The dioxin receptor is a ligand-dependent transcription factor that mediates the biological effects of dioxin and related environmental pollutants. In the absence of ligand the receptor is present in the cytoplasmic compartment of the cell associated with the hsp90-dependent chaperone complex. This complex regulates several functions of the receptor such as ligand binding and nuclear import. Furthermore, intracellular localization of the receptor is modulated by multiple factors such as the export protein CRM-1 and the hsp90-associated immunophilin XAP-2. We have identified the mechanism of XAP-2-induced cytoplasmic localization of the receptor and studied the potential cross-talk between CRM-1 and XAP-2. We show that XAP-2 anchors the ligand-free receptor to cytoskeletal structures. This effect is blocked upon treatment with the actin inhibitor cytochalasin B, whereas the tubulin inhibitor colchicine had no effect on receptor localization. In addition, we show that the receptor interacts with CRM-1 both in the presence and absence of ligand. CRM-1-mediated nuclear export occurs independently of XAP-2. Our data provide evidence that CRM-1 and XAP-2 act in parallel through different mechanisms and target different interfaces of the receptor. These results suggest that two pathways cooperate to localize the non-activated receptor in the cytoplasmic compartment of the cell.

In mammalian cells, multiple systems such as ligand binding, dimerization, and post-translational modifications have evolved to regulate the function of transcription factors. Recently, intracellular compartmentalization has emerged as a major pathway to modulate transcription factor activity. For instance, the dioxin receptor is a ligand-dependent transcription factor which, in the absence of ligand, is found in the cytoplasmic compartment of the cell or, depending on cell type, evenly distributed between the cytoplasm and the nucleus (1, 2). This non-activated form of the receptor interacts with the hsp90-dependent molecular chaperone complex (3) and its associated proteins, such as the hsp90-associated factor p23 (4, 5) and the dioxin receptor specific immunophilin XAP-2 (6–8). The interaction between the dioxin receptor and hsp90 is mediated by two distinct structural determinants, the DNA binding bHLH domain (9), and the ligand binding region within the PAS-B subdomain of the receptor (10). In the presence of ligand the receptor accumulates in the nucleus (1, 11) where it interacts with the general dimerization partner factor ARNT (12). This event induces release of the hsp90 complex (4, 13). Nuclear accumulation of the receptor is regulated by a nuclear localization signal present in the bHLH domain (14) and by the hsp90 complex which regulates the interaction between the import machinery and the activated form of the receptor (1). In addition, we have recently shown that the non-activated form of the receptor is shuttling between the nucleus and the cytoplasm (2). In this respect, nuclear export of the dioxin receptor is likely to be mediated by the export factor CRM-1. This protein interacts with short hydrophobic recognition motifs and thereby induces nuclear export of a large variety of nuclear proteins (15). Treatment with the specific CRM-1 inhibitor leptomycin B induces nuclear accumulation of the receptor suggesting a functional role for CRM-1 in inducing nuclear export of the receptor (2). Subsequent studies have shown that the interaction between CRM-1 and the dioxin receptor is dependent on two NES motifs present in the bHLH and PAS domains of the receptor. These two NES sequences are differentially employed depending on the activation status of the receptor. Nuclear export of the non-activated dioxin receptor is mediated via the NES present in the PAS domain, whereas export of the ligand-activated receptor is mediated by the NES located within the bHLH domain (2).

Additional proteins that influence the intracellular localization of the receptor include the immunophilin-like protein XAP-2. XAP-2 is a hsp90-associated factor that shares a high degree of sequence similarity to classical immunophilins such as FKBP52 (12). Immunophilins interact with hsp90-associated steroid hormone receptors, most notably the glucocorticoid receptor, and regulate ligand-induced nuclear accumulation. In contrast to FKBP52, XAP-2 induces cytoplasmic redistribution of the dioxin receptor by an unknown mechanism (16–18). In addition, XAP-2 binds to the non-activated dioxin receptor-hsp90 complex and enhances receptor transactivation (19, 20) by protecting the ligand-free form against ubiquitination and subsequent degradation (1). The interaction with XAP-2 requires the intact PAS B domain of the receptor (16, 20). These observations suggest that multiple regulatory proteins including CRM-1 and XAP-2 act on the receptor to uphold cytoplasmic localization of the non-activated dioxin receptor and that several receptor interfaces are targeted by different mechanisms. The potential interplay between these different mechanisms(s) remains to be elucidated.

