Defining the Role for XAP2 in Stabilization of the Dioxin Receptor

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The dioxin receptor (DR) is a ligand-activated transcription factor that is activated upon binding of dioxins or structurally related forms of xenobiotics. Upon binding ligand the DR translocates from the cytoplasm to the nucleus where it complexes with the partner protein Arnt to form a DNA binding heterodimer, which activates transcription of target genes involved in xenobiotic metabolism. Latency of the DR signaling pathway is maintained by association of the DR with a number of molecular chaperones including the 90-kDa heat shock protein (hsp90), the hepatitis B virus X-associated protein (XAP2), and the 23-kDa heat shock protein (p23). Here we investigated the role of XAP2 in DR signaling and demonstrated that reduced levels of XAP2 stabilize the DR, arguing for a function of XAP2 beyond its reported role as a cytoplasmic retention factor. In addition, we showed that a constitutively nuclear DR is degraded in the nucleus and does not require nuclear export for efficient degradation. We also provided evidence implicating the ubiquitin ligase protein C-terminal hsp70-interacting protein (CHIP) in the degradation of the DR, and we demonstrated that this degradation can be overcome by overexpression of XAP2. XAP2 protection of CHIP-mediated degradation is dependent on the tetratricopeptide repeat domain of XAP2 and suggests a mechanism whereby competition for the C-terminal tetratricopeptide repeat acceptor site of hsp90 guides the protein triage decision, the point of determination for either maturation of DR folding or DR degradation.

The DR or aryl hydrocarbon receptor (AhR) is a member of the bHLH/PAS (basic Helix-Loop-Helix/Per-Arnt-Sim) family of transcription factors. Members of this protein family are classified by the presence of PAS domains that can accept cellular or environmental signals and/or function as secondary dimerization interfaces (1, 2). PAS family members respond to various environmental cues including xenobiotics (DR), hypoxia (hypoxia-inducible factors (HIFs)), as well as participating in the light entrainment process to regulate the circadian cycle (CLOCK and BMAL). As a consequence of the important signal sensing roles placed upon the bHLH/PAS factors, intricate levels of regulation have been incorporated to control protein activity and transcriptional output. For example, the HIFs are regulated by several hydroxylation events, performed by up to five separate enzymes (prolyl hydroxylases 1–4 and factor inhibiting HIF-1), to regulate HIF protein stability and transactivation capacity (3–10). The circadian rhythm factors undergo regulated nuclear import and export in response to specific phosphorylation events (11). However, the DR is unique in the family so far in that it is the only member regulated by ligand, which acts to transform the cytosolically localized latent receptor complex into a nuclear DNA-bound complex with the bHLH/PAS heterodimeric partner protein Arnt (aryl hydrocarbon receptor nuclear translocator). This transformation process is regulated in large part by the association of the DR with the molecular chaperone hsp90, which is thought to maintain cytosolic localization by masking the N-terminal nuclear localization sequence of the DR (12). hsp90 association with the ligand binding domain is also crucial for maintaining a high affinity ligand binding form of the DR (13–16). Following ligand activation, the DR/Arnt heterodimer binds xenobiotic response elements (XREs) in the enhancer regions of genes encoding xenobiotic metabolizing enzymes and growth regulatory factors (17, 18). Subsequent to ligand activation, the DR is targeted for destruction by the ubiquitin/proteasome degradation system (19–21).

The latent DR chaperone complex is composed of two molecules of the molecular chaperone hsp90 (22), the co-chaperone protein, p23 (23), and an immunophilin-like protein XAP2/Arq9/AIP (hepatitis B virus X-associated protein/AhR-associated protein/AhR-interacting protein, respectively) (24–26). XAP2 is a tetratricopeptide repeat (TPR)-containing protein, displaying homology to the immunophilin proteins FKBP51 and FKBP52 present in steroid hormone receptor complexes. TPR motifs are 34-amino acid residue repeat regions that form a predominantly a-helical structural unit that acts as a protein-protein interaction domain (27). As yet, no definite function has been assigned to the immunophilin proteins present in the steroid hormone receptor complexes, although current models posit a role for nuclear trafficking via the cytoskeletal protein dynein, which is reliant on the peptidylprolyl isomerase domain of the protein (28, 29). Similarly, the role that XAP2 plays in DR signaling is unclear. Original reports demonstrated an increase in DR signaling in XAP2 overexpression studies in both yeast and mammalian systems. This has sub-

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TPR Containing Proteins XAP2 and CHIP Regulate DR Stability

sentially been shown to be a result of increased DR stability (30, 31). Overexpression of XAP2 in transient assay systems leads to decreased levels of DR ubiquitination (32); however, this mechanism has yet to be fully elucidated. Further studies have suggested a role for XAP2 in maintaining cytoplasmic localization of the DR (31, 33, 34).

