidly ensue (7). ARA9 may facilitate one or both of these events although the data supporting this are indirect and based primarily on reporter gene assays (15–18). Concurrently, ligand binding also induces DR depletion in a variety of cell lines (9), suggesting that translocation and depletion may be linked. In general, protein levels can be down-regulated at any stage of their...
production, ranging from transcriptional to post-translational mechanisms. However, the DR is otherwise stable until the addition of ligand, suggesting that proteolysis is most likely to lead to receptor loss. Because the translocation and depletion of the DR occur simultaneously, it is impossible to separate the interdependence of these two events without the use of recombinant DNA. To address this issue, the DR was tagged with the nucleoplasmic NLS such that it is constitutively nuclear in the absence of ligand. Using this modified DR shows that translocation to the nucleus is necessary for DR proteolysis, with ligand binding and ARNT dimerization having no influence on susceptibility to proteolysis. We have also characterized the pathway of DR proteolysis, which involves ubiquitination and proteasome degradation, preceded by nucleophosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**A mouse monoclonal antibody (RPT1) specific for the DR was generously supplied by Dr. G. Perdew (Department of Veterinary Science, Pennsylvania State University). 12CA5 (anti-hemagglutinin) antisera was obtained from Roche Molecular Biochemicals. Anti-ARNT IgG was a gift from Dr. Y. Fujii-Kuriyama (Department of Chemistry, Tohoku University, Sendai, Japan). Anti-ubiquitin IgG was obtained from Sigma. MG132 and E-64 were obtained from Biomial and Roche Molecular Biochemicals, respectively. Calyculin was obtained from Life Technologies, Inc. Nickel-affinity-agarose was obtained from Qiagen. Ubiquitin cDNA was a gift from Dr. R. Baker (John Curtin School of Medical Research, Australian National University, Canberra, Australia). Alkaline phosphatase was purchased from Sigma. All other chemicals and reagents were of the highest quality available commercially.

**Cell Culture and Transfections—**Hepa1c1c7 cells, Hepa1c4 cells, Y1 cells, and 293 T cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Y1 cells stably transfected with DRNLs were grown under the aforementioned conditions and supplemented with 50 μg/ml G418. Transfections were carried out using DOTAP (Roche Molecular Biochemicals) liposomal transfection medium in 35-mm culture dishes using 1–2 μg of DNA per dish. Experiments were conducted 24 h after transfection. Experiments involving the transfection of ubiquitin mutants used wild type ubiquitin cDNA as a control in the same expression vector.

**Constructs and Oligonucleotides for Polymerase Chain Reaction-based Mutagenesis—**Primers for ubiquitin mutagenesis were 5′-TTGGCG-GTAGCGCACTGTC3′/5′GAGCTGCTTCCGGGAA3′ (to generate UbK48R) and 5′-TAAAGGGCTGTTGAGTAC3′/5′GTTACCCCAACGGACCTTTAG3′ (to generate UbK48R/G76A). All ubiquitin constructs were made in a pGem7 cassette prior to being subcloned into pCMV5 (20). In.all cases polymerase chain reaction mutagenesis was performed using the Quick-change kit (Stratagene) according to the manufacturer’s instructions. All constructs were verified by sequencing. The primers and construction of DRNLs have been described previously (21); the NLS consists of the nucleoplasmic NLS (5′/KRPAT-KKAGQQKKKKR3′) followed by the hemagglutinin (HA) epitope. Briefly, the NLS-Δa tag sequence was cloned in frame into an Xhol site on mouse DR cDNA previously generated at the 3′ end of the DR coding region by a polymerase chain reaction (21) and followed by a His6 tag and a stop codon. When expressed in mammalian cells, the subsequent cDNA encoded DR with the NLS-His6 fusion sequence at its carboxyl terminus (21). The subcellular localization of this construct has been shown to be exclusively nuclear in mammalian cells (21).

