The dioxin (aryl hydrocarbon) receptor is a ligand-dependent transcription factor that induces expression of a number of genes encoding drug metabolizing enzymes. The nonactivated form of the dioxin receptor is associated with heat shock protein (hsp) 90, the co-chaperone p23, and the immunophilin-like protein XAP2. Whereas hsp90 has a role in maintenance of the high-affinity ligand binding conformation of the dioxin receptor complex, and p23 stabilizes receptor-hsp90 interaction, the exact role of XAP2 is largely unknown. Here we show that XAP2 protected the ligand-free form of receptor against ubiquitination, resulting in increased dioxin receptor protein levels. Upon exposure to ligand, nuclear translocation of the dioxin receptor was markedly delayed by XAP2, indicating an additional role of XAP2 in regulation of the subcellular localization of the receptor by a mechanism of cytoplasmic retention. In order to mediate these effects, XAP2 required stable association with the hsp90-p23 molecular chaperone complex. The association of XAP2 as well as p23 with the dioxin receptor was determined by the functional state of hsp90. These data indicate a novel mode of regulation of dioxin receptor signaling by the hsp90-dependent molecular chaperone machinery.

The biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and related environmental pollutants are mediated by the dioxin (aryl hydrocarbon) receptor, a member of the bHLH/PAS family of transcription factors. bHLH/PAS proteins are characterized by two conserved domains, the N-terminal bHLH DNA-binding domain and the PAS domain which spans two hydrophobic repeats termed PAS-A and PAS-B. In the case of the dioxin receptor, the PAS-B motif is contained within the ligand-binding domain (1). In the absence of ligand, the latent form of the dioxin receptor is associated with the molecular chaperone hsp90 (2), the co-chaperone p23 (3, 4), and the immunophilin-like protein XAP2, also known as ARA9 and AIP (5–7). Upon ligand binding the dioxin receptor is accumulated within the nucleus where it forms a transcriptionally active complex with the bHLH/PAS transcription factor Arnt which, in turn, induces the release of hsp90 from the receptor (4, 8, 9). The dioxin receptor-Arnt heterodimer activates transcription of target genes by specifically binding to dioxin-inducible transcriptional control elements, xenobiotic response elements, which are located in regulatory regions of a network of genes encoding drug metabolizing enzymes such as cytochrome P-450A1A, glutathione S-transferase Ya, and quinone oxi-}

**The Immunophilin-like Protein XAP2 Regulates Ubiquitination and Subcellular Localization of the Dioxin Receptor**

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The abbreviations used are: hsp, heat shock protein; TCDD, 2,3,7,8 tetrachlorodibenzo-p-dioxin; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; NLS, nuclear localization signal; TPR, tetrapetide repeat.
erase chain reaction using the pGEMXAP2 vector as a template (primers: 5’-CGAACGTTATGGGATATCGGCAAAG-3’ and 5’-GGCTAATGTTGGAAGATCC-3’). Following digestion with HindIII and NheI, the polymerase chain reaction product was inserted into HindIII-XhoI-digested pCMV2/FLAG (Kodak) in-frame with the FLAG epitope. The GST fusion receptor expression vector encoding pBC/DR was kindly provided by Pilar Carrero (Karolinska Institutet, Sweden). The hemagglutinin (HA)-tagged ubiquitin expression plasmid, pMT123, was kindly provided by Dirk Bohmann (European Molecular Biology Laboratory, Germany).

**Protein Expression and Immunoprecipitation—** *In vitro* translation of the dioxin receptor was performed using coupled transcription/translation reactions in rabbit reticulocyte lysate (Promega Biotech) according to the manufacturer’s recommendations. Immunoprecipitation of *in vitro* translated proteins using anti-p23 (JJS) (generously provided by David O. Toft (Mayo Clinic, Rochester, MN), anti-ha90 3G3 (Affinity Bioreagents, Inc.), or anti-dioxin receptor (10) antibodies were performed as described earlier (4). The ATP-regeneration system used in some experiments consisted of 5 mM ATP (Sigma), 50 units/ml creatine phosphokinase (Sigma), and 15 mM phosphocreatinine (Sigma).

