Differential Usage of Nuclear Export Sequences Regulates Intracellular Localization of the Dioxin (Aryl Hydrocarbon) Receptor

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The dioxin receptor belongs to the basic helix-loop helix/Per-Arnt-Sim (bHLH/PAS family of proteins and functions as a ligand-dependent transcription factor to activate target genes. The function of the PAS domain of the dioxin receptor is only partially understood. Whereas the C-terminal half of the PAS domain has been shown to harbor ligand binding activity and to function as an accessory dimerization interface, the precise functional role of the N-terminal half of the PAS domain remains unclear. We have previously shown that this domain confers dimerization specificity to the dioxin receptor. Here we report the identification and characterization of a novel nuclear export sequence (NES) motif, located in the N-terminal portion of the PAS domain, in addition to the previously identified NES in the bHLH domain. By point mutagenesis, we have generated a dominant positive form of the PAS domain NES motif that inhibits accumulation of the dioxin receptor in the nuclear compartment of the cell. This mutant form of the receptor was furthermore unable to sustain reporter gene activation. Importantly, we demonstrate that the ligand-free and ligand-occupied forms of the dioxin receptor differentially employ the two NES motifs. In the absence of ligand, nuclear export is sustained via the PAS domain NES, whereas following ligand-dependent activation nuclear export of the receptor is mediated by the NES in the bHLH domain.

In response to dioxin and related environmental pollutants, the dioxin (aryl hydrocarbon; Ah) receptor activates transcription of a battery of genes encoding drug-metabolizing enzymes such as cytochrome P4501A1 and glutathione S-transferase Ya. The dioxin receptor functions as a ligand-dependent transcription factor and belongs to the bHLH/PAS family of proteins (1). Other members of this family include the hypoxia-inducible factors HIF-1α (2) and EPAS/HLF (3, 4), the circadian regulatory factors Clock (5), Per (6), and Cycle (7), and the neuroregulatory protein Sim (8). Whereas the bHLH domain mediates DNA binding and dimerization, the PAS domain has been shown to function as an accessory dimerization interface and to determine dimerization specificity (9–11). In addition, a portion of the PAS domain, the PAS B subdomain, harbors the ligand binding interface of the dioxin receptor (12). However, the exact role of the PAS domain in regulation of the function of bHLH/PAS factors is not yet fully understood.

In the absence of ligand the dioxin receptor is commonly found evenly distributed in both the cytoplasmic and nuclear compartments of target cells in a complex with hsp90 and several hsp90-associated proteins. In the presence of ligand, the dioxin receptor accumulates in the cell nucleus, where it dimerizes with its partner factor Arnt (1). This event induces dissociation of hsp90 (13) and hsp90-associated proteins (14) from the dioxin receptor. Previous studies have identified both nuclear localization and nuclear export (NES) sequence motifs in the bHLH domain of the dioxin receptor (15). The role of the NES motif located in the bHLH domain is poorly characterized. Mutation of this motif does not affect the intracellular localization of the dioxin receptor in the absence of ligand (16). On the other hand, nuclear export of the receptor following ligand removal appears to be negatively influenced by alterations of this NES motif (16).

Interestingly, a C216W mutation in the PAS domain of the mouse dioxin receptor has previously been reported to impair transcriptional activation (17). In the present study, we show that a novel NES motif is present in the PAS domain of the dioxin receptor, centered at cysteine 216. A C216S mutation yielded a constitutively active NES, resulting in exclusive cytoplasmic localization of the receptor. The mutant form of the receptor was unable to initiate transcription of a reporter gene construct in transient transfection assays.

We show that nuclear localization of the dioxin receptor is differentially regulated via the two NES motifs. The inactive form of the dioxin receptor is actively exported from the nucleus via the NES motif in the PAS domain. In contrast, nuclear export of the ligand-activated dioxin receptor is mediated by the NES motif present in the bHLH domain. In conclusion, we have identified a novel function of the PAS domain in modulating intracellular localization of the dioxin receptor via nuclear export.

MATERIALS AND METHODS

Plasmid Constructs—The pCMVmDR (13), pTX1X1 (18), pSP72 mDR (13), pCMX-DR-GFP (19) GFP-DR 287–421 (20), and GFP-DR/GR-BBD (19) plasmid constructs have been described elsewhere. The C216S point mutation was inserted into pGEX 4T3 DR 1–287 (9) bacterial expression vector, and the fidelity of the resulting point mutation was verified by sequencing. The DR 1–287 and the DR 1–287 C216S fragments were subsequently cloned into pCMVmDR and pSP72 mDR using conventional subcloning techniques.