In our studies we aimed to characterize the molecular mech
anism that regulate cytoplasmic localization of the receptor. We show that CRM-1-mediated nuclear export of the dioxin receptor occurs independently of the cytoplasmic retention activity mediated by XAP-2. Reciprocally, the ability of XAP-2 to induce cytoplasmic localization of the receptor does not require CRM-1. In addition, our results demonstrate that XAP-2-induced cytoplasmic localization of the dioxin receptor involves anchoring of the receptor to certain cytoskeletal proteins, namely actin filaments. Treatment of cells with compounds that disrupt these structures inhibits XAP-2-mediated cytoplasmic localization of the dioxin receptor.

MATERIALS AND METHODS

Recombinant Plasmids—The vectors pCMX/mDR-GFP, pCMX/mDR-ΔPASB-GFP, pCMX/mDR-ΔPASB-GFP C216S, pGEM7m/DR-ΔPASB, pCMX/mDR-ΔPASB C216S, pCMV2.FLAG-mDR, pCMV2.FLAG-XAP-2, pB3.mDR, and pSG5/XAP-2 have been described previously. Plasmids pCMX/GRDBD-mDR-GFP and pCMX/CRM-1 were constructed by standard cloning techniques. Details regarding construction of the different plasmids are available from the authors upon request.

Determination of Intracellular Localization of GFP-tagged Proteins in Living Cells—HeLa cells were propagated in Dulbecco’s minimum essential medium supplemented with 5% fetal calf serum, 25 mM HEPES (pH 7.4), 100 units/ml penicillin, 100 units/ml streptomycin, 10% FBS, and antibiotics. Transfection was performed by electroporation, using a BioRad GenePulser apparatus according to the manufacturer’s instructions. Following transfection, cells were harvested twice with cold PBS, collected by centrifugation, and suspended in 0.5% Tween 20 using enhanced chemiluminescence reagents (Amersham Biosciences) according to the manufacturer’s recommendations.

RESULTS

Characterization of the Molecular Mechanisms That Regulate Cytoplasmic Localization of the Receptor—Intracellular localization of the dioxin receptor is regulated via multiple pathways which include the immunophilin XAP-2 and the hsp90 complex, the CRM-1-dependent nuclear export pathway, and importin-dependent nuclear import. Interestingly, both XAP-2 and CRM-1 mediate cytoplasmic localization of the receptor. Therefore, we decided to investigate whether these two pathways cooperate or if they act independently of each other. For this purpose we co-transfected HeLa cells with expression vectors of a GFP-dioxin receptor fusion protein (mDR-GFP) and XAP-2 as indicated. For comparison the mDR-GFP was introduced into HeLa cells in the absence of XAP-2. Following transfection the cells were treated with vehicle only, 10 nM leptomycin B, 10 nM TCDD, or a combination of TCDD and leptomycin B. As shown previously (1, 2) the mDR-GFP fusion protein was equally distributed both in the cytoplasmic and nuclear compartments of the cell (Fig. 1A, upper panel). Treatment with 10 nM TCDD induced nuclear localization of the receptor (data not shown). Incubation with 10 nM leptomycin B resulted in ligandindependent nuclear accumulation of the receptor (Fig. 1A, lower panel). Co-expression of the receptor-specific immunophilin XAP-2 together with the mDR-GFP fusion protein resulted in marked redistribution of the receptor toward the cytoplasm (Fig. 1B, upper left panel) as expected (16). In the presence of XAP-2, addition of ligand leads only to partial nuclear accumulation of the receptor in the presence of XAP-2 (Fig. 1B, lower right panel). These experiments suggest that two parallel mechanisms cooperate to mediate cytoplasmic localization of the dioxin receptor, because inhibition of CRM-1 by leptomycin B or addition of ligand alone is not sufficient to induce nuclear localization of the receptor in the presence of XAP-2.