**Visualization of Intracellular Localization of GFP-tagged Proteins in Living Cells—** HeLa and COS7 cells were routinely propagated in Dulbecco’s minimum essential medium supplemented with 10% fetal calf serum, 10% horse serum, 1-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 units/ml) at 37 °C at 5% CO2. For intracellular localization assays, cells were grown on 20 × 20 mm glass coverslips in 30-mm diameter dishes. Transient transfections were performed by introducing 0.5 μg of plasmids encoding green fluorescent protein (GFP)-fused dioxin receptor, various receptor chimeras, and/or XAP2 into cells by using LipofectAMINE (Life Technologies, Inc.) according to manufacturer’s recommendations. The total concentrations of DNA were kept constant by compensation with empty expression vectors. After transfection, cells were grown in Dulbecco’s modified essential medium for 24–30 h, and the cells were treated with TCDD (Chensyn, Lenexa, KS), geldanamycin (Life Technologies, Inc.), or combinations thereof, as detailed in the figure legends. The intracellular localization of GFP fusion proteins was monitored using a Nikon LABOPHOT microscope equipped with a fluorescein isothiocyanate-filter set and a camera. Quantitative evaluations of green fluorescent cells were performed as described (22, 23). Briefly, the cells were classified into four categories according to the intracellular localization of GFP fusion proteins: C > N, for predominantly cytoplasmic fluorescence; C = N, when fluorescing proteins were equally distributed both in the cell cytoplasm and nucleus; N > C, for nuclear dominant fluorescence, and N, for exclusive nuclear fluorescence. On average, 200 fluorescing cells were routinely evaluated on each coverslip.

**Cellular Extracts and Immunoblot Assays—** For immunoblot assays, COS7 cells were grown in 10-cm diameter dishes. GST-tagged dioxin receptor (4 μg), HA-tagged ubiquitin (4 μg), and various amounts of FLAG-XAP2 (2–8 μg) were transiently expressed by using LipofectAMINE. Cells were treated with MG132 (Calbiochem), TCDD, geldanamycin, or combinations, as indicated in the figure legends. To prepare whole cell extracts, cells were washed twice with cold phosphate-buffered saline, collected by centrifugation, and lysed for 20 min on ice in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor cocktail (“Complete-Mini,” Roche Molecular Biochemicals), 25 μM MG132, 1 μM dithiothreitol, and 20 mM N-ethylmaleimide (Sigma). Lysates were cleared by centrifugation for 30 min at 14,000 rpm at 4 °C. 600–800 μg of total cellular protein was incubated with anti-GST (Amersham Pharmacia Biotech) antibodies at 4 °C overnight. Immunocomplexes were precipitated by adding 40 μl of a 50% slurry of Protein A-Sepharose (Amersham Pharmacia Biotech) followed by incubation at 4 °C under slow rotation. After rapid centrifugation, the resulting pellets were washed four times with 1 ml of cold RIPA buffer. Precipitated proteins were analyzed by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Immobilized proteins were incubated overnight at 4 °C with mouse anti-HA (F-7) antibodies (Santa Cruz Biotech), diluted 1:500 in blocking solution (5% of nonfat milk in phosphate-buffered saline). Horseradish peroxidase-conjugated anti-mouse antiserum (Amersham Pharmacia Biotech) was used as a secondary antibody diluted 1:500 in blocking solution. After extensive washing in phosphate-buffered saline, 2% Tween 20, the dioxin receptor-ubiquitin conjugates were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) according to the manufacturer’s recommendations. To analyze total GST-dioxin receptor and FLAG-XAP2 protein levels in cells, 25 μg of whole cell extract protein was used for immunoblot assays. Rabbit polyclonal anti-dioxin receptor antibodies (Biomol) diluted 1:500 were used to detect the dioxin receptor fusion proteins, and mouse monoclonal anti-FLAG antibodies (Sigma) diluted 1:1000 were used to detect FLAG-tagged XAP2.

**RESULTS**

**XAP2 Inhibits Ubiquitination of the Dioxin Receptor—** Ligand-dependent activation of the dioxin receptor markedly enhances the turnover of the receptor protein by increasing dioxin receptor ubiquitination (24–26). Given the background that XAP2 has been reported to stabilize dioxin receptor protein levels (16, 18), we examined whether XAP2 interferes with the ubiquitination process of the dioxin receptor. In these experiments, COS7 cells were transiently co-transfected with expression vectors encoding GST-tagged dioxin receptor, HA-tagged ubiquitin, or FLAG-tagged XAP2 or with FLAG peptide alone. The blot was re-probed using anti-FLAG antibodies to monitor XAP2 expression levels (lower panel). B, dioxin receptor ubiquitination assay. 0.8 μg of WCE protein was used in immunoprecipitation experiments using anti-GST antibodies. The dioxin receptor-ubiquitin conjugates were detected by anti-HA immunoblots (top panel). To analyze GST-dioxin receptor protein levels recovered after the immunoprecipitation, the membrane was re-blotted with anti-dioxin receptor antibodies (bottom panel). The positions of molecular weight markers, GST-tagged dioxin receptor (GST-DR), receptor-ubiquitin conjugates (GST-DR-HA-Ub), FLAG-XAP2, and immunoglobulins (Ig) are indicated.