Cell Culture and Transient Transfection Experiments—293 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 8% FBS, penicillin, and streptomycin. Prior
to transient transfection, cells were seeded in six-well plates at ~50% confluency. The cells were subsequently transfected using LipofectAMINE according to the manufacturer’s (Life Technologies, Inc.) suggestions. Typically, 500 ng of a luciferase reporter plasmid (pTX1X1) and 100 ng of a β-galactosidase reporter plasmid under control of the cytomegalovirus promoter as internal control were transfected together with appropriate dioxin receptor expression vectors. The expression vector concentrations ranged between 1 and 50 ng. Following transfection, the cells were washed and maintained for 48 h in the presence or absence of 10 nM TCDD. Luciferase and β-galactosidase activities were subsequently measured using the Gene Glow system (BioTema) or GalactoLight system (Tropics) according to the recommendations of the suppliers.

**Determination of the Intracellular Localization of the Dioxin Receptor**—To determine intracellular localization of the dioxin receptor or chimeric dioxin receptor constructs, HEK293 cells were grown on 20 × 20-mm glass coverslips in 30-mm dishes. Transfections were performed by introducing 0.5–1 µg of plasmids encoding green fluorescent protein (GFP)-tagged dioxin receptor or dioxin-glucocorticoid receptor chimera constructs into cells using LipofectAMINE (Life Technologies) according to the manufacturer’s recommendations. Following transfection, cells were treated as detailed in the figure legends and examined by using a Nikon LABPHOT microscope equipped with a fluorescein isothiocyanate filter set and a photomultiplier. Quantitative evaluations of the intracellular distribution of GFP-tagged proteins were performed as described before (21). Briefly, green fluorescent cells were classified into five categories according to the subcellular distribution of the GFP fusion proteins: C for exclusive cytoplasmic localization; C > N, for predominantly cytoplasmic fluorescence; C = N, for fluorescence equally distributed between the cell cytoplasm and nucleus; N = C, for nuclear dominant fluorescence; N, for exclusive nuclear fluorescence. On average, 200 fluorescing cells (~20 fields with 5–20 fluorescing cells/field) were evaluated per transfection. The experiments were repeated three times, and representative fields are presented.

**In Vitro Translation and Co-immunoprecipitation Assays—Co-immunoprecipitation procedures have been performed in detail elsewhere (14). In short, dioxin receptor was in vitro translated in a transcription-translation coupled reticulocyte lysate system (Promega) according to the manufacturer’s recommendations. Following translation, the dioxin receptor and Arnt were co-immunoprecipitated in the presence of 10 nM TCDD or vehicle alone for 1 h at 25 °C. Subsequently, the protein mixture was incubated with hsp90, dioxin receptor, or p23 antibodies for 45 min at 0–4 °C. Antibody-protein complexes were precipitated by the addition of 50 µl of a 50% phosphate-buffered saline-protein A-Sepharose slurry. The precipitated complex was washed extensively and removed from the protein-A-Sepharose resin by elution with SDS-running buffer. The proteins were resolved in a 7.5% SDS-polyacrylamide gel, the gel was dried and visualized overnight on intensifying films.

**RESULTS**

**Identification of a Putative Novel NES Motif in the PAS Domain of the Dioxin Receptor**—A single amino acid substitution within the PAS domain, C216W, has been reported to inhibit dioxin receptor-mediated transcriptional activation, without affecting the ligand binding activity of the receptor (17). To gain additional information regarding the role of the PAS domain, we examined why a single point mutation in the PAS domain abrogated reporter function. Since the described mutation may induce a rather drastic change in dioxin receptor structure given the large degree of structural differences between Cys and Trp residues, we introduced a more conservative point mutation where the original Cys residue was replaced with a Ser residue. Compared with a Cys-Trp mutation, a substitution of a Cys for a Ser residue would be expected to have only a mild effect on the structural organization of the dioxin receptor. During the initial characterization of the functional properties of the mutant C216S receptor form, we observed that the C216S dioxin receptor mutant interacted efficiently with the hsp90 complex and with in vitro translated Arnt, similar to the wild type receptor (data not shown). These experiments suggest that there are no gross differences in structure between the different receptor forms, which would explain the severe impairment of the dioxin receptor to transactivate properly. Close examination of sequences surrounding the critical Cys216 residue revealed a substantial similarity to an NES motif: LX_2–3LX_2–3LX_2–3 (22). As illustrated in Fig. 1A, this sequence motif of the mouse dioxin receptor is conserved between several species and shows similarity to nuclear export sequences identified in proteins such as the protein kinase inhibitor PKI (23) and the human immunodeficiency virus regulator REV (24). In PKI and REV, the corresponding motif has been shown to mediate interaction with the export protein CRM-1, which actively export of these proteins from the cell nucleus (23).