Molecular chaperones play a crucial role in the maturation of a large proportion of cellular proteins. It is proposed that they act by chaperoning hydrophobic patches on client proteins to inhibit aggregation within the cell, in addition to assisting a chaperone to achieve correct folding into a functional threedimensional structure (35). Client proteins can either transiently associate with an ordered succession of components of the chaperone system and then be released upon successful maturation of the protein, or alternatively they can maintain a permanent association with a chaperone (typically hsp90) once the substrate protein has reached maturation. The DR is placed within the latter class of hsp90 substrate proteins. Recently a TPR-containing protein, CHIP (C-terminal hsp70-interacting protein), was isolated from a yeast twohybrid screen by its ability to interact with the C-terminal region of the molecular chaperone protein hsp70 (36). In addition to containing TPR motifs, CHIP contains a U box domain that has E3 ubiquitin ligase activity (37) to mediate the degradation of substrate proteins, so far including the GR (38), cystic fibrosis transmembrane regulator (39), Parkin (40), and ErbB2 (41). CHIP is proposed to act at the level of protein triage decision making during the maturation of molecular chaperone substrates, i.e. it acts as a crucial component of the process to either continue chaperone-mediated folding of a substrate protein or, if a protein is unlikely to reach the correctly folded structure, it acts to degrade the substrate by ubiquitin-mediated degradation (42, 43). However, the mechanisms that control this decision making process are poorly understood.

Here we investigate the role of XAP2 in the DR activation process. By using cytoplasmic and nuclear localized forms of the DR, in conjunction with antisense XAP2, we show that XAP2 depletion ablates DR transcriptional output by means of increased degradation of the DR. In addition we provide evidence of a role for CHIP in ubiquitin-mediated turnover of the DR, and we show this process requires the TPR domain of CHIP. Overexpression of CHIP inhibits this degradation, whereas a TPR deletion mutant of XAP2 fails to do so. These data suggest a model for which the protein triage decision process is mediated by a competition between opposing TPR proteins.

EXPERIMENTAL PROCEDURES

Plasmids—The DR-NLS/RES/35879/puro (Promega) plasmids have been described previously. The XAP2myc, Ub/CMV5, and UbK48R/CMV5 were obtained from Ben Roberts (Promega) plasmids have been described previously. The XAP26myc, EcoRI/I/H9251 and was subcloned as a restriction site, whereas the CHIP constructs were subcloned into the pCDNA3 vector (Invitrogen) using EcoRI/RI/H9262 and XhoI/XbaI restriction sites, respectively. The mycCHIP constructs were subcloned into the pCDNA3 vector using EcoRI/XhoI restriction sites. Constructs in the pCDNA vectors were sequenced to verify sequence integrity.

Cell Lines and Cell Culture—Mouse adrenal Y1 cells and the human embryonic kidney transformed cell line 293T were routinely grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. The 293/T/DR cell line was generated in the same manner as the previously described 293/T/DR-NLS cell line (44).

Transient Transfections—The transient transfection protocol has been described previously (44) for a period of 24 h followed by an overnight treatment with 10 nM TCDD or vehicle control, after which time extracts were analyzed using the Dual Luciferase Reagent assay system (Promega), according to the manufacturer’s instructions, or analyzed by SDS-PAGE to determine XAP2myc expression. The amount of plasmid DNA transfected into each well was normalized by the transfection of empty pEF6/BOS vector. Transfections were performed a minimum of three times. For transfection of CHIP and XAP2 mutant constructs, 293T cells were transfected in 6well trays using LipofectAMINE-2000 (Invitrogen) according to the manufacturer’s instructions for a period of 24 h. DNA content was normalized using empty pcDNS vector.