The Dioxin Receptor Interacts with the Export Protein CRM-1 Both in the Presence and Absence of Ligand—Our observation that TCDD alone is not sufficient to induce nuclear accumulation of the receptor in cells overexpressing XAP-2 prompted us to investigate if the activated receptor still maintained its ability to interact with CRM-1. We have shown previously (2) that following ligand withdrawal the receptor is exported out of the nucleus by a leptomycin B-sensitive mechanism, suggesting the involvement of CRM-1. In addition, nuclear export of the receptor following ligand withdrawal was inhibited by deletion of the NES present in the bHLH domain (2). However, the possibility remains that the ligand-bound form of the receptor still interacts with CRM-1. To clarify this issue we co-transfected HeLa cells with expression vectors for the export protein CRM-1 and the mDR-GFP fusion protein, and we studied the effect on intracellular localization of the receptor. In the presence of CRM-1, the intracellular localization of the ligand coupled transcription-translation product in rabbit HEK293 cells was markedly shifted toward the cytoplasmic compartment of the cell (Fig. 2A, upper left panel). However, we also observed a significant amount of the receptor in the nuclear compartment, suggesting that the shuttling of the receptor between cytoplasm and nucleus was intact and that the increase in cytoplasmic localization of the receptor was a result of the elevated CRM-1 levels in the cell. Consistent with this...
notion, addition of leptomycin B even in the presence of overexpressed CRM-1 induced nuclear localization of the receptor (Fig. 2A, lower left panel). Interestingly, however, addition of ligand to cells where the mDR-GFP fusion protein was coexpressed together with CRM-1 leads only to a minor increase of nuclear localized receptor (Fig. 2A, upper right panel). These results suggest that CRM-1 can interact with the receptor regardless of its activation state and induce nuclear export. In addition, these results show that ligand binding does not affect the ability of the receptor to interact with CRM-1.

To verify that the interaction between the receptor and CRM-1 occurs both in the absence and presence of ligand, we performed co-immunoprecipitation experiments. We transiently expressed a GST-tagged dioxin receptor expression vector (GST-mDR) in HeLa cells and used 1H9251-GST antibodies to precipitate different receptor-associated proteins. The precipitated material was extensively washed and separated using SDS-PAGE. The fractionated proteins were transferred to a nitrocellulose membrane, and CRM-1 antibodies were added to visualize the presence of CRM-1 in the precipitated material. This experiment shows that the dioxin receptor efficiently interacts with CRM-1 both in the absence and presence of ligand (Fig. 2B, compare lanes 2 and 3). Co-expression of XAP-2 together with the receptor did not perturb this interaction (Fig. 2B, compare lanes 4 and 5); rather, we observed increased levels of CRM-1 interacting with the receptor. However, it is important to note that that XAP-2 stabilizes the dioxin receptor by inhibiting receptor ubiquitination (16). The seemingly higher interaction between the receptor and CRM-1 may simply reflect increased receptor levels. In conclusion, these results show that the dioxin receptor interacts efficiently with the CRM-1-mediated nuclear export machinery both in the presence or absence of ligand. These experiments further suggest that nuclear localization of the receptor may depend on increased nuclear retention, possibly via ligand-induced recruitment of nuclear factors such as co-activators. Interaction with these proteins may inhibit binding between the ligand-activated form of the receptor and CRM-1 and result in stable nuclear localization.

**The Ligand-activated Dioxin Receptor Interacts with the Export Protein CRM-1 through the bHLH Domain**—The results presented above suggest that regardless of its activation state, the receptor maintains its ability to interact with CRM-1. Earlier studies have identified two NES sequences in the receptor, one located in the bHLH (14) domain and one in the PAS A (2) domain. We decided to study if export of the latent or active forms of the receptor is mediated by different receptor domains. We used the GRDBD-mDR-GFP fusion construct where the
bHLH domain has been replaced with the N-terminal region of the glucocorticoid receptor. This protein does not interact with CRM-1 (23). We introduced this construct into HeLa cells by means of transient transfections together with expression plasmid for CRM-1. Following transfection, cells were treated with 10 nM TCDD, and the intracellular localization of the mDR was determined. B, HeLa cells were transfected with the GST-mDR expression plasmid and XAP-2 expression vectors as indicated. Following transfection cells were allowed to grow for 48 h and were subsequently treated with 10 nM TCDD as indicated for 2 h. After this treatment, whole cell extracts were prepared, and co-immunoprecipitation experiments were performed using α-CRM antibodies. Precipitated material was fractionated on a 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was subjected to Western blot analysis using α-CRM-1 antibodies. LMB, leptomycin B.

