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Protein–protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor–Arnt transcription factor complex

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Gene regulation by dioxins is mediated by the dioxin receptor–Arnt heterodimer, a ligand generated complex of two basic helix–loop–helix (bHLH)/Per–Arnt–Sim (PAS) transcription factors. By using dioxin receptor chimeras where the dimerization and DNA binding bHLH motif has been replaced by a heterologous DNA binding domain, we have detected an ability of Arnt to interact with the dioxin receptor via the PAS domain in a mammalian 'hybrid interaction' system. By co-immunoprecipitation assays, we have confirmed the ability of PAS domains of the dioxin receptor and Arnt to mediate independent heterodimerization *in vitro*. Selectivity for PAS dimerization was noted in our hybrid interaction system, as dioxin receptor or Arnt PAS-mediated homodimers were not detected. Surprisingly, however, the PAS domain of Per could dimerize with both the dioxin receptor and Arnt subunits *in vitro*, and disrupt the ability of these subunits to form a DNA binding heterodimer. Moreover, ectopic expression of Per blocked dioxin signalling in mammalian cells. The PAS domains of the dioxin receptor and Arnt are therefore novel dimerizing regions critical in formation of a functional dioxin receptor–Arnt complex, while the PerPAS domain is a potential negative regulator of bHLH/PAS factor function.

Key words: bHLH factors/PAS domain/protein dimerization/repression/transcriptional regulation

Introduction

Signal transduction by dioxins and related polycyclic aromatic hydrocarbons is mediated by the dioxin receptor (also termed the aryl hydrocarbon receptor), a ligand-activated transcription factor known to enhance expression of gene products involved in xenobiotic metabolism. The dioxin receptor is held in a latent complex with the molecular chaperone hsp90 (Denis *et al.*, 1988; Perdew, 1988; Wilhelmsson *et al.*, 1990; Chen and Perdew, 1994), which is subsequently disrupted during ligand-induced activation. The partially characterized activation process results in transformation of the dioxin receptor to a heterodimeric DNA binding species with a specific partner protein, Arnt (Hoffman *et al.*, 1991; for recent reviews, see Swanson and Bradfield, 1993; Whitlock, 1994). Immunocytochemical studies have revealed Arnt to be a nuclear protein (Hord and Perdew, 1994; Pollenz *et al.*, 1994), while in hepatoma cells the dioxin receptor is reported to translocate from cytoplasm to nucleus in response to ligand (Pollenz *et al.*, 1994). It is presently unclear at which point in the transformation process that the hsp90–dioxin receptor complex is disrupted, although *in vitro* studies have shown that Arnt or an Arnt-like factor can play an active role in ligand-induced release of hsp90 (McGuire *et al.*, 1994), suggesting that this event may occur in the nucleus. Hsp90 is an intrinsic component of the dioxin receptor ligand signalling mechanism, as demonstrated by experiments in a genetically manipulated strain of *Saccharomyces cerevisiae*, where ligand responsiveness of dioxin receptor chimeras has been abrogated in depleted hsp90 environments (Carver *et al.*, 1994; Whitelaw *et al.*, 1995).

The activated dioxin receptor–Arnt heterodimer recognizes a specific enhancer element, the XRE (xenobiotic response element), present in upstream regions of dioxin-regulated genes such as cytochrome P450IA1 and glutathione S transferase Ya (Fujisawa-Sehara *et al.*, 1987; Paulson *et al.*, 1990). XRE sequences have been shown to function as dioxin-responsive enhancers in heterologous reporter genes (Gradin *et al.*, 1993; Whitelaw *et al.*, 1993b and references therein), and studies designed to investigate the transcription activating capabilities of the dioxin receptor and Arnt subunits have revealed transactivation domains to reside in the C-terminus of each protein (Jain *et al.*, 1994; Li *et al.*, 1994; Whitelaw *et al.*, 1994). Furthermore, dioxin signalling through the activated receptor–Arnt complex may be dependent on phosphorylation status, as modulation of protein kinase activities has proven to influence dioxin-induced XRE binding activities and responsiveness of reporter genes (Carrier *et al.*, 1992; Okino *et al.*, 1992; Berghard *et al.*, 1993; Gradin *et al.*, 1994).

The dioxin receptor and Arnt belong to the basic helix–loop–helix (bHLH) family of transcription factors (Hoffman *et al.*, 1991; Burbach *et al.*, 1992; Ema *et al.*, 1992), a class of key modulators for cellular processes such as determination of cell lineages and oncogenic cell growth. A common theme for this class of proteins is the formation of heterodimers to allow binding on E box (CANNTG) enhancer sequences, thereby affecting regulation of promoter activity (for a recent review, see Kadesh, 1993). The involvement of MyoD/E2-A and aschaete–scute complex/daughterless gene products in myogenic and neurogenic programmes, respectively, are well characterized examples of bHLH proteins initiating cell differentiation (for a review, see Jan and Jan, 1993), while heterodimeric Myc/Max and TAL1/E47 are implicated oncogenic bHLH complexes (Begley *et al.*, 1989; Amati *et al.*, 1993; Hsu *et al.*, 1994). Crystal structure determinations for E box-bound homodimers of the bHLH regions

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of Max, USF, MyoD and E47 have shown general similarities in the basic region amino acids that make contact with DNA, while the helix–loop–helix segments form the dimerization interface (for a review, see Wolberger, 1994). The dioxin receptor and Arnt belong to a subclass of bHLH proteins which contain PAS domains (Per–Arnt–Sim homology regions) juxtaposed to their bHLH regions. PAS domains are common to the bHLH *Drosophila* factor Sim, essential for development of midline cells in the embryonic central nervous system (Nambu *et al.*, 1991), and the *Drosophila* factor Per, a component of the circadian pacemaker (reviewed by Takahashi, 1992). These PAS domains show amino acid similarities over 250–300 residues, and contain two degenerate hydrophobic repeats, designated PAS A and PAS B, of ~50 amino acids each. The PAS domain of Per forms a homodimerization interface (Huang *et al.*, 1993) while, in the case of the dioxin receptor and Arnt, the PAS domain of Arnt has been shown to stabilize HLH-mediated dimerization between these two subunits (Reisz-Porszasz *et al.*, 1994). We have also recently provided evidence that the PAS domain of the dioxin receptor may play a crucial role in site selection and/or affinity of the receptor–Arnt complex for the cognate DNA recognition sequence (Antonsson *et al.*, 1995).