To examine the role of the putative NES motif in regulation of dioxin receptor function, we transiently co-transfected human 293 kidney cells with a dioxin receptor-regulated luciferase reporter gene construct. In the presence of increasing concentrations of transiently expressed wild-type dioxin receptor, we observed a robust dose-dependent activation of reporter gene activity. In contrast, the C216S mutant receptor was unable to activate XRE-dependent reporter gene transcription (Fig. 1B). We then examined whether the intracellular localization of the dioxin receptor was affected by the C216S mutation. We fused both the wild-type and C216S mutant forms of the dioxin receptor to GFP and monitored intracellular

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**FIG. 1.** A C216S point mutation in the PAS A domain inhibits the transcriptional activity of the dioxin receptor. A, alignment of the primary amino acid sequence around Cys216 to known nuclear export sequences. Presented below in lowercase letters are the mutant forms of the dioxin receptor used later in this study. B, human 293 kidney cells were co-transfected with 1–50 ng of wild type (mDR) or the C216S mutant dioxin receptor expression vectors together with 500 ng of an XRE-driven luciferase reporter construct as described under “Materials and Methods.” Cells were treated with 10 nM TCDD or vehicle alone for 48 h. Whole cell extracts were prepared and analyzed for luciferase activity. As internal transfection control, 100 ng of a cytomegalovirus-driven β-galactosidase expression plasmid was used. Luciferase values were normalized against β-galactosidase activity. Fold induction was calculated relatively to values obtained in vehicle-treated cells. The average results of three transfactions, performed in duplicates, are shown. Open bars show fold-activation in the absence of 10 nM TCDD; gray bars show fold-activation in the presence of 10 nM TCDD.
localization of the proteins following transient transfection of HeLa cells. For quantitative purposes, no fewer than 200 cells where arranged into five different categories as described under "Materials and Methods." In the absence of ligand, the wild-type dioxin receptor, GFP-DR, was detected evenly distributed both in the cytoplasmic and nuclear compartments, whereas upon ligand treatment, 75% of the cells showed a complete or predominant nuclear localization (Fig. 2A). Remarkably, in the absence of ligand the C216S mutant dioxin receptor, GFP-DRC216S, was present almost exclusively in the cytoplasmic compartment of the cell, with very low levels of nuclear fluorescence. Moreover, in the presence of ligand, the mutant C216S receptor failed to accumulate in the cell nucleus (Fig. 2B).

NES motifs have been shown to mediate export from the cell nucleus of a large number of different proteins by recruiting the nuclear export receptor CRM-1 (22). This CRM-1-dependent nuclear export activity has been shown to be selectively inhibited by leptomycin B, a compound that binds specifically to CRM-1 (25). We have previously shown that the wild-type dioxin receptor accumulates in the nucleus in response to leptomycin B treatment in the absence of ligand (19) suggesting that the receptor interacts with the export protein CRM-1. We next tested the effect of leptomycin B on the intracellular localization pattern of the C216S receptor. For this purpose, HeLa cells were transiently transfected with GFP-DR or GFP-DRC216S expression vectors and incubated for 2 h with 50 nM leptomycin B, 10 nM TCDD, or a combination of both, prior to analysis of the intracellular localization patterns of the proteins. In the absence of ligand, the wild-type dioxin receptor-GFP fusion protein was redistributed to a predominantly nuclear localization by leptomycin B treatment. In the presence of both ligand and leptomycin B, similar levels of the GFP-dioxin receptor were observed in the nucleus (Fig. 2A). In contrast, the ligand-free GFP-DRC216S fusion protein failed to accumulate in the nucleus in the presence of leptomycin B or TCDD alone (Fig. 2B). However, in cells incubated with both 10 nM TCDD and 50 nM leptomycin B, we observed a clear translocation of the mutant protein GFP-DRC216S to the nuclear compartment (Fig. 2B). These results suggest that the dioxin receptor is actively exported out of the cell nucleus in a CRM-1-dependent fashion both in the presence and absence of ligand. Mutation of the C216S residue in the PAS domain of the receptor generates a constitutively active export sequence, which drives the receptor toward the cell cytoplasm. Furthermore, in contrast to the wild type receptor, nuclear redistribution of the C216S mutant form of the receptor required both inhibition of the CRM-1 export protein and presence of TCDD.