**Fig. 2.** The dioxin receptor interacts with CRM-1 both in the presence and absence of ligand. A, HeLa cells were co-transfected with the mDR-GFP construct as in Fig. 1 together with 500 ng of expression vector for CRM-1. Following transfection, cells were treated with 10 nM TCDD, and the intracellular localization of the mDR was determined. B, HeLa cells were transfected with the GST-mDR expression plasmid and XAP-2 expression vectors as indicated. Following transfection cells were allowed to grow for 48 h and were subsequently treated with 10 nM TCDD as indicated for 2 h. After this treatment, whole cell extracts were prepared, and co-immunoprecipitation experiments were performed using α-GST antibodies. Precipitated material was fractionated on a 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was subjected to Western blot analysis using α-CRM-1 antibodies. LMB, leptomycin B.

The data presented above show that the receptor interacts with CRM-1 both in the presence and absence of ligand. Interestingly, addition of XAP-2 resulted in a clear increase in the amount of co-precipitated CRM-1. However, because XAP-2 has been shown to stabilize dioxin receptor protein levels by inhibiting receptor ubiquitination (16), this may be an effect of higher receptor levels in the XAP-2 expressing cells. We decided to investigate whether the apparent increase in receptor-CRM-1 interaction noted in co-immunoprecipitation assays was a result of higher intracellular receptor levels or whether the interaction between CRM-1 and the receptor is stabilized in the presence of XAP-2. For this purpose we transiently transfected HeLa cells with an mDR-PASB-GFP receptor construct where the PAS B domain has been deleted. This construct lacks the hsp90 binding PAS B domain and does not interact with XAP-2. In previous studies (2, 16) we have shown that this mutant receptor is localized constitutively in the nuclear compartment of the cell due to failure to interact with XAP-2. In addition, we have identified previously a critical CYS residue in the NES present in the PAS domain of the receptor, which upon mutation to SER induces a prominent shift toward the cytoplasm, possibly due to the formation of a NES with increased activity (2). We now examined whether the mDR-PASB receptor form maintained its ability to interact with CRM-1. For this purpose, we
Cytoplasmic Localization of the Dioxin Receptor

The hsp90-associated Protein XAP-2 Utilizes Cytoskeletal Structures to Redistribute the Dioxin Receptor to the Cytoplasma—The results described above strongly suggest that the CRM-1-dependent export machinery regulates nuclear export both of the latent and active forms of the dioxin receptor. This cellular shuttling occurs both in the presence and absence of ligand, and the hsp90 molecular chaperone complex does not appear to be involved in these events. However, these results do not shed any light on how XAP-2 induces cytoplasmic localization of the receptor. Clearly, this is not dependent on the CRM-1 export complex because leptomycin B treatment only induced a minor shift toward the cell nucleus when the receptor is co-transfected with XAP-2 (Fig. 1). However, the fact that co-treatment of cells with both leptomycin B and TCDD induces complete nuclear localization of the receptor in the presence of XAP-2 suggests that ligand-sensitive mechanisms regulate cytoplasmic localization of the receptor. Earlier studies have shown that FKBP52, a steroid receptor binding immunophilin which displays high degree of sequence similarity with XAP-2 (6), interacts with tubulin cytoskeletal networks (24). Based on sequence similarity between XAP-2 and FKBP52 we speculated that actin or tubulin filaments could be involved in the XAP-2-mediated regulation of cytoplasmic localization of the receptor. To test this hypothesis we used compounds known to specifically disrupt actin or tubulin structures in order to examine whether intracellular localization of the receptor was affected. We expressed mDR-GFP by transient transfection and subsequently treated the cells with colchicine or cytochalasin B. Colchicine has been shown to inhibit polymerization of tubulin networks (25–27), whereas cytochalasin B negatively affects actin polymerization (28).