Immunoblotting and Antibodies—Whole cell extracts and the generation of cytosolic and nuclear extracts for immunoblotting were prepared as described (13, 44). The amount of protein loaded for 7.5 (for DR-NLS) or 10% (for CHIP and XAP2 constructs) SDS-PAGE analysis is indicated in the figure legends. Following transfer, membranes were blocked for 1 h at room temperature in 10% milk, PBS, 0.1% Tween 20 (Sigma). Membranes were then incubated with primary antibody overnight followed by washing with PBS, 0.1% Tween 20 (3 times for 5 min) and a 45min incubation with either a goat anti-rabbit or rabbit anti-mouse horseradish peroxidase-linked secondary antibody (Dako). Membranes were blocked for 3 times (5 min) and visualized by enhanced chemiluminescence. Anti-CHIP polyclonal antibodies (40) were a gift from R. Takahashi (Riken Brain Institute, Japan); anti-DR RPT-1 mAb was a gift from Garry Perdew (Pennsylvania State University), and 9E10 (αmAb) and 9E11 (αHA) were from the animal facility at IMVS Adelaide, Australia. Rabbit polyclonal anti-Anti-antibodies were generated against thioredoxin fusion proteins containing the final 100 amino acids of human Arnt.

Fluorescence Microscopy—for analysis of RevGFP localization, Y1/DR-NLS cells were seeded into 6well trays 24 h before being transfected with 5 μg of the Rev-GFP plasmid using 10 μl of LipofectAMINE 2000 per well, cells were transfected for a period of 6 h, followed by treatment with leptomycin B (LMB) (20 μg/ml final) for 4 h. Cells were then washed twice with PBS, stained with Hoechst stain 33342 diluted in water (2.5 μg/ml final), and visualized with a Nikon TE 300 microscope.

In Vitro Interaction Assays—To investigate the interaction between the DR-NLS complex and CHIP/ATPR, [35]Smethionine-labeled proteins were generated using the T7-coupled reticulocyte translation system (Promega) according to the manufacturer’s instructions. Reactions contained 10 μl of CHIP or CHIP/ATPR and were incubated with 10 μl of the DR-NLS protein and 80 μl of PBS. Mg132 was added to a final concentration of 50 μM, and reactions were left for 30 min at room temperature with occasional agitation. Following the incubation, the reactions were immunoprecipitated with the 12CA5 mAb (5 μg in a 500μl volume of IP buffer (final concentrations of 10 mM Tris, pH 7.5, 2 mM MgCl2, 150 mM NaCl, 10 μg/ml Leupeptin, and 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% milk powder) for 2 h at 4 °C. The complexes were separated by protein A-Sepharose (40 μl of a 1:1 slurry), followed by 2 times for 5 min washes with 500 μl of IP buffer. Proteins were removed from the Sepharose by boiling in SDS sample buffer and analyzed by SDS-PAGE. Gels were then dried and exposed to film.
RESULTS

Characterization of Stable Cell Lines Expressing Cytosolic and Nuclear Localized Forms of the DR—Previous studies investigating the role of XAP2 in DR signaling have only analyzed DR function during overexpression of XAP2. In order to explore the role of XAP2 in the DR activation pathway, we...
decided to deplete levels of endogenous cellular XAP2 using an antisense strategy and to investigate the effects on wild type DR and a constitutively nuclear form of the DR (DR-NLS). The constitutively nuclear DR contains a heterologous NLS and maintains its latent state bound to hsp90, thus requiring ligand for activation (44). To compare potential differences between the cytosolic and nuclear localized forms of the DR, 293T stable cell lines expressing wild type DR or DR-NLS were generated (Fig. 1 A). Immunoblots of whole cell extracts from the engineered cells demonstrate that the DR proteins were successfully expressed (Fig. 1 B), and cell fractionation procedures show that the DR-NLS protein is recovered entirely in the nuclear fraction of the cell, whereas the wild type DR is present in both in the cytosolic and nuclear fractions (Fig. 1 C). This observation could result as a function of overexpression of the DR overriding the normal cytosolic retention mechanism or, alternatively, could be due to the recent observation that the DR can shuttle between the cytosolic and nuclear compartments of the cell in a variety of cell types (48, 49), a phenomenon which is overcome in the DR-NLS protein by virtue of the heterologous nucleoplasmin nuclear localization sequence.