To identify functional domains within the dioxin receptor and Arnt, we previously separated these two subunits of the activated dioxin receptor complex into individually active transcription factors by creating fusion proteins with a heterologous DNA binding domain. Thus, replacement of the N-terminal bHLH domains of the dioxin receptor and Arnt with the zinc finger DNA binding domain of the glucocorticoid receptor provided independent transcription factors which were capable of activating reporter genes containing glucocorticoid response elements (GREs) (Whitelaw *et al.*, 1993a, 1994). Dioxin receptor chimeras based on this design are activated by dioxin in Arnt-deficient mutant hepatoma C4 cells, thus proving that receptor activity can be uncoupled from Arnt (Whitelaw *et al.*, 1993a). Surprisingly, we have now found that dioxin receptor chimeras of this type, although lacking the HLH dimerization motif, display enhanced transactivation properties when co-expressed with Arnt. We show that this potentiation is due to a dimerization with Arnt via the PAS domain, thus creating a mammalian 'hybrid interaction' system, where the strong transactivating domain of Arnt can be recruited to a promoter via protein–protein interaction to enhance activity on a reporter gene. We have used this assay to show that the core ligand and hsp90 binding domain of the dioxin receptor, lying between amino acids 230 and 421, is essential for PAS–PAS interaction between the dioxin receptor and Arnt. Moreover, unlike the PAS domain of Per, which shows homodimerization capacity, the PAS domains of the dioxin receptor and Arnt display no tendency to homodimerize in this system. Intriguingly, in support of the PAS domains having critical functions in the native dioxin receptor–Arnt complex, the ability of this heterodimer to recognize its DNA target sequence *in vitro* was abrogated in the presence of the PerPAS domain. We further show the PerPAS domain to be capable of interaction with both the dioxin receptor and Arnt, revealing the PerPAS region to mediate a broad spectrum of PAS–PAS interactions. Finally, these data indicate that Per has the potential to perform a negative regulatory role with respect to bHLH/PAS factor function.

Results

Activity of a dioxin receptor chimera lacking the bHLH domain is potentiated by Arnt

In a previous strategy to map functional domains within the dioxin receptor, we constructed chimeras between the N-terminal 'zinc finger' DNA binding region of the glucocorticoid receptor and varying C-terminal segments of the dioxin receptor. These fusion proteins lack the very N-terminal bHLH dimerization and DNA binding motif of the dioxin receptor and, when expressed in mammalian cell lines, show dioxin-induced activity on GRE-containing reporter genes (Whitelaw *et al.*, 1993a, 1994). The ability of dioxin receptor chimeras to act as independent transcription factors is exemplified by chimera GRDBD/DR83–805, which contains 90% of dioxin receptor residues (all but the N-terminal bHLH domain, Figure 1). When a GRDBD/DR83–805 expression vector was co-transfected into the Arnt-deficient mutant C4 hepatoma cell line (Hoffman *et al.*, 1991) with a reporter plasmid consisting of the GRE-containing mouse mammary tumour virus (MMTV) promoter upstream of the alkaline phosphatase gene (pMMTV-AF, Whitelaw *et al.*, 1993a), dioxin-induced expression of alkaline phosphatase was observed (Figure 2). Surprisingly, while chimera GRDBD/DR83–805 could be uncoupled from Arnt by virtue of replacement of the bHLH dimerization domain, co-transfection of expression vectors containing native Arnt and GRDBD/DR83–805 resulted in a large enhancement of reporter gene activity (Figure 2). This enhancement was not peculiar to C4 hepatoma cells, as co-expression of Arnt also potentiated GRDBD/DR83–805 activity in CHO cells (Figure 2). Importantly, expression of Arnt alone failed to activate the reporter gene in these cell lines, while co-expression of Arnt with the glucocorticoid receptor portion of the chimera, GRDBD, failed to elevate activity beyond that observed when GRDBD was expressed alone (Figure 2). These results indicate that Arnt was still capable of interaction with this dioxin receptor chimera, despite the chimera being devoid of the HLH dimerization motif, resulting in subsequent formation of a potent transactivation complex.

The PAS domain mediates interaction between the dioxin receptor and Arnt in a mammalian 'hybrid interaction' system

The ability of Arnt to potentiate activity of chimera GRDBD/DR83–805 suggested that Arnt may be able to interact with the dioxin receptor in regions outside the HLH domain. As we have recently discovered Arnt to be a potent transcriptional activator, and chimera GRDBD/DR83–805 to be a weak activator (Whitelaw *et al.*, 1994), the coupling of these two proteins within a cell to give an enhanced transcriptional response is akin to the yeast 'two-hybrid' system commonly used to detect *in vivo* interaction between two proteins. In the yeast two-hybrid system, a DNA binding domain (DBD, e.g. GAL4 or LexA) is fused to a polypeptide for which a dimerization partner is sought. This 'baited' DNA binding fusion