In cells treated with cytochalasin B the intracellular localization of the receptor displayed an even localization pattern within the nucleus and the cytoplasm (Fig. 5A, upper left panel) with a slight increase in nuclear localization. Addition of TCDD to the cells in the presence of cytochalasin B resulted in partial nuclear accumulation of the receptor (Fig. 5A, upper right panel) when compared with untreated cells. In a similar manner, treatment of cells with colchicine led to minimal changes in the intracellular localization pattern of the non-activated dioxin receptor (Fig. 5A, lower left panel). Addition of ligand to colchicine-treated cells led to a clear inhibition of nuclear accumulation of the receptor (Fig. 5A, lower right panel). In contrast, in cells co-transfected with XAP-2 and the receptor, addition of cytochalasin B resulted in considerable changes in intracellular localization of the receptor. Whereas treatment of cells with cytochalasin B resulted in clear inhibition of the cytoplasmic retention activity induced by XAP-2 (compare Fig. 5B, upper left panel, with Fig. 1B, upper left panel). In addition, we observed minimal or no nuclear accumulation of the receptor upon addition of ligand, suggesting that ligand-induced

transfected HeLa cells with expression vectors for mDR-ΔPASB-GFP in the presence or absence of CRM-1. In the absence of CRM-1 the mDR-ΔPASB-GFP fusion protein displayed a constitutively nuclear localization pattern (Fig. 4A, left top panel). In the presence of CRM-1, however, the intracellular localization shifted dramatically toward the cytoplasmic compartment (Fig. 4A, top right panel). These experiments show that, despite its seemingly nuclear appearance, the mDR-ΔPASB protein apparently retains its ability to interact with the export protein CRM-1. In control experiments we used mDR-ΔPASB-GFP C216S, where a point mutation was introduced into the PAS domain of the receptor. This C216S mutated form of the receptor displays drastically amplified cytoplasmic appearance, due to increased nuclear export activity of the NES motif present in the PAS A domain (2). In transient transfection experiments we observed a clear shift from the nucleus to the cytoplasm for the mDR-ΔPASB-GFP C216S (Fig. 4A, lower left panel). When co-expressed with CRM-1, intracellular redistribution of mDR-ΔPASB-GFP C216S to the cytoplasm was further intensified, resulting in very low levels of nuclear localization (Fig. 4A, lower right panel). This observation suggests that the ability of this construct to be exported out of the nucleus was not dependent on interaction with XAP-2. To corroborate this observation, we performed co-immunoprecipitation experiments where the association between CRM-1 and the mDR-ΔPASB constructs was investigated. The mDR-ΔPASB constructs and CRM-1 were in vitro translated using rabbit reticulocyte lysate. To visualize the interaction between CRM-1 and the receptor, CRM-1 was labeled with [35S]methionine. Following translation the proteins were mixed and precipitated using α-dioxin receptor polyclonal antibodies. As shown in Fig. 4B, CRM-1 interacted with the mDR-ΔPASB receptor construct (Fig. 4B, compare lanes 1 and 3). Interestingly, when the C216S form of this receptor was used, we observed a clear increase in the ability of the receptor to bind CRM-1 (Fig. 4B, compare lanes 1 and 2). We conclude that the dioxin receptor interacts with the CRM-1-mediated export machinery even when the hsp90-XAP-2 interacting domain is deleted.

Fig. 3. CRM-1 mediates export of the ligand-activated dioxin receptor through the bHLH domain. HeLa cells co-transfected with GRDBD-mDR-GFP and CRM-1 expression plasmid are depicted. Following transfection cells were treated with 10 nM TCDD, and the intracellular localization pattern of the GRDBD-mDR-GFP fusion protein was determined.
accumulation of the receptor is dependent on intact cytoskeletal structures. In a similar fashion, nuclear import of either the glucocorticoid receptor or the tumor suppressor protein p53 requires intact cytoskeletal structures (29). These results suggest that XAP-2-dependent cytoplasmic relocalization of the receptor involves anchoring of the hsp90-receptor complex to actin structures. This mechanism appears to be mediated by the receptor immunophilin XAP-2. Treatment of cells with cytochalasin B, an agent known to repress polymerization of actin filaments, inhibits XAP-2-mediated anchoring of the receptor to actin and inhibits XAP-2-mediated cytoplasmic localization of the receptor.