Following confirmation of expression of both of the constructs in the stable lines, the cells were characterized for their responsiveness to the prototypical DR ligand, TCDD (dioxin). The cell lines were transfected with the XIXI luciferase reporter plasmid containing XRE sequences from the Cyp1A1 promoter (Fig. 1 D). The stable cell lines containing either the wild type or nuclear localized DR gave a robust response to ligand on the XRE-driven reporter (Fig. 1 E). In addition, the basal activity of the luciferase reporter was not significantly different between the DR and the DR-NLS construct, reaffirming the inability of the DR-NLS construct to act in the absence of ligand (44).

**XAP2 Depletion Abrogates DR Signaling**—XAP2 has been suggested previously to play a role in stabilization of overex-
pressed DR. This model has been derived from several observations, including co-expression of XAP2 and DR in transient transfection assays leading to a 2-fold increase in DR protein levels and induction from DR target genes compared with expression of DR alone (30, 31). In addition, co-expression of XAP2 has been demonstrated to lead to a decrease in ubiquitinated forms of the DR (32). Moreover, XAP2 has been suggested to be involved in the maintenance of a correctly chaperoned DR complex in order for the DR to be capable of interacting with the nuclear import machinery (50). We decided to address the function of XAP2 in DR signaling by using the 293T/DR-NLS cell line in combination with an antisense-based XAP2 depletion approach, to investigate whether XAP2 depletion can affect signaling through a nuclear localized DR. To this end we generated an expression vector that expressed the non-coding strand of XAP2 mRNA driven by the powerful elongation factor 1α promoter. Increasing amounts of the vector were transfected into the 293T/DR or 293T/DR-NLS cell lines, in combination with the XRE-luciferase reporter gene, pXIXI, for 24 h followed by overnight treatment with TCDD or vehicle control. In the absence of the antisense expression vector, TCDD gave a robust induction for both the DR and DR-NLS constructs (Fig. 2, A and B). To verify that these observations were specific for the DR, a chimeric protein that fuses the bHLH region of the DR to the PAS/C-terminal portion of HIF-1α was employed (Fig. 3A). This chimeric construct is subject to hypoxic regulation and regulates gene output via XRE sequences instead of hypoxia-response element sequences. In transient transfection experiments, using identical levels of the antisense XAP2 construct to those performed for the DR constructs, hypoxic induction of the DRbHLH/HIF chimeric protein was not influenced by XAP2 depletion, demonstrating that the effect on the DR is not a global influence on transcriptional output and is specific for a region of the DR C-terminal to the bHLH region (Fig. 3B). As a means of verifying that the antisense vector was capable of depleting the cell of XAP2, the 293T cells were simultaneously transfected with trace amounts of an XAP2 sense construct, which contained a C-terminal 6Myc epitope. Immunoblots of cell extracts from the transient transfections displayed a consistent dose-dependent decrease in levels of the tracer XAP26myc construct with increasing levels of antisense XAP2 (Fig. 2C).
displayed a decreased response, we reasoned that XAP2 depletion was acting at a step beyond nuclear translocation, as the exogenous NLS is located at the C terminus and should be impervious to the presence or absence of a DR-specific co-chaperone, which acts within the LBD of the DR (59), and thus the focus of the following experiments reports the effects observed with the DR-NLS protein. An alternative possibility to the effects on DR localization is that cellular depletion of XAP2 affects the stability of the DR, which takes into account the reported observations between the nuclear localized and cytosolic forms of the protein. Following co-transfection of the