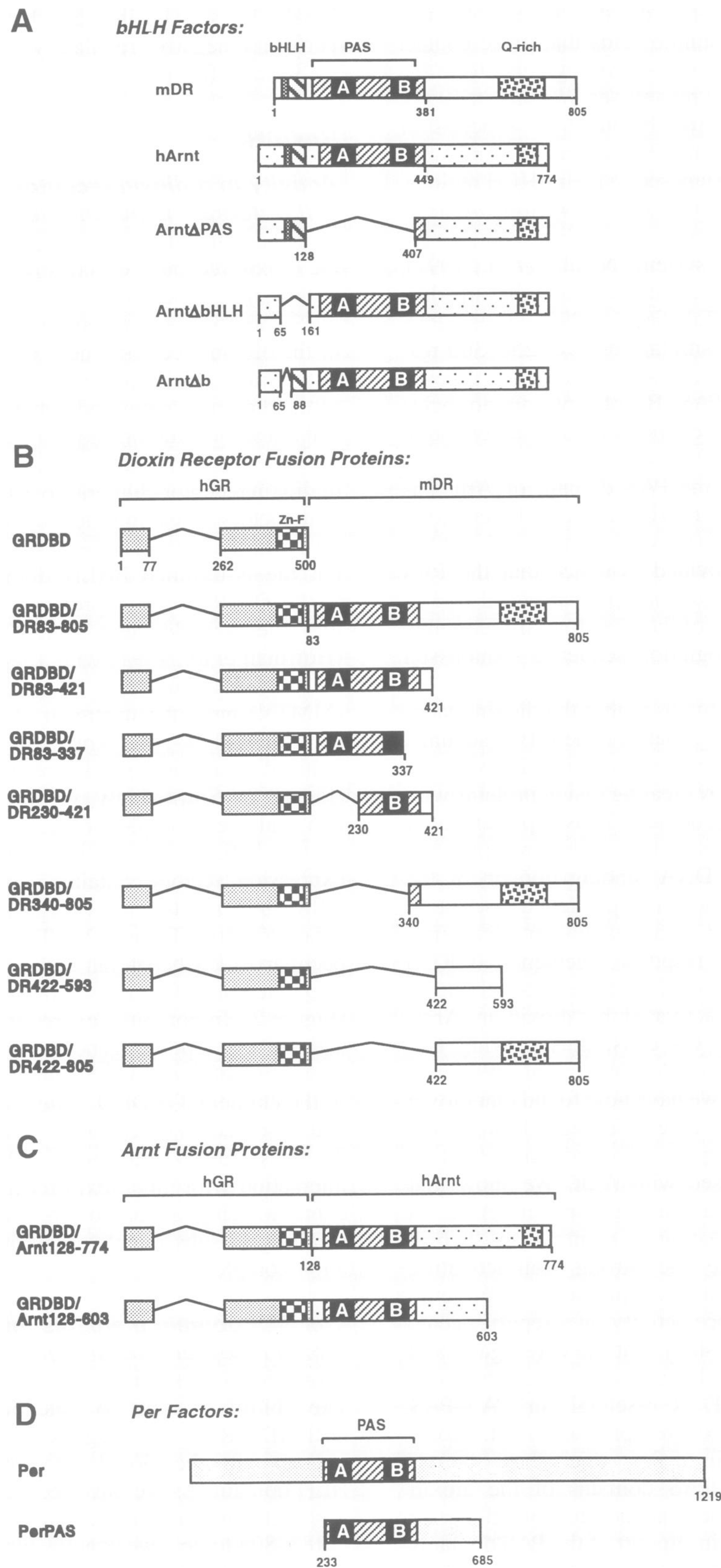


Fig. 1. Schematic representation of motifs within native and mutant derivatives of the dioxin receptor, Arnt, chimeric glucocorticoid receptor/dioxin receptor and glucocorticoid receptor/Arnt fusion proteins, and the *Drosophila* PAS protein Per. (**A**) Structural organization of the mouse dioxin receptor and native and deletion mutant human Arnt, showing bHLH motifs, regions of PAS homology and glutamine rich (Q-rich) segments. (**B**) Organization of human glucocorticoid receptor/mouse dioxin receptor fusion proteins, consisting of the N-terminus of the glucocorticoid receptor (GRDBD) containing the zinc finger (Zn-F) DNA binding domain, but deleting the N-terminal GR transactivation domain (amino acids 78–261), fused to various C-terminal segments of the dioxin receptor. (**C**) Organization of GRDBD/Arnt fusion proteins analogous to those described in (B). (**D**) The native *Drosophila* PAS protein Per and an isolated internal fragment, PerPAS, showing the location of the PAS domains.

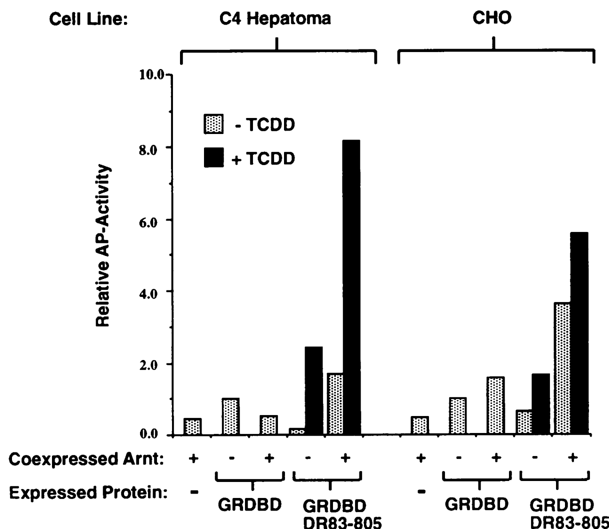


Fig. 2. Activity of a glucocorticoid receptor/dioxin receptor chimera lacking the bHLH domain is potentiated by Arnt in transient transfection assays. CHO and Arnt-deficient mutant C4 hepatoma cells were co-transfected with reporter gene pMMTV-AF and expression vectors for GRDBD, GRDBD/DR83-805, Arnt or blank expression vector (–) as indicated. After treatment with dioxin (10 nM) or vehicle (0.1% DMSO), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. Reported values are normalized against reporter gene activity of GRDBD in the absence of Arnt, which was arbitrarily set to 1.0 for each cell line.

protein is introduced into cells along with a reporter gene containing a response element recognized by the DBD of the fusion protein, together with another chimera consisting of a second polypeptide fused to a strong transactivation domain. Thus, if the two chimeric polypeptides dimerize within the cell, the strong transactivation domain becomes recruited to the promoter and activates the reporter gene, providing a sensitive assay for detecting *in vivo* protein–protein interaction (for a recent review, see Fields and Sternglanz, 1994).