**Cell Extract Preparation and Immunoblotting and Immunoprecipitation—**Cells were harvested, and whole cell extracts were prepared as described previously (21). When extracts were prepared for analysis of ubiquitin conjugates or phosphorylation, 5 mM N-ethylmaleimide or 5 mM NaF were added to the respective buffers. Nuclear extracts were prepared as follows. Cells were lysed in a buffer containing 10% Ficol1-400, 0.1 M Tris-HCl, pH 7.4, 0.1% Nonidet P-40, and 150 mM NaCl. After 5 min at 4°C, extracts were centrifuged at 10,000 × g for 15 min. The supernatant (cytoplasmic fraction) was removed, and the pellets were washed once with 1 ml of phosphate-buffered saline prior to centrifugation at 10,000 × g for 10 min. The resulting pellet was resuspended in a buffer containing 0.1 M Tris-HCl, pH 7.5, 0.1% Tween 20, 400 mM NaCl, 0.1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol and mixed for 20 min at 4°C. The suspension was centrifuged at 10,000 × g for 10 min, and the nuclear supernatant was removed and boiled in SDS-PAGE sample buffer. All procedures were carried out at 4°C. Immunoprecipitation of DRNLs was performed using 12CA5 IgG. Briefly, whole cell extracts (60 μl) were mixed with 5 × volumes of a binding buffer containing 100 mM KCl, 0.1% Tween 20, 10 mM phenylmethylsulfonyl fluoride, and Tris-HCl, pH 7.5. 12CA5 IgG was added, and the mixture was incubated for 2 h at 4°C. A 50-μl suspension of Protein A-Sepharose (Amersham Pharmacia Biotech) was added, and the mixture was incubated for an additional hour. The suspension was washed 3 times with 1 ml of binding buffer, and the remaining slurry was boiled for 5 min in SDS-PAGE sample buffer prior to immunoblotting. For assays using alkaline phosphatase, 20 μg of whole cell extract was resuspended in 1 ml of Tris-HCl, pH 9.5 and 11 μg of alkaline phosphatase (20 diethanolamine units per assay) in a 50-μl volume. Reactions were conducted at 37°C and terminated with the addition of SDS-PAGE sample buffer.

Treatment of Cells with Protease Inhibitors and Calyculin—Cells were incubated with the calpain inhibitor E-64 or the proteasome inhibitor MG132 at concentrations of 10 μM and 2.5 μM, respectively. Both chemicals were dissolved in MeSO and administered to cells 1 h before the commencement of experiments. Calyculin was dissolved in MeSO and used in cells at a concentration of 50 nM. In all cases controls received vehicle solvent.

**Nickel Affinity Purification of DRNLs and DRNLs-Ubiquitin Conjugates—**Nickel affinity purification was performed using the method previously described by Lees and Whitelaw (21) with modifications by Abell et al. (22). His-tagged DRNLs was eluted from the nickel-agarose with 300 mM imidazole in a buffer consisting of 0.1% Tris-HCl, 0.1% Triton X-100, and 200 mM NaCl. SDS-PAGE sample buffer was added to the DRNLs eluate prior to electrophoresis and immunoblotting.

**RESULTS**

Ligand-induced Degradation of the Dioxin Receptor in Hepatoma Cells—When the DR ligand TCDD is administered to murine hepatoma Hepa1c1c7 cells, analysis of whole cell extracts by immunoblotting reveals that the DR is depleted in a time-dependent manner (Fig. 1A). Preparation of nuclear and cytoplasmic extracts shows that the DR enters the nucleus following TCDD administration (Fig. 1B). Co-incubation of the cells with the proteasome inhibitor MG132 and TCDD leads to an accumulation of the DR in the nucleus only (Fig. 1B, lane 6),
Hepa1c4 cells were incubated with TCDD (10 nM) for the times indicated. Whole cell extracts were prepared, separated by 8% SDS-PAGE, and immunoblotted using anti-DR mAb. B, cytosolic and nuclear extracts were prepared from Hepa1c4 cells after a 2.5-h incubation in the presence or absence of TCDD (10 nM), MG132 (2.5 μM), E64 (10 μM), or Me₂SO (DMSO) as shown. Extracts were analyzed as in A.