DR-NLS construct with the antisense XAP2 construct, DR-NLS protein expression was noticeably depleted in the presence of the antisense construct compared with transfection with the control vector (Fig. 4A), suggesting that depletion of XAP2 leads to destabilization of the DR resulting in the subsequent decreased output in the luciferase reporter assay. This depletion effect is dose-dependent as transfection with the highest amount of the antisense construct displayed a decreased response, we reasoned that XAP2 depletion was acting at a step beyond nuclear translocation, as the exogenous NLS is located at the C terminus and should be impervious to the presence or absence of a DR-specific co-chaperone, which acts within the LBD of the DR (59), and thus the focus of the following experiments reports the effects observed with the DR-NLS protein. An alternative possibility to the effects on DR localization is that cellular depletion of XAP2 affects the stability of the DR, which takes into account the reported observations between the nuclear localized and cytosolic forms of the protein. Following co-transfection of the DR-NLS construct with the antisense XAP2 construct, DR-NLS protein expression was noticeably depleted in the presence of the antisense construct compared with transfection with the control vector (Fig. 4A), suggesting that depletion of XAP2 leads to destabilization of the DR resulting in the subsequent decreased output in the luciferase reporter assay. This depletion effect is dose-dependent as transfection with the highest amount of the antisense construct led to the greatest depletion of both DR-NLS expression (Fig. 4B) and a Myc-tagged XAP2 tracer protein (Fig. 4C). The Nuclear Localized DR Is Degraded in the Nucleus—In order to determine whether the DR-NLS construct requires nuclear export to be degraded, we employed the CRM-1 exportin inhibitor LMB, which has been shown previously to inhibit nuclear export of the DR (51). Nuclear localization of the DR stabilizes the DR in the absence of ligand (20); however, debate exists in the literature as to whether the DR requires nuclear export in order to be degraded (19, 20, 52, 53). Y1/DR-NLS cells were treated with LMB, TCDD, the hsp90 inhibitor geldanamycin (GA), the 26 S proteasome inhibitor MG132, or a combination of each followed by separation by SDS-PAGE and immunostaining with the anti-hemagglutinin mAb. Inhibition of the CRM-1 nuclear export pathway failed to stabilize the DR-NLS construct, whereas MG132 treatment successfully stabilized the protein in the absence of TCDD (Fig. 5A, compare lanes 1–3). This effect was unchanged following co-treatment with TCDD (Fig. 5A, compare lanes 4–6). Importantly, GA treatment, which has been shown previously to dissociate the hsp90 complex and potentially expose nuclear export sequences within the DR, also failed to stabilize the DR in the presence of LMB, suggesting that the chaperone-free DR does not require nuclear export in order to be degraded (Fig. 5A, compare lanes 7–9). Equal protein loading was verified by an identical blot probed with anti-Arnt antibodies (Fig. 5B). As a further control to ensure that LMB treatment successfully inhibited nuclear export, Y1/DR-NLS cells were transiently transfected with the GFP-NES construct which encodes a GFP protein containing an NES identical to that from the IxB protein. As shown in Fig. 5C, the NES-GFP construct displayed a diffuse staining pattern in the absence of LMB. However, in LMB treatments identical to those used in the treatment of the Y1/DR-NLS stable cell line, NES-GFP localization was specifically confined to the nuclear compartment of the cell confirming that LMB is inhibiting CRM-1-mediated export (Fig. 5C). These data confirm that incorrectly chaperoning the DR-NLS protein, by either treating with GA (to dissociate hsp90) or XAP2 depletion (antisense experiments), leads to degradation of the DR-NLS and that this degradation can occur in the nucleus.