![Fig. 1. XAP2 inhibits ubiquitination of the dioxin receptor.](image-url)
Fig. 2. Nuclear accumulation of the ligand-activated dioxin receptor is delayed by XAP2. A, 0.5 mg of pCMX/DR-DFP was introduced into HeLa cells by transient transfection together with 0.5 mg of pSG5/XAP2 or the same amount of carrier vector (pSG5) alone. Cells were treated with 10 nM TCDD or the vehicle Me2SO alone for 2 h. Intracellular localization of the GFP-dioxin receptor was examined by fluorescent microscopy. Representative images of the receptor-GFP expressing cells are shown following the indicated different treatments. B, quantitative analysis of compartmentalization of the GFP-dioxin receptor fusion protein. GFP-dioxin receptor and XAP2 were transiently expressed as in A, and cells were incubated with ligand for increasing periods of time up to 8 h, as indicated. Dioxin receptor-GFP expressing cells were classified into four categories as described under “Materials and Methods.” One representative experiment was used to illustrate the dynamics of nuclear accumulation of the GFP-dioxin receptor, shown as percentage of cells belonging to the categories C > N, C = N, C < N, and N, respectively.


detection of an increase in high molecular weight GST-dioxin receptor-ubiquitin conjugates. Strikingly, co-expression of XAP2 markedly reduced in a concentration-dependent manner the levels of ubiquitinated GST-dioxin receptor complexes both in the absence or presence of ligand (Fig. 1B, upper panel, lanes 1–8). In excellent agreement with our observations using unfractionated whole cell extracts (Fig. 1A), we detected an increase in dioxin receptor protein levels when we re-analyzed the precipitated material with anti-dioxin receptor antibodies (Fig. 1B, lower panel, lanes 1–8). Under very similar experimental conditions, we also observed XAP2-dependent protection against ubiquitination of GAL4-dioxin receptor fusion proteins (data not shown). Interestingly, in the absence of XAP2, ligand-dependent ubiquitination of the GAL4-dioxin receptor was more prominent as compared with ubiquitination of the GST-dioxin receptor fusion protein, indicating that the GST moiety by itself may partially stabilize the receptor. In conclusion, these experiments strongly suggest that XAP2 protects the dioxin receptor against ubiquitination, resulting in stabilized dioxin receptor levels both in the presence or absence of ligand.

Ligand-dependent Nuclear Accumulation of the Dioxin Receptor is Delayed by XAP2—Although our results indicate that XAP2 protects both the non-activated and ligand-activated dioxin receptor forms against ubiquitination, it has been reported that XAP2 is not associated with the ligand-activated dioxin receptor-Arnt complex (5–7). This complex, in turn, is targeted for degradation by ligand-dependent ubiquitination (24, 26). These data suggest that XAP2 may be involved in regulation of additional steps of the ligand-dependent dioxin receptor activation pathway. For instance, it is plausible that the XAP2-dependent decrease in dioxin receptor ubiquitination levels may be caused by a stabilization of the non-activated form of the receptor. We have earlier observed that the hsp90 chaperone complex is an important determinant of the subcellular compartmentalization of the dioxin receptor.2 We therefore examined the effect of XAP2 on ligand-dependent nuclear accumulation of the dioxin receptor. To this end, the intracellular localization of the dioxin receptor was monitored by fluorescence microscopy upon transfection of HeLa cells with GFP-dioxin receptor constructs. For quantitative purposes, about 200 green fluorescent cells were routinely classified into four categories: C > N, predominantly cytoplasmic fluorescence; C = N, equal distribution in both the cytoplasm and nucleus; C < N, predominantly nuclear fluorescence; and N, exclusively nuclear localization (22, 23). Representative images of green fluorescent cells and the statistical evaluation of the subcellular distribution of GFP-dioxin receptor are presented in Fig. 2, A and B, respectively. In untreated cells, the GFP-dioxin receptor fusion protein was homogeneously distributed in both the cytoplasmic and nuclear compartments of the cell. Upon treatment of cells with 10 nM TCDD for 2 h, we observed a very distinct accumulation of the receptor within the cell nucleus with very low or no detectable levels of cytoplasmic fluorescence by the GFP-dioxin receptor (Fig. 2A). Remarkably, co-expression of XAP2 resulted in a marked redistribution of the compartmentalization of the dioxin receptor. In the absence of ligand, the majority of the transfected cells (>90%) showed an exclusively cytoplasmic localization of the GFP-dioxin receptor (Fig. 2A). Moreover, XAP2 produced a similar redistribution