Sequences outside the bHLH Domain of the Dioxin Receptor Mediate Nuclear Export—We investigated whether the NES motif present in the PAS A domain was able to function independently of the NES motif present in the bHLH domain. To this end, we generated a dioxin receptor chimeric construct where we replaced the bHLH domain of the dioxin receptor with the DNA binding domain of the glucocorticoid receptor. Recent studies show that the glucocorticoid receptor is insensitive to the effects of leptomycin B treatment and is probably not a CRM-1-regulated protein (26). The resulting chimeric construct, GR-DBD/DR83–805, resembles the wild-type dioxin receptor in that it also contains a strong N-terminal nuclear localization signal motif. To determine the intracellular localization of this chimeric construct HeLa cells were transiently transfected using the same protocol as employed above. Following transfection, HeLa cells were treated with vehicle alone, 10 nM TCDD, or 10 nM leptomycin B. Interestingly, in the absence of ligand, GR-DBD/DR83–805 showed virtually the same intracellular localization pattern as the wild-type dioxin receptor (Fig. 3A). In analogy to the effects on the intracellular localization of the wild-type dioxin receptor, the addition of leptomycin B or TCDD induced nuclear accumulation of the GR-DBD/DR83–805 chimeric construct. These observations suggest that deletion of the bHLH domain does not alter the shuttling properties of the dioxin receptor contrasting the current model of intracellular transport of the dioxin receptor, which postulates that nuclear export is mediated by a NES motif present in the bHLH domain (15, 27). The ability of the GR-DBD/DR83–805 construct to shuttle clearly demonstrates that additional export sequences are present in the dioxin receptor. Moreover, the sensitivity displayed by the GR-DBD/DR83–805 fusion protein to leptomycin B reiterates that CRM-1 is involved in the
intracellular redistribution of the dioxin receptor through sequences outside the bHLH domain.

To generate further evidence that the putative NES motif in the PAS domain is functional, we mutated four hydrophobic residues (Phe\textsuperscript{214}, Leu\textsuperscript{218}, Leu\textsuperscript{221}, and Leu\textsuperscript{222}) to alanine (the 4 Ala mutant). In other proteins, these four conserved residues have been shown to represent critical determinants for the interaction between CRM-1 and different CRM-1 target factors (24). We fused the 4 Ala mutant form of the dioxin receptor to GFP and analyzed its intracellular localization pattern following transient expression in HeLa cells. Interestingly, mutation of the four conserved hydrophobic residues resulted in a dioxin receptor form that was predominantly nuclear in the absence of ligand (Fig. 3B). Importantly, the addition of leptomycin B did not significantly alter the dominant nuclear localization of this mutant form of the dioxin receptor. These results indicate that exchange of the hydrophobic residues compromises the interaction between CRM-1 and the PAS domain of the receptor. The addition of TCDD to cells transfected with the 4 Ala-GFP form of the receptor resulted in a modest increase in nuclear accumulation, suggesting that these mutations do not alter the ability of this protein to bind TCDD. These experiments support our hypothesis that the PAS domain contains a functional NES motif that mediates nuclear export of the dioxin receptor in the absence of ligand. Furthermore, the activity of this NES can be enhanced by replacements of Cys\textsuperscript{216} with Ser or conversely inhibited by mutating the hydrophobic residues as indicated.