The DR Is Degraded by the E3 Ubiquitin Ligase CHIP—Many similarities have been identified between the DR signaling pathway and the glucocorticoid receptor pathway, such as maintenance of a cytosolically localized complex in the latent state, association with the hsp90 molecular chaperone complex including the presence of p23 and immunophilin type...
proteins, and ligand invoking a nuclear translocation and initiation of the transcriptional pathway (54, 55). Recently, the GR has been identified as a substrate for the molecular chaperone protein CHIP (38). Based on the many similarities between the DR and GR already observed, we sought to investigate whether the DR, like the GR, can be degraded by CHIP.

We transiently transfected 293T cells with an expression vector for the DR-NLS construct in combination with the pcDNA3/CHIP expression vector which expresses wild type CHIP (Fig. 6A). When the DR-NLS was co-expressed in the presence of the CHIP expression vector, the DR-NLS protein was consistently expressed at lower levels than in the absence of CHIP, as assessed by immunoblotting with an anti-hemagglutinin mAb (Fig. 6B). The expression of human CHIP was confirmed by analyzing extracts and probing immunoblots with an anti-CHIP polyclonal Ab (Fig. 6C). As observed previously (36, 40), overexpression of CHIP results in higher migrating forms of CHIP, which is presumably a result of CHIP being a target for auto-ubiquitination or other forms of post-translational processing. As a verification of whether CHIP-induced degradation of the DR-NLS protein is mediated via the ubiquitin pathway, we transiently transfected the DR-NLS construct with a wild type ubiquitin expression vector, or the K48R ubiquitin mutant which expresses a form of ubiquitin which prevents poly-ubiquitination of substrate proteins and leads to stabilization (20). In these experiments, the K48R ubiquitin construct resulted in higher levels of DR-NLS observed in whole cell extracts following analysis by immunoblots in comparison to the wild type ubiquitin, suggesting that CHIP-mediated degradation of the DR-NLS occurs via ubiquitin-mediated degradation (Fig. 6D, compare lanes 3 and 4). The presence of the Myc epitope-tagged version of CHIP used in this experiment was visualized by immunostaining with an anti-Myc mAb (Fig. 6E) acting to both verify equal expression of CHIP and confirm that an N-terminally tagged version of CHIP successfully destabilizes the DR-NLS. Overexpression of CHIP can also destabilize the wild type cytosolic DR in a Hepa 1c1c7 stable cell line (data not shown).

CHIP-induced Degradation of DR-NLS Is U Box-dependent and Acts via Competition with the TPR Domain of XAP2—CHIP has been proposed to act as an E3 or E4 ubiquitin ligase containing an U box domain within its C-terminal region based on homology to the yeast U box containing protein UFD2 (37). To define whether the U box of CHIP was essential in mediating the degradation of the DR-NLS substrate, a deletion mutant was generated that lacked the U box (CHIP(1–197)) (Fig. 7A). When transfected in combination with the DR-NLS construct, the U box mutant failed to destabilize the DR-NLS protein in contrast to full-length CHIP (Fig. 7B). Both proteins were expressed to similar levels as judged by immunoblotting with an anti-Myc mAb. These data, in conjunction with the experiments using the K48R ubiquitin mutant, strongly suggest a role for the ubiquitin proteasome pathway in CHIP-mediated degradation of the DR-NLS.

A critical protein-protein interaction domain mediating the interactions of the various chaperone proteins with their respective substrates is the TPR domain. TPR domains form a structural unit composed of a pair of anti-parallel α-helices able to recognize a specific substrate motif (27, 56). For example the hsp70/hsp90 organizing protein, Hop, has two critical TPR domains, of which TPR2A recognizes the C-terminal pentapeptide MEEVD motif of hsp90 (56–58). A separate TPR motif of Hop, TPR1, has been shown to recognize a specific substrate motif (27, 56). For example the hsp70/hsp90 organizing protein, Hop, has two critical TPR domains, of which TPR2A recognizes the C-terminal pentapeptide MEEVD motif of hsp90 (56–58). A separate TPR motif of Hop, TPR1, has been shown to recognize a specific substrate motif (27, 56). For example the hsp70/hsp90 organizing protein, Hop, has two critical TPR domains, of which TPR2A recognizes the C-terminal pentapeptide MEEVD motif of hsp90 (56–58). A separate TPR motif of Hop, TPR1, has been shown to recognize a specific substrate motif (27, 56).