2 A. Kazlauskas, L. Poellinger, and J. Pongratz, manuscript in preparation.
pattern of GFP-dioxin receptor in COS7 cells (data not shown), indicating that this effect is not cell type- specific. Consistent with these observations, the XAP2 homologue ARA9 has recently been reported to induce a similar cytoplasmic re-distribution of dioxin receptor in COS1 cells, as assessed by immunocytochemical techniques (18).

Upon ligand treatment of HeLa cells transiently expressing both XAP2 and GFP-dioxin receptor, we observed a very pronounced delay in nuclear accumulation of the receptor (Fig. 2B, compare upper and lower panels). In control cells (i.e. in the absence of overexpressed XAP2), ligand treatment induced a rapid accumulation of the GFP-dioxin receptor in the nucleus, inducing predominating nuclear (category C < N) or exclusively nuclear (category N) localization of the receptor in the majority (60 to 80%) of the cells within 1–2 h of treatment (Fig. 2, A and B, upper panel). In contrast, in the presence of coexpressed XAP2, nuclear accumulation of the dioxin receptor was significantly delayed, yielding in the majority of cells an equally cytoplasmic and nuclear distribution pattern (category C5N) of the GFP-dioxin receptor even after prolonged (8 h) ligand treatment (Fig. 2B, lower panel). In this context it was interesting to note that, in the absence of overexpressed XAP2, we observed a gradual decrease in the fluorescence of the GFP-dioxin receptor in the nucleus. Thus, in cells treated with TCDD for longer time periods than 8 h we were able to detect only a few weakly green fluorescing cells, all of them displaying an exclusively nuclear localization of the receptor. However, in the presence of transiently expressed XAP2, we did not observe any decrease in fluorescence of the dioxin receptor, even if the cells were treated for up to 12 h with ligand (data not shown). These results are in excellent agreement with the model that ligand treatment enhances the turnover of the dioxin receptor, and that XAP2 stabilizes dioxin receptor protein levels. Taken together, these data indicate that XAP2 mediates in living cells cytoplasmic retention of the dioxin, resulting in a significant delay in nuclear accumulation of the ligand-activated form of receptor.

XAP2-induced Cytoplasmic Redistribution of the Dioxin Receptor Is Mediated by the PAS Domain of the Receptor—As schematically outlined in Fig. 3A, the dioxin receptor contains an N-terminal nuclear localization signal (NLS) within the DNA-binding bHLH domain, whereas the C-terminal half of the PAS domain (spanning the PAS-B motif) harbors the minimal ligand and hsp90-binding domain. It has recently been shown that the association of XAP2 with the dioxin receptor requires both the PAS-B motif and the region between the PAS-A and PAS-B motifs (16). To identify the functional motifs of the dioxin receptor mediating XAP2-dependent cytoplasmic retention, we used GFP fusion proteins spanning various deletion mutants of the dioxin receptor. GRDBD/DR 83–805-GFP (A), GRDBD/DR 422–805-GFP (B), and GFP-DRΔ287–421 (C) fusion proteins (schematically represented together with full-length dioxin receptor in the top of each panel) were transiently expressed in HeLa cells in the absence or presence of XAP2 as described in the legend of Fig. 2. GRDBD/DR 83–805-GFP fusion protein-expressing cells were incubated in the absence or presence of 10 nM TCDD for 2 h. The intracellular localization of GFP fusion proteins was monitored by fluorescent microscopy as described above. Representative images of green fluorescing cells are shown (middle panels). Categorization and quantitative evaluation of the intracellular localization of the GFP-fused proteins were performed as described in the legend of Fig. 2 (bottom graphs). In panel B, nearly 100% of the GRDBD/DR 422–805-GFP fusion protein expressing cells displayed an exclusively nuclear localization pattern which was observed both in the absence and presence of XAP2. DR, dioxin receptor; GR, glucocorticoid receptor; Zn-F-DBD, zinc finger DNA-binding domain; NLS, nuclear localization signal. DMSO, Me2SO.
ceptor, the GRDBD/DR 83–805-GFP chimeric construct was evenly distributed in both the cell cytoplasm and nucleus when expressed in HeLa cells. Upon exposure to ligand, GRDBD/DR 83–805-GFP was efficiently accumulated in the nucleus with a nuclear translocation rate similar to that of the wild-type dioxin receptor, resulting in bright nuclear fluorescence in about 80% of the analyzed cells. In the presence of co-expressed XAP2, the ligand-free form of GRDBD/DR 83–805-GFP showed exclusively cytoplasmic localization. Moreover, ligand-dependent nuclear translocation of this protein was significantly delayed with <5% of cells showing predominantly nuclear localization of the protein (Fig. 3A). Thus, these results demonstrate that the bHLH domain and the endogenous NLS motif of the dioxin receptor were not required to mediate XAP2-dependent cytoplasmic retention.