**Deletion of the PAS B Domain of the Dioxin Receptor Inhibits Cytoplasmic Localization of the Dioxin Receptor Independently of CRM-1**—We have recently demonstrated that deletion of the PAS B subdomain of the dioxin receptor results in formation of a constitutively nuclear protein, presumably due to its inability to interact with hsp90 and the immunophilin-like protein XAP-2. XAP-2 has been shown to possess an important role in mediating cytoplasmic retention of the dioxin receptor (20). To bypass the effects of the hsp90-XAP-2 complex on the intracellular localization of the receptor, we decided to investigate the effect of deletion of the PAS B subdomain within the C216S mutant dioxin receptor with regard to both intracellular localization and the transcriptional activity of the protein. We monitored the intracellular localization pattern of dioxin receptor-GFP fusion proteins following transient transfection in HeLa cells. As expected, the dioxin receptor lacking the PAS B subdomain, DRA287–421 (GFP-wt-ΔPASB), demonstrated a clear nuclear localization pattern (Fig. 4A). A corresponding deletion of the PAS B subdomain in the GFP-DRC216S (GFP-C216SΔPASB) mutant form of the receptor induced a dramatic shift in intracellular localization of the protein toward the cytoplasmic compartment (Fig. 4A). These results suggest that despite its seemingly nuclear appearance, the GFP-wt-ΔPASB protein is actively being exported from the nucleus. However, mutation of Cys\textsuperscript{216} to Ser generates a strong NES with enhanced export function. This enhanced NES is therefore able to drive the GFP-C216SΔPASB receptor from the nucleus to the cytoplasm. In agreement with this model, the addition of leptomycin B to cells transfected with GFP-C216SΔPASB shifted the localization of the protein toward the nuclear compartment (Fig. 4A).

The fact that deletion of the PAS B domain results in a receptor protein that constitutively accumulates in the nucleus provided the possibility of analyzing whether the C216S mutation would affect the transcription activation potential of the DRΔPASB receptor forms. In transient transfection experiments, C216SΔPASB displayed ~50% of the ligand-independent (constitutive) transcriptional activation function that was obtained by the wt-ΔPASB protein lacking the C216S mutation (Fig. 4B), in excellent agreement with the less pronounced constitutive nuclear localization of the GFP-C216SΔPASB protein (Fig. 4A). The ability of this construct to induce XRE-mediated reporter gene expression suggests that XRE binding activity remains unaffected. In conclusion, these results indicate that the C216S mutation within the PAS A subdomain generates an NES motif that is functionally more active than the corresponding NES motif within the wild-type receptor.

**The bHLH Domain Regulates Nuclear Export of the Dioxin Receptor following Ligand-dependent Activation**—Our results indicate that the NES motif within the PAS domain of the dioxin receptor plays an important role in regulation of the intracellular localization of the receptor in the absence of ligand. To assess regulation of dioxin receptor function by nuclear export in the presence of ligand, we transfected HeLa cells with expression vectors for GFP-DR, GFP-DR C216S, or GFP-GR-DBD/DR 83–805 and incubated the cells with a com-
A combination of 10 nM TCDD and 50 nM leptomycin B to induce nuclear accumulation. The cells were then extensively washed, and the medium was replaced with medium containing vehicle alone or leptomycin B (Fig. 5A). The cells where then further incubated for 4 h, in the absence of ligand, and the intracellular localization pattern of the GFP-DR construct was determined. Under these conditions, we observed a clear shift of the GFP-DR construct from the nucleus toward the cytoplasmic compartment, where 50% of the cells displayed an equal distribution between the nuclear and cytoplasmic compartments (Fig. 5B). Interestingly, cells expressing the GFP-DR C216S protein displayed only a minor increase in cytoplasmic localization compared with the wild-type dioxin receptor (Fig. 5B). In contrast, substitution of the bHLH domain of the wild-type dioxin receptor with the DNA binding domain of the glucocorticoid receptor generated a protein that maintained a predominantly nuclear localization even after ligand withdrawal. These results suggest that export of the activated form of the dioxin receptor is predominantly mediated by the bHLH domain. Moreover, treatment of cells with leptomycin B, after ligand withdrawal, inhibited cytoplasmic redistribution (Fig. 5B). Taken together, these experiments indicate that the NES motif present in the bHLH domain of the dioxin receptor mediates export of the ligand-activated receptor, whereas the NES motif within the PAS domain is responsible for nuclear export of the nonactivated receptor.