To test for regions of Arnt that interact with the dioxin receptor chimera GRDBD/DR83–805 in the present mammalian hybrid interaction system, we co-expressed Arnt deletion mutants with GRDBD/DR83–805 in CHO cells and measured activity on the reporter gene pMMTV-AF. Deletion mutant ArntΔbHLH lacks the HLH domain of Arnt, a proven dimerization motif (Mason *et al.*, 1994; Reisz-Porszasz *et al.*, 1994), while ArntΔPAS lacks the PAS domain of Arnt (Figure 1), a motif shown to strengthen the HLH-mediated dimerization between the dioxin receptor and Arnt (Reisz-Porszasz *et al.*, 1994). Deletion mutant ArntΔbHLH was as equally active as native Arnt in enhancing GRDBD/DR83–805 activity, providing evidence that the HLH domain of Arnt is not responsible for interacting with this dioxin receptor chimera, which lacks a HLH domain. TheArntΔPAS deletion mutant, however, was totally devoid of any ability to potentiate activity of GRDBD/DR83–805, indicating that the Arnt PAS domain may mediate an independent interaction with the dioxin receptor, possibly via the dioxin receptor PAS domain (Figure 3A). To test this hypothesis in more detail, we performed the hybrid interaction assay with fusion protein GRDBD/DR83-421, which includes the entire PAS domain and the overlapping core ligand binding domain, but deletes the C-terminal transactivation domain of the dioxin receptor (Figure 1). While fusion proteins containing this portion of the dioxin receptor are capable of binding ligand (Whitelaw *et al.*, 1993a), GRDBD/DR83–421 is unable to activate reporter gene pMMTV-AF due to a lack of transactivating residues that are located in the C-terminus (Whitelaw *et al.*, 1994; Figure 3A). When GRDBD/DR83–421 was co-expressed with Arnt, however, a strong activity on the reporter gene was observed (Figure 3A), consistent with a dimerization between these proteins being mediated by their PAS domains. In confirmation of such a putative PAS–PAS interaction, the deletion mutant ArntΔPAS failed to provide activity, while ArntΔbHLH produced an activity equal to that of wild-type Arnt. When expression vectors for native Arnt and deletion mutants ArntΔbHLH and ArntΔPAS were transiently transfected into CHO cells, immunoblotting of cell extracts showed all proteins to be expressed at similar levels (Figure 3B), ruling out the possibility that lack of expression from the ArntΔPAS plasmid had caused aberrant results.

A striking observation in the above hybrid interaction experiments was the ability of Arnt to potentiate activity of the dioxin receptor chimeras in the absence of ligand. In the normal dioxin-responsive pathway, the dioxin receptor is unable to interact with Arnt in the unliganded state, presumably due to the repressive action of hsp90 (Pongratz *et al.*, 1992; Whitelaw *et al.*, 1993b). However, in situations where Arnt is transiently overexpressed, we and others have previously noted that activity on XRE reporter genes is elevated in the absence of dioxin (Reisz-Porszasz *et al.*, 1994; Whitelaw *et al.*, 1994), suggesting that the need for ligand can be bypassed at high concentrations of Arnt. In support of the hypothesis that Arnt may be able to derepress the dioxin receptor, we have recently provided evidence that dimerization with Arnt can play a role in releasing hsp90 from the ligand-bound receptor *in vitro* (McGuire *et al.*, 1994). A plausible explanation for the Arnt-induced, ligand-independent activities seen in the present experiments might therefore be that overexpressed Arnt can activate the dioxin receptor chimeras by initiating hsp90 release, consequently forming potent, transcriptionally active dioxin receptor chimera–Arnt heterodimers.

PAS-mediated dimerization between GRDBD/DR83–421 and Arnt in vitro

The ability of Arnt to enhance activity of chimera GRDBD/DR83–421 in our hybrid interaction system suggested that a functional interaction between these two proteins was mediated by the PAS domain of Arnt. To test if such a postulated dimerization mechanism could be detected *in vitro*, we performed co-immunoprecipitation experiments with proteins expressed in reticulocyte lysates. GRDBD/DR83–421, Arnt and ArntΔPAS were radiolabelled with [^{35}S]methionine during *in vitro* translations, and radiolabelled proteins were analysed on SDS–PAGE gels. Single bands of the predicted molecular weight were detected and, importantly, Arnt and ArntΔPAS show translation of equal efficiency (Figure 3C). To test for an interaction between GRDBD/DR83–421 and Arnt, we used antibodies directed against an N-terminal epitope of Arnt (Mason *et al.*, 1994) to immunoprecipitate mixtures of the two proteins. After reticulocyte lysates containing