FIG. 2. Characterization of DR depletion in Hepa1c4 cells. A, Hepa1c4 cells were incubated with TCDD (10 nM) for the times indicated. Whole cell extracts were prepared, separated by 8% SDS-PAGE, and immunoblotted using anti-DR mAb. B, cytosolic and nuclear extracts were prepared from Hepa1c4 cells after a 2.5-h incubation in the presence or absence of TCDD (10 nM), MG132 (2.5 μM), E64 (10 μM), or Me₂SO (DMSO) as shown. Extracts were analyzed as in A.

with the calpain inhibitor E-64 having no effect on DR levels either in the cytoplasmic or nuclear compartments (Fig. 1B, lanes 2 and 5). Using a radiolabeled probe containing the xenobiotic response element (XRE; the DNA-binding site of the DR/ARNT heterodimer), a gel shift was performed with extracts from TCDD-treated Hepa1c1c7 cells. When TCDD is added to the cells for 2.5 h, the extracts show a single shifted band corresponding to the DR/ARNT heterodimer (Fig. 1C, lane 4). This band is intensified severalfold in the presence of MG132 but not E-64 (Fig. 1C, lanes 5 and 6), suggesting that proteasome inhibition increases the amount of DR/ARNT heterodimer available for DNA binding. Because an increase in DNA binding could conceivably result from elevated ARNT or DR, MG132 and E-64 were added to Hepa1c1c7 cells for 2.5 h, and levels of ARNT were determined by immunoblotting (Fig. 1D). Neither protease inhibitor had any effect on ARNT concentrations either in the presence or absence of TCDD, suggesting that the increase observed in the gel shift resulted primarily from stabilization of DR by MG132.

In Hepa1c1c7 cells the depletion of the DR and its subsequent stabilization by MG132 depends upon the addition of ligand. Therefore, the proteolysis of the DR could be initiated by (i) translocation to the nucleus, (ii) formation of a heterodimer with ARNT in the nucleus, or (iii) conformational changes induced by ligand. We attempted to address each of these possibilities experimentally using a combination of methods. In mutant Hepa1c4 cells, DR is expressed at similar levels to those described in Fig. 1A, addition of TCDD to Hepa1c4 cells resulted in the rapid depletion of DR (Fig. 2A). When cytoplasmic and nuclear extracts were prepared, DR translocated to the nucleus where it could only be visualized by the addition of MG132 (Fig. 2B, lane 6). Because of the absence of nuclear ARNT in these cells, there is some DR leakage out of the nuclear compartment during extract preparation, accounting for the DR present in the cytoplasmic fraction following the addition of TCDD and MG132. The data from Fig. 2 suggest that the ligand-induced depletion of the DR is not triggered by dimerization with its partner factor ARNT.

Nuclear Instability of the Dioxin Receptor—Because dimerization with ARNT in the nuclear compartment does not appear to initiate DR proteolysis, the role of nuclear translocation per se was investigated using recombinant DNA (Fig. 3). We decided to completely bypass ligand-induced nuclear translocation of the DR to determine the nuclear stability of the DR in both the absence and presence of ligand. Ligand binding could conceivably alter the structure of the latent DR complex such that it could be formatted for nuclear proteolysis, even though this is clearly not the case in the cytoplasmic compartment (Fig. 3). The carboxy terminal of the DR was modified by the addition of the nucleoplasmic NLS followed by an additional epitope recognized by anti-hemagglutinin IgG and a hexahistidine tag (His₆, 21). We have previously generated a derivative cell line from Y1 murine adrenal cells that stably expresses this DRNLS construct. From these cells, DRNLS has been shown to be exclusively nuclear while remaining in a latent HSP90 complex. When Y1/DRNLS cells were incubated with MG132, DRNLS was counter-stained with either anti-HA or anti-ARNT IgG. B, cycloheximide (100 μg/ml) was incubated with Y1/DRNLS cells for 1 h prior to the addition of TCDD or vehicle control. Whole cell extracts were prepared at the time points shown on the figure, separated by 8% SDS-PAGE, and immunoblotted with anti-HA mAb. D, a DRNLS expression vector was transiently transfected into 293 T cells in the presence or absence of ARNT expression vector. 24 h after transfection TCDD (10 nM) was added, and whole cell extracts were prepared 12 h later. Extracts were immunoblotted with anti-HA mAb, membranes were stripped, and immunoblots were performed using anti-ARNT polyclonal antibodies. As shown in the lanes for ARNT marked (–), 293 T cells contain a small amount of endogenous ARNT protein.