XAP-2 Induces Interaction between the Dioxin Receptor and Actin—The presented experiments suggest that the receptor immunophilin XAP-2 mediates cytoplasmic retention of the dioxin receptor in the absence of ligand. To test whether the effect of XAP-2 involved direct binding to actin, we decided to perform co-immunoprecipitation experiments. We transfected COS cells and precipitated receptor-associated proteins as described in Fig. 2 using a GST-tagged dioxin receptor fusion protein (GST-mDR) and α-GST antibodies. Precipitated material was extensively rinsed, and the proteins were fractionated through SDS-PAGE. Following electrophoresis, proteins were transferred to a nitrocellulose membrane that was probed with α-actin antibodies. In cells transfected with empty GST-expressing plasmid, only background levels of actin immunoreactivity were detected (Fig. 6, lane 1). In addition, similar results were obtained when the GST-tagged receptor plasmid or XAP-2 were expressed in COS cells individually (Fig. 6, lanes 2–4). However, co-expression of both XAP-2 and the GST-tagged receptor in COS cells induced a considerable increase in co-precipitated actin (Fig. 6, lanes 4). Interestingly, addition of TCDD to cells co-transfected with XAP-2 and GST-tagged receptor induced a substantial decrease in the amount of precipitated actin (Fig. 6, compare lanes 5 and 6). This experiment shows that the non-activated dioxin receptor interacts with actin filaments in the absence of TCDD and that this interaction is mediated by the receptor-associated immunophilin XAP-2. Furthermore, incubation with ligand induces release of the receptor complex from actin and probably subsequent nuclear accumulation.
XAP-2-mediated Protection of the Dioxin Receptor Is Not Affected by Disruption of Cytoskeletal Structures—Given the fact that cytochalasin B is able to inhibit cytoplasmic retention of the dioxin receptor, we tested whether other functions of XAP-2 were impaired under these conditions. XAP-2 has been shown to protect the dioxin receptor against ubiquitination and thereby increase the intracellular levels of the dioxin receptor (16). To test whether XAP-2-mediated protection of the receptor was influenced by cytochalasin B, we transfected COS7 cells with expression vectors for the dioxin receptor and FLAG-tagged XAP-2 and treated the cells as described in Fig. 5. After transfection the cells were harvested, and whole cell extract was prepared and fractionated by SDS-PAGE. Following electrophoresis, proteins were transferred to a nitrocellulose membrane, and Western blotting was performed using α-dioxin receptor or α-FLAG antibodies. As shown in Fig. 7A, addition of XAP-2 stabilized the cellular levels of the receptor (Fig. 7A, compare lanes 2 and 3). Interestingly, addition of colchicine or cytochalasin B did not inhibit the stabilizing effect of XAP-2 on the dioxin receptor. This result was not due to altered or lowered intracellular levels of XAP-2, because Western analysis showed that XAP-2 protein levels remained constant throughout our experimental conditions (Fig. 7A, lower panel). Thus, destabilization of cytoskeletal networks does not interfere with the stabilizing effects of XAP-2 on the dioxin receptor.

These results indicate that disruption of cytoskeletal networks by cytochalasin B inhibits XAP-2-mediated cytoplasmic retention of the dioxin receptor. However, cytochalasin B does not inhibit stabilization of receptor protein levels by XAP-2. Because it is possible that stabilization of the receptor by XAP-2 might not necessarily involve direct interaction between the receptor and XAP-2, we investigated whether the interaction between the receptor and XAP-2 was disrupted in the presence of colchicine or cytochalasin B. For this purpose, COS7 cells were transiently transfected with expression vectors encoding the mDR-GST and FLAG-tagged XAP-2. Following transfection, cells were treated with or without TCDD, and whole cell extracts were prepared. Extracts were subsequently incubated with α-GST antibodies, and immunoprecipitation experiments were performed. Precipitated material was fractionated through a 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. Western experiments were performed using α-actin antibodies.
blotting experiments were performed using FLAG antibodies. As shown in Fig. 7B, the GST-tagged receptor interacted with FLAG-tagged XAP-2 in the presence of both with colchicine or cytochalasin B. In fact, the experiments suggest that the interaction between the receptor and XAP-2 is stabilized upon treatment of cells with colchicine or cytochalasin B (Fig. 7B, compare lanes 4–6).