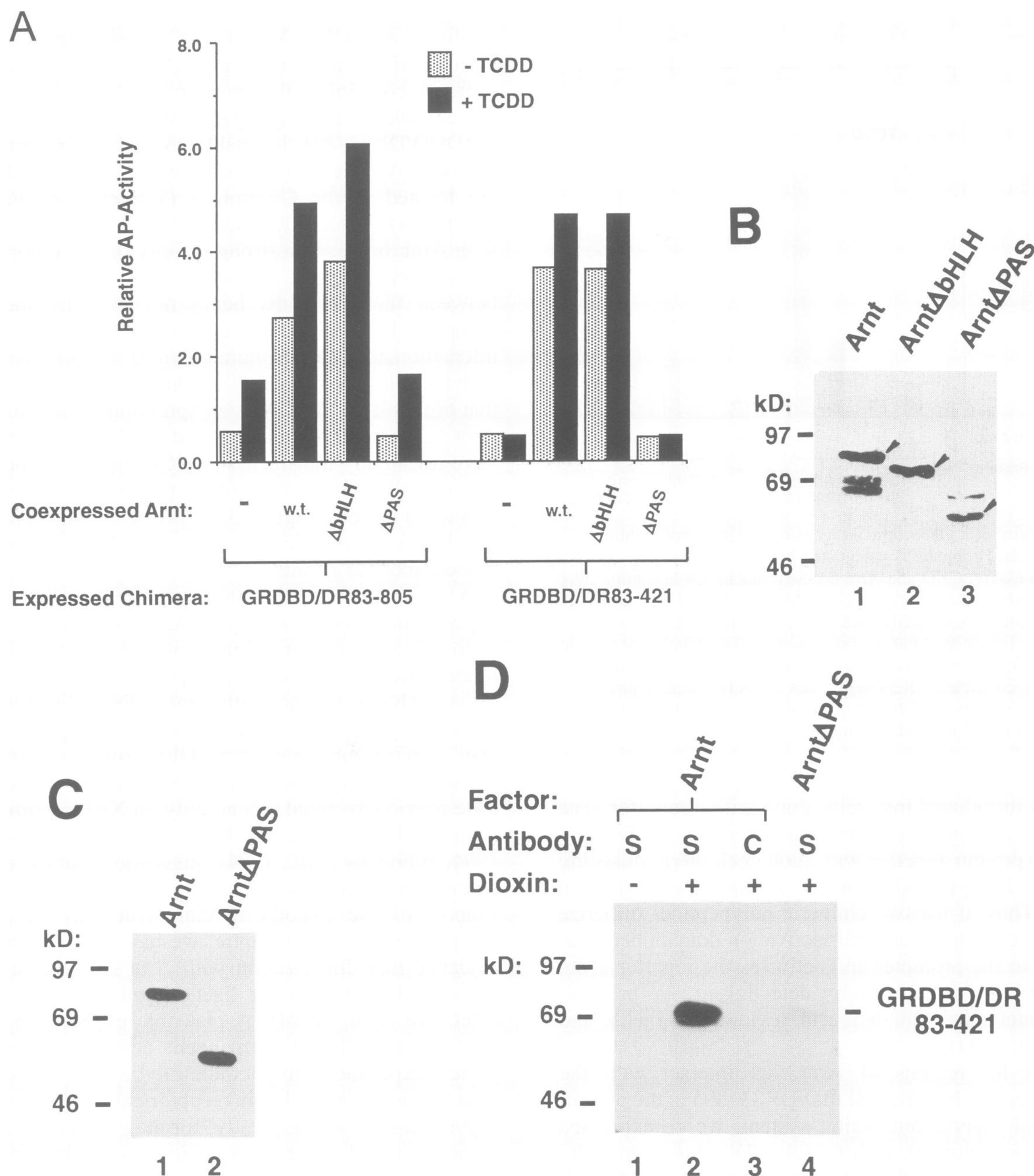


Fig. 3. Interaction between GRDBD/dioxin receptor chimeras and Arnt is mediated by the PAS domains. (**A**) CHO cells were co-transfected with reporter gene pMMTV-AF and expression vectors for the indicated GRDBD/dioxin receptor chimera and one of either wild-type Arnt or Arnt deletion mutants lacking the bHLH or PAS domains. After treatment with dioxin (10 nM) or vehicle (0.1% DMSO), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. Reported values are normalized against the reporter gene activity observed for the GR portion of the chimera, GRDBD, which was arbitrarily set to 1.0. (**B**) Detection of native Arnt, ArntΔbHLH and ArntΔPAS proteins expressed during transient transfection of CHO cells. Whole cell protein extracts were separated by 7.5% SDS–PAGE, transferred to nitrocellulose and expression levels monitored by immunodetection with an antiserum directed against an Arnt N-terminal epitope, as indicated by the arrowheads. (**C**) Analysis of native Arnt and deletion mutant ArntΔPAS translated *in vitro*. Samples of [^{35}S]methionine-labelled proteins in reticulocyte lysates (2 μl) were separated by 7.5% SDS–PAGE and visualized by fluorography. (**D**) Arnt, but not ArntΔPAS, co-immunoprecipitates with the PAS-containing dioxin receptor chimera GRDBD/DR83–421. *In vitro* translation mixtures containing equivalent amounts of Arnt or ArntΔPAS were incubated with [^{35}S]methionine-labelled, *in vitro* translated GRDBD/DR83–421 in the presence of dioxin (20 nM) or vehicle alone (1% DMSO). After further incubation with either pre-immune serum or antiserum directed against an N-terminal epitope of Arnt, antibody-bound proteins were immunoprecipitated with protein A–Sepharose. After extensive washing, immunoprecipitated proteins were analysed by SDS–PAGE and fluorography. Lanes S contain proteins immunoprecipitated with antiserum specific for Arnt, and lane C immunoprecipitates from pre-immune serum.

[^{35}S]methionine-labelled GRDBD/DR83–421 and unlabelled Arnt were incubated with dioxin, Arnt antibodies were able to co-immunoprecipitate the dioxin receptor chimera, while a control precipitation with pre-immune serum was devoid of any radiolabelled precipitate (Figure 3D, compare lanes 2 and 3). Interestingly, this interaction was ligand dependent, as Arnt antibodies failed to precipitate the dioxin receptor chimera after an equivalent incubation in the absence of ligand (compare lanes 1 and 2). These results are consistent with dioxin receptor residues 83–421 being able both to bind ligand and to interact with hsp90 (Whitelaw *et al.*, 1993a), thus

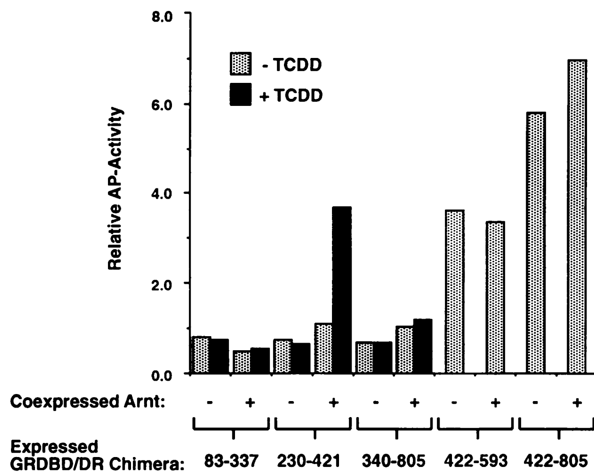


Fig. 4. The core ligand and hsp90 binding domain of the dioxin receptor, encompassing PAS B between amino acids 230 and 421, is essential for PAS-mediated interaction with Arnt. CHO cells were co-transfected with reporter gene pMMTV-AF, expression vectors for the indicated GRDBD/dioxin receptor chimeras, and either blank (–) or Arnt expression vector as indicated. After treatment with dioxin (10 nM) or vehicle (0.1% DMSO), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. Reported values are normalized against the reporter gene activity observed for the GR portion of the chimera, GRDBD, which was arbitrarily set to 1.0.

encompassing a domain postulated to suppress an interaction with Arnt in the unliganded state (Whitelaw *et al.*, 1993b). When co-immunoprecipitation experiments were performed with ArntΔPAS, no ligand-induced interaction with GRDBD/DR83–421 was seen (Figure 3D, compare lanes 2 and 4), consistent with the inability of ArntΔPAS to enhance activity of this chimera on a reporter gene in transient transfection assays (Figure 3A), and strongly supporting the notion that interaction between Arnt and dioxin receptor chimeras occurred through the PAS domain.