Fig. 5. The DR-NLS protein is degraded in the nucleus and does not require export to the cytosol to be degraded. A and B, the DR-NLS is degraded in the nucleus. Y1/DR-NLS cells were transfected with TCDD (10 nM), LMB (20 μg/ml), MG132 (7.5 μM), or GA (2 μM) in the indicated combinations for 4 h prior to harvesting for whole cell extracts followed by immunoblotting with the anti-hemagglutinin mAb (A) or αArnt Abs to control for equal loading (B). C, LMB abrogates nuclear export of a GFP-NES construct. Y1/DR-NLS cells were transiently transfected with the GFP-NES construct for 6 h followed by treatment for 4 h with LMB (20 μg/ml).
sion vector. CHIP-(128–303) failed to destabilize the DR-NLS, in contrast to full-length CHIP (Fig. 7B), even though both proteins were expressed at similar levels as assessed by immunoblotting (Fig. 7C). To assess whether CHIP interacts with the DR complex, in vitro translated DR-NLS was mixed with either full-length CHIP or the CHIP-(128–303) construct prior to immunoprecipitation with the anti-hemagglutinin mAb (B) or 10% SDS-PAGE and analyzed using an αCHIP polyclonal Ab (C); the positions of the relevant proteins are indicated. D and E, CHIP-mediated degradation of the DR-NLS occurs via the ubiquitin pathway. 293T cells were transiently transfected with pcDNA or pcDNA/CHIP in combination with a DR-NLS expression vector as indicated. 20 μg of whole cell extracts were separated by 7.5% SDS-PAGE and analyzed for DR-NLS expression with the anti-hemagglutinin mAb (B) or 10% SDS-PAGE and analyzed using an αCHIP polyclonal Ab (C); the positions of the relevant proteins are indicated. D and E, CHIP-mediated degradation of the DR-NLS occurs via the ubiquitin pathway. 293T cells were transiently transfected with pcDNA or pcDNA/CHIP in combination with wild type ubiquitin or the K48R ubiquitin mutant. Whole cell extracts were harvested, and 10 μg of lysate was analyzed for DR-NLS expression (D) or CHIPmyc expression (E).

DISCUSSION

The role of immunophilin proteins in signaling pathways has yet to be clearly defined. Current models posit that the FKBP51/52 components of the steroid hormone receptor sys-
tems modulate trafficking of the hormone receptor from the cytoplasm to the nucleus. This is achieved by an interaction of the peptidylprolyl isomerase (PPIase) domain of the immunophilin with molecular motor proteins such as dynein (28, 29). However, this effect appears to be functionally distinct from the catalytic activity of the PPIase domain as treatment with FK506 (a PPIase inhibitor) failed to inhibit nuclear targeting of the glucocorticoid receptor (28). Initial hypotheses for XAP2 predicted that this DR co-chaperone acted in a similar manner to mediate trafficking of the DR chaperone complex, as overexpression of XAP2 leads to redistribution of overexpressed DR from a diffuse staining pattern into a predominantly cytoplasmic expression pattern. This phenomenon appears to be acting through cytoplasmic retention rather than increased nuclear

**Fig. 7.** XAP2 overexpression can overcome CHIP-mediated DR-NLS degradation. A, schematic representation of CHIP deletion mutants. B and C, CHIP-mediated degradation of the DR-NLS requires the U box and TPR domains of CHIP. 293T cells were transfected for 24 h with a DR-NLS expression vector in combination with full-length mycCHIP1–303, CHIPΔUBox1–197, or CHIPΔTPR128–303. Whole cell extracts (10 μg) were analyzed for DR-NLS expression (B) or for verification of expression of the transfected CHIP constructs (C). D, CHIP interacts with the DR-NLS complex through the N-terminal TPR domain. In vitro translated [35S]Met-labeled DR-NLS was incubated with [35S]Met-labeled CHIP or the CHIPΔTPR deletion mutant, followed by immunoprecipitation with the 12CA5 anti-hemagglutinin mAb and separation by SDS-PAGE. 10% of the protein included in the interaction assay is depicted in lanes 1–3. Lanes 4 and 5 depict complexes immunoprecipitated with the 12CA5 anti-hemagglutinin mAb and displayed is an overnight exposure in addition to an overexposed image of the gel to visualize the interaction between the DR-NLS complex and CHIP. E, schematic representation of the transfected XAP2 constructs. F and G, overexpression of XAP2 but not XAP2ΔTPR can outcompete CHIP-mediated degradation of the DR-NLS. 293T cells were transfected for 24 h with a DR-NLS expression vector in the presence or absence of mycCHIP1–303 in combination with the indicated amounts of full-length mycXAP21–330 or XAP2ΔTPR1–263. Whole cell lysates (10 μg) were analyzed for DR-NLS expression (F) or expression of Myc-tagged CHIP and XAP2 constructs (G).
export of the DR as treatment with LMB failed to restore nuclear localization (50). XAP2, which lacks PPIase activity (61), appears to mediate trafficking of the DR in a different manner to that of FKBP52, which transports the GR from the cytoplasm in a complicated fashion requiring a switching event between the FKBP51 and FKBP52 (62). Taken together, this evidence suggests that XAP2 is a cytosolic retention agent. However, XAP2 has also been shown to interact with the nuclear viral protein EBNA-3. In these experiments, co-expression of EBNA-3 with a GFP-tagged XAP2 construct was sufficient to localize the normally diffuse staining pattern of XAP2 to the nuclear compartment, arguing against a role for XAP2 as a general agent for cytosolic localization of partner proteins (63). Furthermore, it has been shown recently by using a DR-yellow fluorescent protein fusion containing a heterologous NLS that overexpression of XAP2 is incapable of redistributing a similar DR-NLS construct used in the present study to the cytoplasm (50). What then is the role for XAP2 in DR signaling? By using a stable cell line expressing a form of the DR that is constitutively localized to the nucleus in the absence of ligand, we demonstrate by using XAP2 depletion studies that XAP2 is important for function of this nuclear receptor, in addition to XAP2 overexpression being able to stabilize DR-NLS protein levels. Thus, it would appear that XAP2 is required for tasks in DR signaling beyond mere cytosolic localization of the DR chaperone complex. These data are consistent with an important role for XAP2 in stabilizing the DR-containing chaperone complex, a result which leads to increased protein levels of the DR.