The Inhibitory Effects of Cytochalasin B on Cytoplasmic Redistribution Are Mediated by the PAS B Domain of the Dioxin Receptor—Next we studied if the inhibitory effects of colchicine were mediated by the PAS B domain of the receptor or, alternatively, if CRM-1-mediated export was affected by this treatment. For this purpose we expressed in HeLa cells the mDR/ΔPASB-GFP or mDR/ΔPASB-GFP C216S fusion proteins. Following transfection, the cells were treated with colchicine or cytochalasin B, and the intracellular localization pattern of the GFP fusion proteins was determined. Interestingly, treatment with cytochalasin B did not affect the overall intracellular localization pattern of mDR/ΔPASB (Fig. 8, upper left panel). These results suggest that the inhibitory effects of cytochalasin B are mediated by the XAP-2-hsp90 interacting PAS B domain of the receptor. In contrast, addition of colchicine to the cells decreased the levels of nuclear localized mDR/ΔPASB protein. This effect is probably due to inhibition of nuclear import (Fig. 8, lower left panel), suggesting that, in analogy to transcription factors like p53, the dioxin receptor may require intact tubulin filaments to accumulate in the nucleus. In control experiments we expressed in HeLa cells mDR/ΔPASB C216S which displays increased cytoplasmic localization due to enhanced nuclear export (2). As observed with the mDR/ΔPASB construct, treatment with colchicine lowered the amount of cells with nuclear staining. On the other hand, cytochalasin B treatment had no effect on the enhanced cytoplasmic localization pattern of this construct, consistent with our model that the effects of cytochalasin B are mediated by XAP-2 and the PAS B domain of the dioxin receptor.

DISCUSSION

Activation of the bHLH-PAS dioxin receptor represents a complex process where several regulatory mechanisms act in a sequential manner (12). These mechanisms include ligand binding, nuclear translocation, and specific binding to DNA enhancer elements located in the vicinity of dioxin-inducible genes (12). Cellular compartmentalization appears to play a critical role in regulation of dioxin receptor activity. We have shown that two NES motifs located in the PAS (2) and bHLH domains (14) of the receptor mediate interaction between the receptor and CRM-1. CRM-1 has been shown to interact with a number of transcription factors and mediate nuclear export (30, 31). CRM-1-mediated nuclear export can be inhibited upon addition of the fungal metabolite leptomycin B (22). We have shown previously that incubation of cells with leptomycin B results in nuclear localization of the dioxin receptor both in the absence or presence of ligand, suggesting an important role for CRM-1 in nuclear export of the dioxin receptor (2). Interest-
ingly, the dioxin receptor utilizes the two different NES in a differential manner depending on the activation state of the receptor. In the absence of ligand the receptor is exported from the nucleus via the NES located in the PAS domain, whereas the NES situated in the bHLH domain is utilized for nuclear export of the ligand-activated form of the receptor (2, 32). Furthermore, additional pathways are involved in anchoring the receptor in the cytoplasm. We and others (16–18) have shown that the receptor-specific immunophilin XAP-2 induces cytoplasmic localization of the receptor. In the current study we show that the CRM-1 and the XAP-2-dependent pathways function independently from each other and regulate intracellular localization of the receptor via different mechanisms.

In the absence of ligand the receptor is found bound with the hsp90-chaperone complex (3). The receptor interacts with the hsp90 complex via two distinct domains, the DNA binding bHLH and the PAS B domains. Interaction with the hsp90 complex regulates different functional activities of the dioxin receptor. The interaction between the PAS B domain of the receptor and hsp90 is required to maintain the ligand binding activity of the receptor intact (33). In addition, we and others (1, 34, 38) have shown that the hsp90 complex is involved in nuclear accumulation of the receptor. This is accomplished by regulating the interaction between the nuclear localization sequence present in the bHLH domain of the receptor and the nuclear import machinery.

Interestingly, the interaction between the PAS domain and the hsp90 chaperone complex results in recruitment of the dioxin receptor-specific immunophilin XAP-2. XAP-2 interaction is specifically mediated by the PAS B ligand binding domain of the receptor, whereas the bHLH domain does not support binding of XAP-2. Several studies (16–18) have shown that this interaction is responsible for cytoplasmic redistribution of the receptor, although the precise mechanism is presently unclear.