A C-terminal segment of the dioxin receptor PAS domain is critical for the PAS–PAS interaction with Arnt

The PAS domain contains two degenerate hydrophobic repeats, designated PAS A and PAS B (Figure 1). We further investigated the PAS–PAS interaction between the dioxin receptor and Arnt by asking whether one of these repeats was more important than the other in forming the dimerization interface. By constructing chimeras GRDBD/DR83–337 and GRDBD/DR230–421, we have divided the GRDBD/DR83–421 fusion protein into two entities, containing either PAS A and most of PAS B (GRDBD/DR83–337), or the complete PAS B motif within the minimal ligand binding domain (GRDBD/DR230–421) (Figure 1). The present mammalian hybrid interaction assay showed that the PAS A-containing chimera, GRDBD/DR83–337, was not able to interact with Arnt, while the C-terminal PAS chimera, GRDBD/DR230–421, maintained very strong interaction with Arnt (Figure 4). As observed with previous dioxin receptor chimeras, ArntΔbHLH was equally as strong as native Arnt in enhancing the reporter activity together with GRDBD/DR230–421, while ArntΔPAS was devoid of this ability (data not shown). These results indicate that the C-terminus of the dioxin receptor PAS domain was a critical determinant for PAS-mediated interaction with Arnt, while the N-terminal portion containing PAS A was unable to form an independent interaction with Arnt. The PAS A region of the dioxin receptor may help to stabilize the dimerization mediated by the C-terminal/PAS B region during interaction of GRDBD/DR83–421 with Arnt, however, as the Arnt-potentiated activity of GRDBD/DR230–421 was consistently lower than that of Arnt-potentiated activity of GRDBD/DR83–421, and *in vitro* immunoprecipitation assays show a stronger interaction between Arnt and GRDBD/DR83–421 than between Arnt and GRDBD/DR230–421 (data not shown). A further chimera, GRDBD/DR340–805, which contains dioxin receptor residues C-terminal to PAS B, failed to show enhanced activity when co-expressed with Arnt (Figure 4).

Chimera GRDBD/DR340–805 contains only a portion of the core ligand binding domain located between amino acids 230 and 421, and shows no ligand binding activity (McGuire, unpublished results). The observation that chimeras which disrupt the core ligand binding domain, exemplified by the two fusion proteins GRDBD/DR83–337 and GRDBD/DR340–805, could not sustain an interaction with Arnt indicates that the intact core ligand binding domain is essential for PAS–PAS interaction between the dioxin receptor and Arnt. To test for interaction between Arnt and residues C-terminal to the core ligand binding domain of the dioxin receptor, we also tested chimeras GRDBD/DR422–593 and GRDBD/DR422–805 (Figure 1) in our mammalian hybrid interaction system. We have previously reported that chimera GRDBD/DR422–805 forms a potent transcription factor that, in the absence of the ligand binding domain, functions constitutively (Whitelaw *et al.*, 1994). Co-expression of Arnt with this chimera failed to enhance activity (Figure 4), indicating a lack of stable interaction between Arnt and the dioxin receptor C-terminus *in vivo*. It is formally possible, however, that an interaction between Arnt and the dioxin receptor C-terminus would be obscured in this system by the high constitutive activity of GRDBD/DR422–805, i.e. a maximum transcriptional activity had already been obtained in the absence of Arnt. We therefore tested a further chimera, GRDBD/DR422–593, which lacks a portion of the potent C-terminal transactivation domain, and thus shows a lower level of constitutive activity than GRDBD/422–805 (Figure 4). Co-expression of either Arnt (Figure 4), ArntΔbHLH or ArntΔPAS (data not shown) with GRDBD/422-593 did not alter the activity of the dioxin receptor chimera, indicating a lack of interaction between this dioxin receptor C-terminal fragment and Arnt.

Failure to detect homodimerization processes mediated by the PAS domains of the dioxin receptor or Arnt in vivo

Having demonstrated that the PAS domains of the dioxin receptor and Arnt are capable of mediating heterodimerization independently of the HLH domains, we sought to establish whether these domains could also mediate the formation of homodimeric complexes. Using the same mammalian hybrid interaction assay, we co-expressed the full length native dioxin receptor with the dioxin receptor PAS domain-containing chimera GRDBD/DR83–421. Despite experiments being performed in either the absence

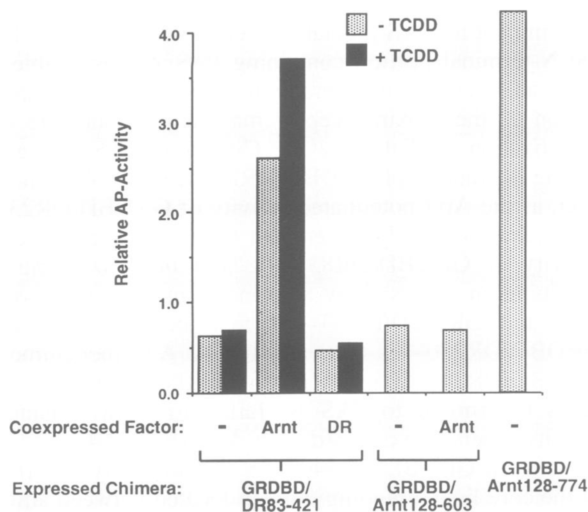
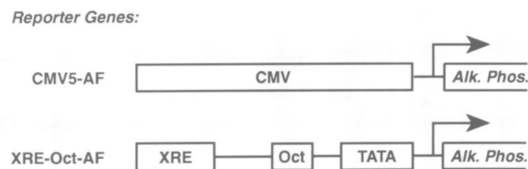


Fig. 5. PAS domain-mediated homodimerization is not detected for the dioxin receptor or Arnt in the hybrid interaction assay. CHO cells were co-transfected with reporter gene pMMTV-AF and expression vectors for either dioxin receptor or Arnt PAS-containing chimeras, along with blank (–), native dioxin receptor or Arnt expression vectors as indicated. After treatment with dioxin (10 nM) or vehicle (0.1% DMSO), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. Reported values are normalized against the reporter gene activity observed for the GR portion of the chimera, GRDBD, which was arbitrarily set to 1.0.