In order to investigate further the mechanism behind DR degradation, we became interested in the molecular chaperone CHIP. CHIP is a protein that interacts with components of the molecular chaperone machinery and acts in a poorly understood process to govern the decision for a protein to either continue folding with the assistance of molecular chaperones or to instigate degradation of the client protein through the ubiquitin proteasome pathway, a process known as “protein triage” (42, 43). As the DR is an hsp90 client protein, we were intrigued to determine whether CHIP was involved in the processes that regulate DR degradation. By using both the DR-NLS and wild type DR in Hepa1c1c7 cells (data not shown), we have evidence suggesting that CHIP mediates ubiquitin-mediated degradation of the latent receptor, as overexpression of CHIP decreases DR-NLS protein levels (Fig. 6B), a process that can be halted by co-expression of a mutant form of ubiquitin that inhibits ubiquitin chain elongation (Fig. 6D). It should be noted, however, that this mechanism of degradation is presumably distinct from ligand-mediated degradation of the DR. Rather, these data are consistent with a model where the DR, like other CHIP substrates, undergoes a protein folding decision, and if a favorable outcome appears unlikely then the protein is shunted toward the degradative pathway. This is due to the fact that in the ligand-activated state, the DR sheds its molecular chaperones and heterodimerizes with the Arnt partner factor. By shedding the chaperone complex, the DR would lose the link provided by CHIP to the degradative machinery, necessitating an alternative pathway for ligand-mediated degradation.

Given the role for XAP2 in DR stabilization and the observation that XAP2 can overcome CHIP-mediated degradation, and that this process is mediated by the TPR domain of XAP2 (Fig. 7F), this poses the interesting model in which XAP2 is a key mediator in the protein triage decision for the DR pathway, thus providing a unique role for immunophilin type molecules in regulating hsp90 substrate activity. Furthermore, this is the first evidence of a control of the poorly understood mechanism of the protein triage decision, that being the presence of XAP2 in the DR chaperone complex denotes a “successfully folded” protein, whereas protein complexes lacking XAP2 can potentially be targeted by CHIP for degradation. By altering the balance of CHIP and XAP2 within 293T cells, we were able to direct this pathway to either degradation (through overexpression of CHIP) or stabilization (through overexpression of XAP2). These data are consistent with the data of others who have demonstrated that overexpression of XAP2 results in decreased ubiquitination of the DR (32).

Thus, it appears that the role of XAP2 in DR signaling is complex but is centered around the ability of this protein to stabilize the interactions between the DR and the chaperone machinery. Consequently, stabilization of the DR-hsp90 complex by XAP2 (30, 59) prevents a transient unmasking of the N-terminally located NLS or, alternatively, by altering the conformation of the NLS to preclude recognition by nuclear import machinery (50), resulting in increased cytosolic localization of overexpressed DR. Furthermore, the presence of XAP2 in the DR-chaperone complex potentially signifies a mature complex that is suitable for the DR signaling pathway and ultimately prevents CHIP-induced degradation.

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