FIG. 3. Stability of a constitutively nuclear dioxin receptor. A, a Y1 adrenal stable cell line expressing DRNLS was incubated with TCDD (10 nM) for 1 h to verify that it dimerized with ARNT. Whole cell extracts were prepared with or without TCDD and DRNLS immunoprecipitated with anti-HA mAb. The immunoprecipitated DRNLS was scanned using a Molecular Dynamics densitometer (Sunnyvale, CA), and the loss of DRNLS was expressed graphically on the y axis as a percentage of DRNLS at time 0. Each time point denotes the mean of duplicate determinations from a representative experiment. B, DRNLS cells were co-incubated with TCDD (10 nM) and/or the protease inhibitors MG132 (2.5 μM) or E64 (10 μM) for 2.5 h. Whole cell extracts were prepared, separated by 8% SDS-PAGE, and immunoblotted with anti-ARNT polyclonal antibodies. As shown in the lanes for ARNT marked (–), 293 T cells contain a small amount of endogenous ARNT protein.
either Ub or UbK48R. TCDD (10 nM) was added 24 h after transfection by 8% SDS-PAGE and analyzed by immunoblotting using anti-HA IgG. TCDD (10 nM) was added 24 h after transfection with cells harvested and whole cell extracts prepared an additional 12 h later. Proteins were separated by 8% SDS-PAGE and immunoblotted with either anti-HA or anti ARNT antibodies. C, gene induction was performed using an XRE-driven luciferase reporter construct. DRNLS was transiently co-expressed with either Ub or UbK48R in the presence of an XRE reporter gene (21) together with an internal control plasmid expressing renilla luciferase. TCDD (10 ns) was added 24 h after transfection, and cells were harvested and assayed for luciferase activity an additional 12 h later. Activity is expressed on the y axis as arbitrary units (AU), which is the ratio of XRE reporter gene luciferase activity/internal control. Each bar represents the mean ± S.D. of four separate determinations.

3C, lane 3). The calpain inhibitor E-64 had no effect in either case (Fig. 3C, lane 2), suggesting that DRNLS is rapidly degraded by a proteasome-mediated pathway. Transient co-expression of DRNLS with or without ARNT in human embryonic kidney 293 T cells also failed to display any ligand-dependent changes in DRNLS levels (Fig. 3D). Thus it appears that DRNLS stability is determined independently of the concentration of its partner proteins.

Role of Ubiquitination in the Degradation of the Dioxin Receptor—Ubiquitin conjugation is a common mechanism by which transcription factor levels are targeted for destruction. Because ubiquitin conjugation precedes 26 S proteasome degradation, chemical inhibitors of the proteasome do not distinguish between ubiquitin-mediated or proteasome-mediated degradation of a protein (although examples of the latter are rare and usually reserved for damaged proteins). When incubating Y1/DRNLS cells or Hepa1c1c7/c4 cells with MG132 we were unable to visualize DR-ubiquitin conjugates as laddering on anti-HA immunoblots; however, the detection of such conjugates often varies, and as such, an alternative method was used to verify a role for ubiquitination in DR proteolysis. A ubiquitin construct with a lysine to arginine mutation at amino 48, a principal acceptor residue for further ubiquitin monomers, was utilized. Expression of UbK48R in eukaryotic cells acts as a dominant negative mutation (25, 26) by inhibiting ubiquitin chain formation, thereby preventing the mult ubiquitination of a target protein and its subsequent recognition and proteolysis by the 26 S proteasome. Co-expression of UbK48R with DRNLS in 293 T cells results in an increase in DRNLS levels when compared with transfection with wild type Ub (Fig. 4A, compare lanes 1 and 2). We attempted to “trap” monoubiquitinated DRNLS by creating another mutation at the carbonyl-terminal glycine of UbK48R (UbK48R/G76A). A glycine to alanine mutation at this residue has been demonstrated to inhibit ubiquitin isopeptidases (27, 28), thus we reasoned that co-transfection with this double mutant would enhance our ability to detect DRNLS-Ub. Although we were still able to effectively inhibit DRNLS proteolysis with this construct (Fig. 4A, lane 5), no DRNLS-ubiquitin conjugate(s) were identified, suggesting that the levels of DRNLS-Ub conjugates are very low and/or unstable under these experimental conditions. When UbK48R was co-expressed with DRNLS and ARNT, no changes were observed in ARNT levels as determined by immunoblotting (Fig. 4B). Therefore, the levels of DR but not ARNT are suggested to be regulated by ubiquitination. In Fig. 4C the effect of UbK48R on DRNLS-signaling was determined in a gene reporter assay. Co-transfection of UbK48R with DRNLS significantly increased DRNLS-mediated reporter activity as compared with DRNLS alone. Overall, the simplest interpretation of the results presented in Fig. 4 is that the nuclear DR is rapidly degraded by ubiquitination that is either complexed with HSP90 or ARNT.