Next we analyzed the nuclear translocation properties of a GFP fusion protein, GRDBD/DR 422–805-GFP, spanning the same region of the glucocorticoid receptor as above fused to the C-terminal half of the dioxin receptor lacking both the bHLH and PAS domains (Fig. 3B). This fusion protein displayed an exclusively nuclear localization pattern in both control and XAP2-overexpressing cells, demonstrating that the C-terminal region of the dioxin receptor is resistant to cytoplasmic retention by XAP2 (Fig. 3F). Finally, the fusion protein GFP-DR Δ287–421 lacking the PAS-B motif of the dioxin receptor (Fig. 3C) was localized mainly in the nuclear compartment of the cell, resulting in about 70% and 25% of GFP-DR Δ287–421 expressing cells falling into categories N and C < N, respectively. Consistent with the deletion of the ligand-binding domain compartmentalization of this protein was not altered by ligand. It was also not affected by coexpression of XAP2 (Fig. 3C). In conclusion, these data demonstrate that an intact PAS-B domain of the dioxin receptor is required for XAP2-dependent regulation of the intracellular localization of the receptor.

Association of XAP2 with the Dioxin Receptor Depends on the Functional Status of Hsp90—XAP2 has been shown to interact with the non-activated form of the dioxin receptor (16). In addition, we and others have observed that the non-activated form of the dioxin receptor interacts with an additional hsp90-associated factor, p23 (3, 4). It is unclear, however, whether XAP2 can function in a hsp90-independent manner or requires an intact hsp90 complex to regulate dioxin receptor function. The observations that a significant fraction of the ligand-free form of GRDBD/DR 83–805-GFP containing a potent NLS signal of the glucocorticoid receptor was retained in the cytoplasmic compartment of the cell and that deletion of the hsp90-interacting PAS-B domain results in a predominantly nuclear localization of the dioxin receptor suggest the involvement of hsp90 in regulation of the intracellular trafficking of the receptor. Moreover we have recently observed that the integrity of the hsp90-p23 complex is an important determinant of the ligand-dependent nuclear translocation process of the dioxin receptor.

Against this background we examined the importance of the functional status of hsp90 in dioxin receptor-XAP2 complex formation. To this end we used the ansamycin antibiotic geldanamycin which is known to specifically bind the ATP-binding pocket of hsp90 (28) and thereby inhibit the conversion of hsp90 from the ADP- to the ATP-bound state. This effect also correlates with the inhibition of the recruitment of p23 to the hsp90 complex (29).