In the absence of XAP-2, the receptor is found evenly distributed in the nuclear and cytoplasmic compartment of the cell (1, 16, 32, 35, 36). Co-expression of XAP-2 induces a dramatic shift in receptor localization toward the cytoplasm (1, 17, 18). Furthermore, XAP-2 expression severely delays nuclear accumulation of the receptor in the presence of ligand or, alternatively, in the presence of the CRM-1-specific inhibitor leptomycin B (22). However, combined treatment of both leptomycin B and ligand induces full nuclear translocation of the receptor even in the presence of XAP-2. These results suggest that two different pathways are responsible for nuclear export and for anchoring the receptor in the cytoplasm. These two different pathways function independently and target different domains of the receptor. Indeed, nuclear export occurs both in the presence and absence of ligand. Co-immunoprecipitation analysis revealed that the receptor interacts with CRM-1 both in the presence and absence of ligand. This interaction is not dependent on the PAS B domain of the receptor and thus not subject to regulation by XAP-2. This observation indicates that the CRM-1-dependent pathway constitutively mediates nuclear export of the receptor and therefore is not likely to be critical to inhibit dioxin receptor transcriptional activation. In contrast, XAP-2-dependent cytoplasmic anchoring of the receptor occurs only in the absence of ligand. We speculate that the difference may represent an important regulatory event in the activation process of the dioxin receptor that may prevent ligand-independent transcriptional activation of the receptor.

XAP-2-mediated cytoplasmic anchoring of the receptor involves a different mechanism. In earlier studies (37), the immunophilin FKBP52 has been shown to interact with several different members of the steroid receptor superfamily, most notably the glucocorticoid receptor. This immunophilin regulates the intracellular localization pattern of the glucocorticoid receptor by facilitating interaction with tubulin filaments (24). This interaction is important to mediate nuclear accumulation of the glucocorticoid receptor. Interestingly, the integrity of tubulin filaments is also important for nuclear accumulation of the tumor suppressor protein p53 (29).

In contrast, little is known regarding the potential involvement of cytoskeletal structures in anchoring transcription factors like the dioxin receptor in the cytoplasmic compartment of the cell. In this study we present evidence for the involvement of actin structures in anchoring the dioxin receptor in the cytoplasm. This activity is mediated by the PAS B domain of the receptor and is inhibited upon treatment of cells with cytochalasin B, a known inhibitor of actin polymerization. Interestingly, XAP-2-induced cytoplasmic redistribution of the receptor was inhibited by cytochalasin B and because XAP-2 interacts via the PAS B domain of the receptor, deletion of this domain should result in a protein that is not affected by cytochalasin B. Indeed this situation was observed. In experiments where we utilized the PAS B deletion constructs these mutants were shown to be refractory to cytochalasin B. These results suggest that the immunophilins XAP-2 and FKBP52 are functionally very different and mediate alternative tasks in regulation of intracellular localization, despite a high degree of sequence homology.

Interestingly, we did not observe any inhibition of the XAP-2-dependent stabilization of the receptor by cytochalasin B or colchicine. In addition, in co-immunoprecipitation experiments we clearly observed that the interaction between the dioxin receptor and XAP-2 was not affected, showing that inhibition of XAP-2-dependent cytoplasmic localization by cytochalasin B did not involve disruption of the dioxin receptor-XAP-2 complex. However, it is interesting to notice that these two mechanisms act on the receptor independently of one another. Nuclear export of the receptor is not dependent on XAP-2 because the mDR-ΔPASB construct was able to interact with CRM-1. In addition, the nuclear dominant appearance of this construct was dramatically shifted toward the cytoplasmic compartment in cells co-transfected with CRM-1 demonstrating that this form of the receptor can be efficiently exported out of the nucleus.

In addition, leptomycin B-sensitive nuclear export of the receptor was not inhibited in the presence of ligand. Coupled to earlier results (32) demonstrating that addition of leptomycin B inhibited the transcriptional activity of the receptor, these results suggest that nuclear export may be required for full transcriptional activity. We speculate that post-transcriptional modifications occur in the cytoplasm and are required to generate a receptor with full transcriptional activation potential. In this respect, cytoplasmic localization of the receptor does not automatically result in a transcriptionally inactive conformation. Clearly, additional experiments are required to clarify this and remaining questions. For example, it will be important to clarify what steps following ligand activation occur in the nucleus or in the cytoplasm. In addition, determination of the potential role for the hsp90 complex in any of these steps will be required.

In conclusion, we have shown that two non-overlapping mechanisms regulate cytoplasmic localization of the bHLH-PAS dioxin receptor. The CRM-1-dependent export complex mediates export of the receptor both in the absence and presence of ligand. In addition, the hsp90 chaperone complex serves as a platform for the immunophilin-like protein XAP-2 and possibly additional auxiliary factors to anchor the non-activated form of the receptor in the cytoplasm by locking the receptor complex to actin filaments.
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