Role of Phosphorylation in the Nuclear Degradation of the Dioxin Receptor—In an effort to determine the mechanism responsible for rapid nuclear proteolysis of the DR we investigated the potential role of phosphorylation. Several transcription factors couple phosphorylation to ubiquitination as an effective trigger for their proteolysis (see Refs. 13, 29, and 30 for examples). Thus, in the absence of any obvious structural destabilization caused by ligand binding, it seemed logical that the DR may also be phosphorylated prior to its degradation. When the phosphatase inhibitor calyculin was added to Y1 cells expressing DRNLS, a time-dependent shift in the migration of DRNLS was observed by immunoblotting (Fig. 5A), most notably after incubation for 1 h. We interpreted the slower migrating of these 2 bands to represent a phosphorylated form of DRNLS. When calyculin-treated extracts were split into separate aliquots and treated with alkaline phosphatase, the doublet was resolved to a single band after 25 min (Fig. 5B), confirming the presence of phosphorylated DRNLS. When DRNLS cells were treated with calyculin and MG132 in the presence or absence of ligand (Fig. 5C) it was apparent that DRNLS was phosphorylated to the same extent whether partnered by HSP90 or ARNT (Fig. 5C, compare lanes 2 and 3). Moreover, when the immunoblots were exposed for longer time periods, several more bands from calyculin-treated cells were evident at higher molecular mass with an accrual of immunoreactive material at a molecular mass range of >200 kDa. Importantly, in the same cell line deficient for DRNLS, no immunoreactivity was observed (lane 4), suggesting that the staining consists of DRNLS and covalently bound derivatives thereof. These results are consistent with phosphorylation-induced ubiquitination of the DRNLS protein. To verify that this staining represented DRNLS-ubiquitin conjugates, we purified the His6-tagged DRNLS receptor from Y1 cells on a nickel affinity column and counter-stained with anti-ubiquitin IgG (Fig. 5D). In extracts from the control cell line, nickel affinity purification showed no immunoreactive material with either anti-HA or anti-ubiquitin IgG in the presence of MG132 and calyculin (Fig. 5D, lane 1). Nickel affinity purification of DRNLS from Y1 cells incubated with MG132 showed minimal staining with anti-ubiquitin IgG, indicative of the very low levels of DR-ubiquitin conjugates normally found within cells (Fig. 5D, lane 2). However, when Y1/DRNLS cells were incubated with MG132 and calyculin, a considerable increase was observed in ubiquitin conjugates compared with MG132 alone (Fig. 5D, lanes 2 and 3). Therefore, the collective data presented in Fig. 5 suggest that calyculin treatment results in the phosphorylation and subsequent ubiquitination of DRNLS.
performed using anti-HA mAb. Samples were separated by 8% SDS-PAGE, and immunoblots were bated with alkaline phosphatase (Ap). MG132 (2.5 M) was pre-incubated with the cells for 1 h prior to the addition of calyculin. Whole cell extracts were prepared, separated by 8% SDS-PAGE, and immunoblotted with anti-HA mAb. The position of DRNLS and the slower migrating phosphorylated (pDRNLS) are marked on the figure. B, protein extracts from Y1/DRNLS cells previously treated with calyculin for 1 h were split into aliquots and incubated with alkaline phosphatase (AP) at pH 9.5 for the times indicated. Reactions were terminated by the addition of SDS-PAGE sample buffer, samples were separated by 8% SDS-PAGE, and immunoblots were performed using anti-HA mAb. C, DRNLS cells or the non-transfected parent Y1 cell line were incubated in the absence or presence of TCDD (10 nM) and/or MG132 (2.5 M) for 1 h prior to the addition of calyculin (50 nM). Following a 1-h incubation with calyculin, cells were harvested, and whole cell extracts were prepared. Extracts were separated by 8% SDS-PAGE and immunoblotted with anti-HA mAb. The two panels represent the same immunoblot at different exposures to highlight phosphorylated (pDRNLS) (Ex 1) and high molecular mass immu- noreactive material (Ex 2). In addition, DRNLS from cells treated with calyculin + MG132 or calyculin + MG132 + TCDD were immunopre-cipitated using anti-HA mAb and counter-stained with anti-ARNT antiseraum. D, DRNLS was purified via its His tag using nickel affinity-agarose. Y1/DRNLS cells or the parent Y1 cell line were incubated with MG132 (2.5 M) for 1 h prior to the addition of calyculin (50 nM). Cells were harvested 1 h later in a denaturing buffer and purified on nickel-agarose (see “Experimental Procedures”). DRNLS was eluted by imid- azole (250 mM) and boiled in SDS-PAGE sample buffer prior to analysis by 8% SDS-PAGE and immunoblotting with anti-HA or anti-Ub antibodies. Molecular mass standards are as shown.