The C216S Mutation in the NES Motif Induces Enhanced Proteolytic Degradation of the Dioxin Receptor—Dioxin receptor stability has been shown to be regulated by ligand, hsp90-associated factors (20), and intracellular localization of the receptor (16). Moreover, degradation of the receptor involves both ubiquitin-dependent and -independent pathways (1). It has recently been proposed that proteasome-mediated degradation of the dioxin receptor occurs predominantly in the cytoplasmic compartment of the cell (16). Considering that the C216S mutant form of the dioxin receptor showed a pronounced increase in cytoplasmic localization (Fig. 2B), we examined if the DR C216S mutant was more sensitive to proteolytic degradation than the wild-type receptor. Using immunoblot analysis, we monitored the expression levels of the wild-type dioxin receptor or the C216S mutant form in cells treated with the proteasome inhibitor MG132, TCDD, or a combination of both compounds. Since prolonged treatment of cells with TCDD results in an increase in dioxin receptor degradation, we treated cells for a limited period of time (2 h) when only modest degradation or no degradation of the wild type receptor can be
detected. Under these conditions, treatment of the cells with MG132, TCDD, or a combination of both compounds did not affect wild-type dioxin receptor expression levels (Fig. 6A, compare lanes 2–5). In contrast, in cells transfected with the C216S mutant form of the receptor, we failed to detect any expression of the ligand-free C216S mutant protein unless cells were treated with the proteasome inhibitor MG132 (compare lanes 6 and 7). MG132, however, failed to stabilize the C216S mutant protein when the cells were treated with TCDD (Fig. 6A, compare lanes 8 and 9). Introduction of the different GFP fusion constructs into cells resulted in clear stabilization of the mutant form of the dioxin receptor protein. Interestingly, we were able to detect the mutant protein in the absence of MG132 treatment. Moreover, the addition of MG132 had little effect on GFP-dioxin receptor protein levels in the absence of ligand. However, treatment of cells with 10 nM TCDD resulted in a clear reduction of the levels of the GFP-tagged mutant receptor protein (Fig. 6C, compare lanes 1 and 2). These results show that the C216S mutant is rapidly turned over by proteolysis and that several proteolytic pathways act on the dioxin receptor. It appears that, in the absence of ligand, the main degradation system that targets the dioxin receptor is the ubiquitin-proteasome system, since inhibition of this system with MG132 is sufficient to stabilize the dioxin receptor. However, the addition of ligand results in a dioxin receptor form that displayed a predominant cytoplasmic localization. Interestingly, the C216S mutation resulted in a dioxin receptor form that exhibited a predominant nuclear localization.

Given the critical role of the hsp90-associated immunophilin XAP-2 in protecting the dioxin receptor against degradation via the ubiquitin-proteasome pathway (20), we examined if overexpression of XAP-2 would increase the stability of the C216S dioxin receptor. In control co-immunoprecipitation assays, using in vitro translated proteins, we observed that both wild-type and C216S dioxin receptors interacted with XAP-2 with similar affinity (data not shown). Next we used the same experimental conditions as described above, where cells were exposed for 2 h to TCDD. For this purpose, COS cells were co-transfected with expression vectors encoding the mutant C216S dioxin receptor, XAP-2, or with the empty vector as a negative control. Interestingly, we observed that in the presence of XAP-2 the C216S dioxin receptor was stabilized in the absence of ligand (Fig. 6C, compare lanes 1 and 2). However, the addition of 10 nM TCDD to the transfected cell destabilized the C216S dioxin receptor, and XAP-2 was not able to fully protect the ligand-activated form against degradation (Fig. 6C, compare lanes 1 and 2 with lanes 3 and 4).