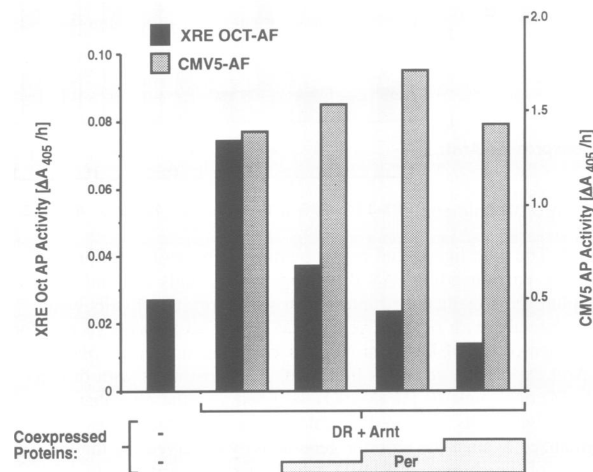


Fig. 6. Ectopic expression of Per can abrogate dioxin responsiveness of an XRE-driven reporter gene. CHO cells were co-transfected with either XRE-Oct-AF or control CMV5-AF reporter genes as indicated, together with a mixture of expression vectors for the dioxin receptor, Arnt, increasing quantities of Per and blank expression vector to a total of 3 μg DNA. After treatment with dioxin (10 nM), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry.

or presence of ligand, we observed no enhancement of reporter gene activity produced by GRDBD/DR83–421, indicating an inability of the PAS domain to mediate homodimerization by the dioxin receptor (Figure 5). As a counterpart to the described dioxin receptor chimeras, we constructed Arnt chimeras where the bHLH domain of Arnt has been replaced by the glucocorticoid receptor DNA binding domain, providing GRDBD/Arnt128–774 and GRDBD/Arnt128–603 (Figure 1). Chimera GRDBD/Arnt128–774 shows a strong, constitutive activity on the GRE-driven reporter gene pMMTV-AF, while GRDBD/Arnt128–603 has minimal activity (Whitelaw *et al.*, 1994; Figure 5). Co-expression of native Arnt with these chimeras failed to elevate activity in either case, indicating that the Arnt PAS domain, in similar fashion to the dioxin receptor PAS domain, is incapable of mediating an independent homodimerization process in the context of our hybrid interaction system.

The PAS domain of PER can disrupt formation of a functional dioxin receptor–Arnt complex

Given the importance of the PAS domains of the dioxin receptor and Arnt in forming an active dioxin receptor–Arnt heterocomplex, we were intrigued by the possibility of exogenous PAS domain-containing proteins being able to interfere with the dioxin receptor signal transduction pathway. The *Drosophila* Per protein contains a PAS domain but is devoid of a bHLH motif, making it a potential negative regulator of bHLH/PAS proteins. It has been proposed previously that the PAS domain of Per can interact with the PAS domain of Arnt (Huang *et al.*, 1993). We therefore analysed the influence of Per on the dioxin responsiveness of reporter gene XRE-Oct-AF, which consists of a promoter containing XRE, octamer and TATA box sequences upstream of the alkaline phosphatase gene (Whitelaw *et al.*, 1994). As expected, the dioxin-induced alkaline phosphatase levels were elevated when expression plasmids for the dioxin receptor and Arnt were co-transfected with the reporter gene into CHO cells (Figure 6). By co-expressing increasing amounts of Per, the dioxin-induced activity was decreased to below the background levels observed by transfection of reporter gene in the absence of any dioxin receptor and Arnt expression plasmids. These results suggest that ectopic expression of Per may abort dioxin signalling in cells. This influence of Per was not due to detrimental effects on cell growth or non-specific disruption of reporter gene activity, e.g. by squelching, as the activity of a constitutively expressed alkaline phosphatase reporter gene, CMV5-AF, driven by the powerful cytomegalovirus promoter, was unaffected by transfection of the Per expression vector (Figure 6).

To test if the inhibitory effects of Per would be apparent at the level of DNA binding of the dioxin receptor–Arnt heterodimer, we performed gel mobility shift assays of the activated dioxin receptor complex using the XRE target DNA recognition sequence as radiolabelled probe. Cytosolic extract from untreated hepatoma cells contains both dioxin receptor and Arnt proteins, which are converted to a DNA binding heterodimeric complex upon exposure to dioxin *in vitro* (Cuthill *et al.*, 1991; Figure 7B, compare lanes 1 and 2). Expression of Per cDNA in reticulocyte lysate provided the *in vitro* translated Per protein which ran as a single band on SDS–PAGE (Figure 7A, lane 2). Inclusion of increasing amounts of an *in vitro* translation mix containing Per to the hepatoma cell cytosol at the time of dioxin treatment decreased binding by