As schematically outlined in Fig. 4, we assembled in parallel two reaction mixtures. In one of these, in vitro translated, [35S]methionine-labeled XAP2 was incubated with in vitro translated dioxin receptor (where about 10% of the dioxin receptor was [35S]methionine-labeled in order to track the presence of the receptor in subsequent immunocomplexes). In the second control reaction mixture an identical amount of [35S]methionine-labeled XAP2 was incubated together with unprogrammed reticulocyte lysate (mock translation). Following incubation for 1 h at 25 °C, equal aliquots of the reaction mixtures were subsequently transferred to a concentrated reticulocyte lysate (3 volumes) supplemented with an ATP regeneration system and incubated in the presence of 25 μg/ml geldanamycin (GA) or vehicle (DMSO) alone for 30 min at 30 °C. Equal amounts of the reaction mixtures were used for immunoprecipitation experiments with anti-hsp90 (α-hsp90), anti-p23 (α-p23), anti-dioxin receptor (α-DR), and control (C) antibodies. Immunocomplexes were precipitated with Protein A-Sepharose and subsequently analyzed by 12% SDS-PAGE. To estimate relative amounts of the dioxin receptor and XAP2 protein levels prior to precipitation, an aliquot from each reaction mixture was directly analyzed by SDS-PAGE (Inputs; lanes 16–19). The positions of molecular weight markers, the dioxin receptor (DR) and XAP2 are indicated.
cifically co-precipitated in a complex with p23 only in the presence of the dioxin receptor (Fig. 4, lanes 6, 7 and 9). However, no anti-p23 antibody-precipitated material was detected in geldanamycin-treated samples (Fig. 4, lanes 8 and 10), consistent with the model that geldanamycin induced release of p23 from the dioxin receptor. Similar levels of the dioxin receptor were specifically recovered using anti-dioxin receptor antibodies (Fig. 4, lanes 11–15). In agreement with the anti-hsp90 immunoprecipitation result, XAP2-dioxin receptor interaction was disrupted by geldanamycin (Fig. 4, compare lanes 12–13).

These results indicate that p23 and XAP2 can co-exist within the same multiprotein complex and that the integrity of this complex is determined by the functional status of the molecular chaperone hsp90.

Geldanamycin Inhibits XAP2-mediated Cytoplasmic Redistribution of the Dioxin Receptor—Given the observation that geldanamycin induced release of XAP2 from the dioxin receptor complex in vitro, we examined if treatment of cells with geldanamycin would affect XAP2-dependent cytoplasmic redistribution of the dioxin receptor. We transiently transfected HeLa cells with wild-type dioxin receptor-GFP together with XAP2 or an empty expression vector. The cells were treated with 0.1 μg/ml geldanamycin or vehicle (Me2SO) alone in the presence or absence of 10 nM TCDD for 1 h. Subcellular localization of the receptor was analyzed by fluorescence microscopy. In the absence of XAP2, geldanamycin treatment did not change the cellular distribution pattern of the ligand-free dioxin receptor, as compared with untreated cells. However, no ligand-induced nuclear accumulation of the dioxin receptor-GFP was observed in the presence of TCDD and geldanamycin (Fig. 5A). Interestingly, XAP2-dependent cytoplasmic redistribution of the dioxin receptor was also impaired by geldanamycin, resulting in a clear shift in compartmentalization of the receptor from a predominantly cytoplasmic one (category C > N in about 90% of the cells; Fig. 5B) to a more equal distribution of the receptor (category C = N in about 70% of the cells). Similar results were obtained using cells exposed to both geldanamycin and TCDD. Importantly, although prolonged geldanamycin treatment has been shown to decrease the protein levels of the dioxin receptor (30), no detectable changes in total fluorescence intensity by the dioxin receptor-GFP and cell morphology were observed when we incubated the cells with the relatively low dose of geldanamycin for 1 h. Taken together, these results strongly suggest that XAP2-mediated cytoplasmic accumulation of the dioxin receptor is mediated by an hsp90-dependent mechanism.

XAP2-mediated Protein Stabilization of the Dioxin Receptor Is Inhibited by Geldanamycin—Since geldanamycin inhibited XAP2-dependent cytoplasmic accumulation of the dioxin receptor we next examined the effect of geldanamycin on XAP2-dependent protection of the dioxin receptor against ubiquitination. To this end we used the in vivo ubiquitination assay.
pretreated with the proteasome inhibitor MG132 (7.5 μM) in the absence or presence of XAP2 as in the legend of Fig. 1. Cells were transfected with GST-dioxin receptor (GST-DR) and HA-ubiquitin (HA-Ub) in the upper panel. Cells were transfected with GST-dioxin receptor and HA-ubiquitin in the presence of geldanamycin. These results indicate that the interaction of XAP2 with the dioxin receptor is dependent on the functional state of hsp90 within the dioxin-receptor complex.