FIG. 5. Phosphorylation of DRNLS and its relationship to ubiquitination. A, the phosphatase inhibitor calyculin (50 mM) was added to Y1/DRNLS cells for the time periods indicated. The proteasome inhibitor MG132 (2.5 M) was pre-incubated with the cells for 1 h prior to the addition of calyculin. Whole cell extracts were prepared, separated by 8% SDS-PAGE, and immunoblotted with anti-HA mAb. The position of DRNLS and the slower migrating phosphorylated (pDRNLS) are marked on the figure. B, protein extracts from Y1/DRNLS cells previously treated with calyculin for 1 h were split into aliquots and incubated with alkaline phosphatase (AP) at pH 9.5 for the times indicated. Reactions were terminated by the addition of SDS-PAGE sample buffer, samples were separated by 8% SDS-PAGE, and immunoblots were performed using anti-HA mAb. C, DRNLS cells or the non-transfected parent Y1 cell line were incubated in the absence or presence of TCDD (10 nM) and/or MG132 (2.5 M) for 1 h prior to the addition of calyculin (50 nM). Following a 1-h incubation with calyculin, cells were harvested, and whole cell extracts were prepared. Extracts were separated by 8% SDS-PAGE and immunoblotted with anti-HA mAb. The two panels represent the same immunoblot at different exposures to highlight phosphorylated (pDRNLS) (Ex 1) and high molecular mass immu- noreactive material (Ex 2). In addition, DRNLS from cells treated with calyculin + MG132 or calyculin + MG132 + TCDD were immunopre-cipitated using anti-HA mAb and counter-stained with anti-ARNT antiserum. D, DRNLS was purified via its His tag using nickel affinity-agarose. Y1/DRNLS cells or the parent Y1 cell line were incubated with MG132 (2.5 M) for 1 h prior to the addition of calyculin (50 nM). Cells were harvested 1 h later in a denaturing buffer and purified on nickel-agarose (see “Experimental Procedures”). DRNLS was eluted by imid- azole (250 mM) and boiled in SDS-PAGE sample buffer prior to analysis by 8% SDS-PAGE and immunoblotting with anti-HA or anti-Ub antibodies. Molecular mass standards are as shown.