DISCUSSION

A single amino acid substitution (C216W) in the PAS A domain has been shown to inhibit the ability of the dioxin receptor to activate XRE-dependent gene expression (17). To rule out the possibility of structural rearrangements that would inhibit receptor function, we engineered a more conservative exchange, where the original Cys was changed to Ser. Interestingly, the C216S mutation greatly diminished the ability of the receptor to activate transcription. However, the C216S receptor form retained important functional features such as the ability to interact with the hsp90 chaperone complex, ligand binding, and dimerization with Arnt, ruling out major structural rearrangements as the reason for loss of transcriptional activity. Our data show that the C216S mutation affects the intracellular localization of the dioxin receptor. Interestingly, the C216S mutation resulted in a dioxin receptor form that displayed a predominant cytoplasmic localization. We have previously shown that the latent form of the dioxin receptor is being actively shuttled between the nucleus and the cytoplasm. Nuclear export of the latent form of the receptor is sensitive to leptomycin B (19), a specific inhibitor of the nuclear export protein CRM-1 (22). Close examination of the primary amino acid sequence surrounding the C216S mutation revealed several interesting features. First, this region of the dioxin receptor shows large degree of similarity to a bona fide NES sequence, the target interaction sequence for the nuclear export receptor protein CRM-1 (22). Moreover, the C216S mutation resembles a mutation in the Schizosaccharomyces pombe AP-1 homologue PAP-1. The interaction between PAP-1 and CRM-1 is regulated by the redox regulatory factor REF-1 which modulates the activity of PAP-1 by regulating the redox status of a critical cysteine residue in the PAP-1 NES sequence. Enhanced interaction between PAP-1 and CRM-1 reduces PAP-1...
then treated with 10 nM TCDD or vehicle alone for 4 h. Thereafter, cells were allowed to recover for 48 h and were transfected with expression vectors encoding C216S receptor mutant and XAP-2 as indicated. After transfection, the cells were harvested, and whole cell extracts were prepared. Equal amounts of whole cell extract protein were then fractionated through a 7.5% SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane. Western blot analysis was performed using a polyclonal dioxin receptor antiserum. The position of the C216S receptor is indicated by an arrow. Western blotting experiments were performed using dioxin receptor antibodies. The position of the C216S receptor protein was able to prevent degradation of the receptor in the absence of ligand and, to some extent, in the presence of ligand. XAP-2 has been shown to inhibit degradation of the dioxin receptor by interfering with receptor ubiquitination. However, we cannot rule out the possibility that XAP-2 (and possibly other chaperone-associated proteins) can protect the dioxin receptor against degradation.

The C216S mutation in the dioxin receptor displays a large degree of functional similarities to PAP-1. The original cysteine is flanked by two arginine residues, as observed in recognition sequences for the redox regulatory factor REF-1 (28). Mutation of this Cys to Ser may generate a “constitutively reduced” cysteine, where the interaction with CRM-1 would elude redox regulation. Although it is not known whether the activity of the dioxin receptor is modulated by REF-1, XRE-mediated transcriptional activation has been observed in cells treated with chemicals that induce oxidative stress (29). Taken together, these results suggest that the PAS A domain of the dioxin receptor harbors a functional NES motif that regulates dioxin receptor function. Furthermore, exchange of Cys to Ser generates a constitutively active NES that directs the receptor to the cytoplasm.

The present experiments suggest that the NES motif in the PAS domain regulates intracellular localization of the dioxin receptor in the absence of ligand (see model in Fig. 7). Thus, the cytoplasmic localization of the receptor is regulated, at multiple levels, both by CRM-1 and the immunophilin-like protein XAP-2 (19, 20). In contrast, nuclear export of the ligand-activated form of the receptor appears to be mediated by the NES motif present in the bHLH domain (Fig. 7). Replacement of the bHLH domain with the DNA binding domain of the glucocorticoid receptor, which lacks functional nuclear export activity (26), generated a fusion protein that fails to be exported from the cell nucleus following ligand withdrawal. Moreover, the nuclear export kinetics of the ligand-activated wild-type and C216S dioxin receptors displayed similar rates, suggesting that the C216S mutation did not affect the export rate of the ligand-activated receptor. These experiments suggest that nuclear export of the ligand-activated dioxin receptor requires the NES motif that regulates dioxin receptor function. Additionally, the nuclear export of the ligand-activated form of the receptor appears to be mediated by the NES motif present in the PAS A domain of the dioxin receptor harbors a functional NES motif that regulates dioxin receptor function. Further-
via ubiquitin-independent pathways.

In conclusion, our results show that the intracellular localization of the dioxin receptor is determined by multiple regulatory pathways. In the absence of ligand, the receptor is actively being transported between the nucleus and the cytoplasm. While the molecular mechanism that regulates nuclear import in the absence of ligand remains unknown, nuclear export of the nonactivated receptor is mediated by the NES motif present in the PAS domain of the receptor. We show that modifications that alter the activity of this motif have profound functional consequences for the stability and activity of the receptor. Furthermore, the activity of the individual NES motifs appears to be closely linked to the functional status of the receptor. It appears that, in the absence of ligand, nuclear export of the receptor is mediated by the PAS domain, while export of the active receptor, following ligand removal, is mediated by the bHLH domain. Clearly, additional studies are required to clarify the complex interplay of mechanisms that regulate the intracellular localization of the dioxin receptor.

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