In immunoprecipitation experiments we recovered XAP2 in a complex together with the dioxin receptor, hsp90 and p23. The non-activated form of the dioxin receptor shows in several aspects similarities to non-activated forms of steroid hormone receptors. For instance, the non-activated forms of the glucocorticoid or progesterone receptors are known to form a multiprotein complex consisting of hsp90, p23, and one of the tetratricopeptide repeat (TPR) (31) motif-containing immunophilin class proteins such as FKBP52 and CyP-40 (32). Interestingly, XAP2 shares strong homology regions with FKBP52 (5–7). In this context it is noteworthy that the C-terminal part of the XAP2 which encompasses three TPR motifs appears to be required to interact with the C-terminal part of hsp90 (16). In a similar fashion, the TPR motifs of FKBP52 have been demonstrated to be important for interaction with hsp90 (33) via the conserved MEEVD motif of hsp90 (34). In fact, the MEEVD motif seems to represent a common binding site for TPR proteins. In addition to FKBP52, TPR proteins such as FKBP51, CyP-40, p60 (HOP), and PP5 all target this motif to interact with hsp90 (34, 35).

Here we have observed that XAP2 has the ability to retain the non-activated dioxin receptor in the cytoplasmic compartment of the cell by a mechanism that is mediated by the PAS domain of the dioxin receptor. A similar cytoplasmic re-distribution pattern of dioxin receptor was recently observed by immunocytochemical assays in COS-1 cells following coexpression of the dioxin receptor with the XAP2 homologue ARA9 (18). Although the mechanism of this mode of regulation remains unclear, it is plausible that the association of the dioxin receptor with components of the nuclear import machinery described above. GST-dioxin receptor and HA-tagged ubiquitin were transiently expressed in COS7 cells together with or without XAP2. The cells were treated with the proteasome inhibitor MG132 alone for 2 h followed by incubation for 4 h with MG132 in the presence of 0.5 μg/ml geldanamycin or vehicle (Me2SO) alone. Immunoblot analysis shows that the levels of XAP2 were not affected by geldanamycin (Fig. 6, lower panel), whereas, as expected (30), dioxin receptor levels were reduced in whole cell extracts prepared from geldanamycin-treated cells (Fig. 6, lower panel). Ubiquitin conjugates of the GST-dioxin receptor were monitored by anti-HA immunoblot analysis of anti-GST immunoprecipitated material. Although geldanamycin treatment induced a pronounced decrease in dioxin receptor protein levels, we observed no corresponding increase in ubiquitinated dioxin receptor complexes (Fig. 6, upper panel), indicating that the effect of geldanamycin in the presence of the proteasome inhibitor may be to shunt the dioxin receptor into degradation by another pathway. Interestingly, geldanamycin induced down-regulation of dioxin receptor protein levels also in the presence of XAP2, although XAP2 protected the dioxin receptor against ubiquitination both in the absence or presence of geldanamycin (Fig. 6, upper panel). Taken together these data indicate that ligand and geldanamycin induce dioxin receptor degradation by two distinct pathways, and that XAP2-dependent protection of the dioxin receptor against ubiquitination does not stabilize the dioxin receptor in the presence of geldanamycin.

**DISCUSSION**

In the present study we demonstrate that the hsp90-associated immunophilin homolog XAP2 modulates dioxin receptor function by two distinct modes of regulation: (i) it protects the dioxin receptor against proteasome-mediated degradation by inhibition of receptor ubiquitination; and (ii) it has a pronounced effect on cytoplasmic redistribution of the dioxin receptor both in the absence and presence of ligand. Whereas XAP2 induces an exclusively cytoplasmic localization of the ligand-free dioxin receptor, it significantly delayed nuclear translocation of the ligand-activated receptor form. Thus, variations in XAP2 protein levels in various tissues or in various physiological situations may constitute an important level of regulation of the kinetics of activation of the dioxin receptor, and thus an important modulator of receptor-mediated signal transduction.