DISCUSSION

Compartmentalizing transcription factors is a major strategy used by the cell to confer control of activity or spatial proximity to physiological signals (for review see Ref. 31). Over the past few years it has become clear that many nuclear proteins contain specific amino acid nuclear localization sequences that confer their subcellular distribution. In several transcription factors this sequence is “masked” until an appropriate signal exposes it, allowing binding to importin-α and transport through the nuclear pore (for review see Refs. 32–35). The DR is one such factor that, in response to cognate ligands, is rapidly translocated to the nucleus and concomitantly de- pleted from the cell. The NLS of the DR is relatively well characterized and consists of a bipartite cluster of basic amino acids with a mutation-insensitive region between the cluster (7), ARNT (36) and HIF1α (8) both possess similar NLS se- quences to the DR in their amino termini; however, in the case of HIF1α an additional hypoxia-sensitive NLS has recently been shown to control its nuclear translocation (8). In the present study we show that when the DR is sent directly to the nucleus, bypassing ligand-induced exposure of its NLS, it is rapidly degraded in the presence or absence of ligand.

fore, in a proteolytic context, ligand binding acts only in trans- porting the DR to the site of degradation (Fig. 3).

Although ARNT may potentially play a role in regulating HIF1α levels, the results from this study suggest that it does not influence the stability of the DR. The DR is rapidly depleted in both ARNT-containing Hepa1c1c7 cells or ARNT-deficient Hepa1c4 cells (Figs. 1 and 2), suggesting that DR dimerization with ARNT, although essential to transcriptional activity, is not a prerequisite for proteolysis. This finding was in accord- ance with results obtained using the constitutively nuclear receptor DRNLS, which was demonstrated to be unstable when complexed either with HSP90 or ARNT (Fig. 3B). In all cases, ligand-activated DR or DRNLS could be stabilized in the nuclear compartment with the proteasome inhibitor MG132 (Figs. 1–3), suggesting that a common nuclear mechanism ac- counts for DR proteolysis.

Ubiquitination has been demonstrated to down-regulate the bHLH transcription factors HIF1α (37, 38) and MyoD (29, 39, 40), albeit in very different cellular contexts. Both proteins are involved in development and may be required to rapidly switch target genes on and off. Likewise, control of DR levels may also be important in development and as such may require rapid down-regulation. At present, gene disruption studies have not defined a clear role for the DR in development; however, there are deficiencies in growth, and liver size and reduced fertility (see Ref. 41). In the present study we show that co-expression of DRNLS with UbK48R results in stabilization of DRNLS (Fig. 4), suggesting that ubiquitination is a major mechanism for rapidly removing the DR. Under these conditions, levels of the DR partner protein ARNT are not affected by UbK48R, thus we consider the rapid degradation of the DR/ARNT heterodimer to involve DR only (Fig. 4B). An additional level of negative con- trol has been recently identified by Mimura et al. (14), who showed that the DR is able to switch on the synthesis of a repressor protein AHRR, which binds to target DR XRE ele- ments, thus starting a negative feedback loop. In our reporter assays, UbK48R did not increase activity by the amount pre- dicted from its effects on DR levels (Fig. 4C). It is conceivable that without UbK48R the reporter activity is already close to maximal with respect to the transcriptionally active DR/ARNT heterodimer. Alternatively, expression of AHRR may limit the extent to which high levels of DR can stimulate transcription.

Phosphorylation is well established to elicit the structural changes required for ubiquitination. In the case of inhibitor of kB transcription factor α, the kinase and ubiquitin ligating enzyme may be part of the same protein complex (42); however, the relationship between the kinase and the ubiquitin protein isopeptide ligase is less defined for other transcription factors. We show that co-incubation of DRNLS with MG132 and the phosphatase inhibitor calyculin causes the accumulation of a phosphorylated form of DRNLS (Fig. 5). Concurrently, a build-up of high M, DRNLS-Ub occurs (Fig. 5, C and D), sug- gesting that phosphorylation of DRNLS leads to ubiquitina- tion. This process occurs whether HSP90 or ARNT is com- plexed with DRNLS, suggesting that this phosphorylation is a nuclear event that occurs irrespective of the structural differ- ences between these two complexes. The exact location of the phosphorylation site(s) and the nature of the kinase/ubiquitin protein isopeptide ligase responsible for DR proteolysis are the subject of further investigation.

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