Interestingly, XAP2 is associated with hsp90 and the dioxin receptor in a complex with p23. In studies on steroid hormone receptors complex formation between the receptor, hsp90, and p23 has been interpreted as an indication that the hsp90-receptor complex has reached a late or the last step in a maturation process (32). In steroid hormone receptors, the ATP-bound state of hsp90 is required to acquire a high affinity ligand-binding conformation of the hsp90-bound receptors (29). In this complex the role of p23 appears to be stabilization of the interaction between hsp90 and target proteins. It is conceivable that p23 has a similar function within the dioxin receptor-hsp90 complex. The hsp90-dependent maturation process of steroid hormone receptors can be inhibited by geldanamycin (36–38). Geldanamycin is widely used in studies on hsp90-dependent chaperoning of different proteins and is known to specifically bind the ATP-binding pocket of hsp90 (28) and thereby inhibit the conversion of hsp90 from the ADP-bound to the ATP-bound state during the ATP-dependent maturation process (29, 39). Our experiments show that the association of both p23 and XAP2 with the dioxin receptor-hsp90 complex is inhibited by geldanamycin. These results indicate that the interaction of XAP2 with the dioxin receptor is dependent on the functional state of hsp90 within the dioxin-receptor complex.

In immunoprecipitation experiments we recovered XAP2 in a complex together with the dioxin receptor, hsp90 and p23. The non-activated form of the dioxin receptor shows in several aspects similarities to non-activated forms of steroid hormone receptors. For instance, the non-activated forms of the glucocorticoid or progesterone receptors are known to form a multiprotein complex consisting of hsp90, p23, and one of the tetratricopeptide repeat (TPR) (31) motif-containing immunophilin class proteins such as FKBP52 and CyP-40 (32). Interestingly, XAP2 shares strong homology regions with FKBP52 (5–7). In this context it is noteworthy that the C-terminal part of the XAP2 which encompasses three TPR motifs appears to be required to interact with the C-terminal part of hsp90 (16). In a similar fashion, the TPR motifs of FKBP52 have been demonstrated to be important for interaction with hsp90 (33) via the conserved MEEVD motif of hsp90 (34). In fact, the MEEVD motif seems to represent a common binding site for TPR proteins. In addition to FKBP52, TPR proteins such as FKBP51, CyP-40, p60 (HOP), and PP5 all target this motif to interact with hsp90 (34, 35).

Here we have observed that XAP2 has the ability to retain the non-activated dioxin receptor in the cytoplasmic compartment of the cell by a mechanism that is mediated by the PAS domain of the dioxin receptor. A similar cytoplasmic re-distribution pattern of dioxin receptor was recently observed by immunocytochemical assays in COS-1 cells following coexpression of the dioxin receptor with the XAP2 homologue ARA9 (18). Although the mechanism of this mode of regulation remains unclear, it is plausible that the association of the dioxin receptor with components of the nuclear import machinery...
such as importins (40) may be directly or indirectly inhibited by XAP2. Interestingly, the XAP2-dependent cytoplasmic retention effect was abolished in cells treated with geldanamycin, resulting in the re-distribution of the receptor from the cytoplasm to a pattern reflecting equal distribution in both the cytoplasmic and nuclear compartments. Taken together these observations suggest that XAP2-induced cytoplasmic retention of the receptor is mediated via its association with hsps.

In addition to the effect of XAP2 on intracellular compartmentalization of the receptor, it has been shown that XAP2, as well as its homologue ARA9, has the ability to stabilize the non-activated dioxin receptor protein, resulting in both elevated protein levels of the receptor and an increase of ligand-binding sites (16, 18).

Consistent with this model, we demonstrate that XAP2 prevents ubiquitination of the non-activated dioxin receptor form. Moreover, ligand-dependent ubiquitination was also inhibited by increasing amounts of the XAP2.

It has been reported that XAP2 dissociates from the ligand-activated dioxin receptor-Arnt complex (5–7). This suggests that the XAP2-dependent inhibitory effect on ligand-dependent ubiquitination of the dioxin receptor might be the result of XAP2-dependent inhibition of dioxin receptor ubiquitination prior to ligand-dependent activation and dimer formation between the dioxin receptor and Arnt. With regard to the failure of XAP2 to protect the dioxin receptor against geldanamycin-induced degradation, the disruption of the p23-hsp90-XAP2 complex by geldanamycin may induce a malfolded configuration of the receptor which presents the dioxin receptor to an alternative pathway of degradation. In conclusion, XAP2 modulates signal transduction by the dioxin receptor by two distinct modes of regulation: (i) cytoplasmic retention of the receptor, and (ii) stabilization of receptor protein levels by impairing the ubiquitin-dependent turnover of the receptor. Importantly, both these modes of regulation require the integrity of the XAP2-hsp90-p23-dioxin receptor complex, illustrating the mechanistic complexity of the hsp90 chaperone machinery.

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