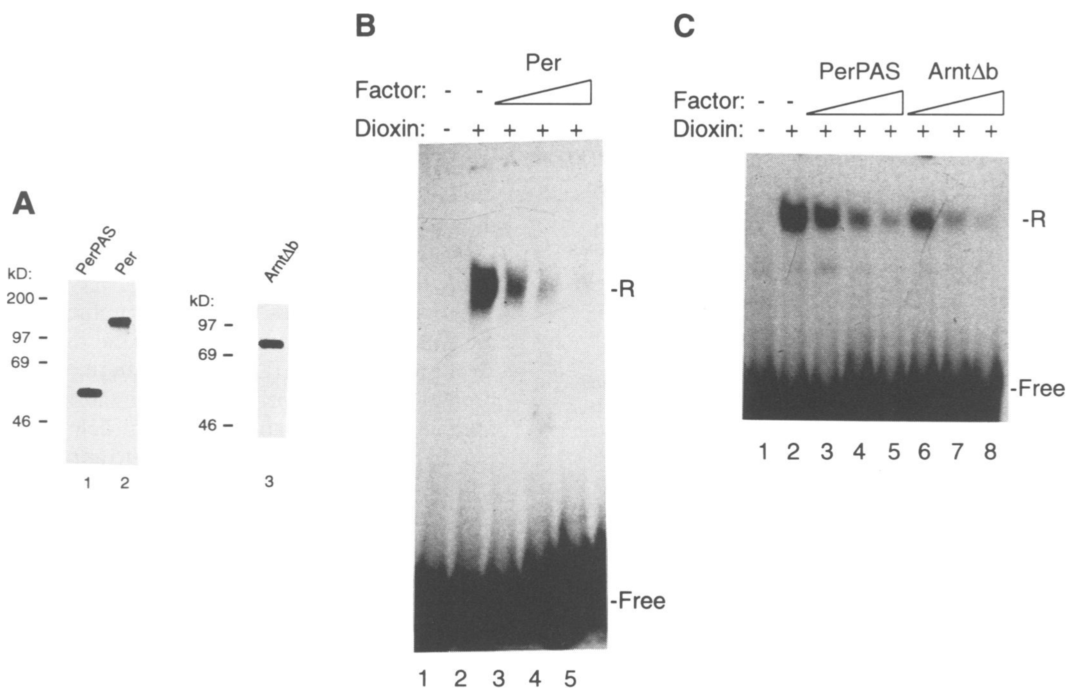


Fig. 7. Both native Per and the PerPAS domain can disrupt formation of a functional dioxin receptor–Arnt heterodimer. (**A**) Analysis of Per, PerPAS and ArntΔb proteins translated *in vitro*. Aliquots (2 μl) of reticulocyte lysates containing [^{35}S]methionine-labelled proteins were separated by SDS–PAGE and visualized by fluorography. (**B**) Native Per can disrupt formation of a DNA binding dioxin receptor–Arnt heterodimer. Gel mobility shift assays were performed with ^{32}P-labelled XRE probe after incubation of Hepa1c1c7 cytosolic extracts (10 μl) with unprogrammed reticulocyte lysate (15 μl, lane 2) or with lysates containing increasing concentrations of Per (total of 15 μl lysates per incubation, lanes 3–5). Dioxin (10 nM) was added to the cytosol concomitant with Per. (**C**) Both the PerPAS domain and ArntΔb can disrupt formation of a DNA binding dioxin receptor–Arnt heterodimer. Gel mobility shift assays were performed as described in (B) with Hepa1c1c7 cytosol (10 μl) in the presence of either unprogrammed reticulocyte lysate (15 μl, lane 2) or with increasing concentrations of PerPAS or ArntΔb (total of 15 μl lysates per incubation, lanes 3–8). Dioxin was added to the cytosol concomitant with *in vitro* translation mixtures. R represents the dioxin receptor–Arnt–XRE complex, Free represents unbound XRE probe. Lane 1 of each figure contains a sample of unincubated probe.

the dioxin receptor complex to the XRE probe in a concentration-dependent manner (Figure 7B, compare lanes 2–5).

Inhibition of the XRE complex generation indicated an ability of Per to disrupt the DNA binding activity by the receptor, possibly by PAS-mediated interaction with either one or both of the dioxin receptor and/or Arnt subunits. To investigate this possibility further, we translated PerPAS *in vitro* (Figure 7A, lane 1), containing the PAS domain of Per within residues 233–685 (Figure 1; Huang *et al.*, 1993), and determined its ability to interfere with DNA binding activity of the ligand-activated dioxin receptor. A similar concentration-dependent abrogation of dioxin-induced DNA binding was observed when PerPAS was added to hepatoma cell cytosol (Figure 7C, lanes 3–5). Efficient inhibition of the DNA binding activity of the ligand-activated dioxin receptor–Arnt complex was observed both if PerPAS was added at the onset of dioxin receptor–Arnt heterodimerization, or upon exposure to an already preformed dioxin receptor complex (data not shown). Furthermore, a corresponding inhibition of DNA binding activity of the dioxin receptor complex (Figure 7C, lanes 6–8) occurred upon addition of *in vitro* translation mixtures containing ArntΔb (Figure 1), an Arnt mutant modelled on the well characterized dominant negative regulator of bHLH proteins, Id, that contains an intact HLH motif but lacks the basic domain (Benezra *et al.*, 1990), thereby depriving residues essential for DNA contact but maintaining those necessary for dimerization (Figure 1).

The PAS domain of Per can interact with both the dioxin receptor and Arnt in vitro

Abrogation of the formation of a dioxin-induced XRE complex in hepatoma cell cytosolic extracts by PerPAS indicates that this region of Per has the potential to disrupt dioxin signalling by precluding generation of an activated dioxin receptor complex capable of recognizing its target DNA sequence. To test for a physical interaction between the PerPAS domain and either the dioxin receptor or Arnt, we performed co-immunoprecipitation assays after incubation of *in vitro* translated, [^{35}S]methionine-labelled PerPAS with crude cell extracts containing high levels of dioxin receptor or Arnt. Infection of rabbit kidney RK13 cells with recombinant vaccinia virus programmed to express either the dioxin receptor or Arnt provided high levels of these proteins in cytosolic cell extracts (Whitelaw, unpublished data). After incubation of PerPAS with a cytosolic extract containing high levels of the dioxin receptor in the presence of ligand, immunoprecipitation with dioxin receptor antiserum revealed that a high level of PerPAS associated with the dioxin receptor, compared to the background levels of PerPAS co-immunoprecipitated by control pre-immune serum (Figure 8A, compare lanes 2 and 3). Furthermore, co-immunoprecipitation of PerPAS by dioxin receptor antiserum was more prevalent in the

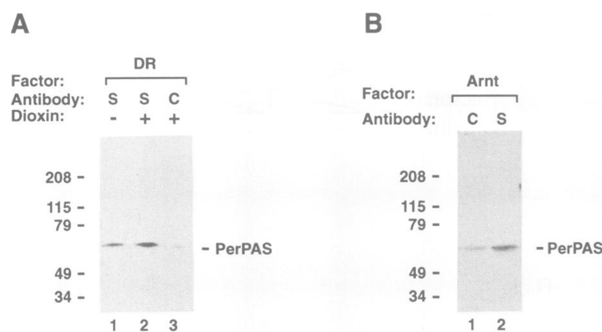


Fig. 8. The PerPAS domain can interact with the dioxin receptor and Arnt. (**A**) Ligand-enhanced co-immunoprecipitation of PerPAS with the dioxin receptor. A cytosolic extract of RK13 cells containing high levels of vaccinia virus-expressed dioxin receptor was incubated with *in vitro* translated, [^{35}S]methionine-labelled PerPAS in the presence of dioxin (20 nM) or vehicle (1% DMSO). After incubations for 2 h at 25°C and 16 h at 4°C, immunoprecipitations were performed with either specific antiserum directed against an N-terminal epitope of the dioxin receptor (lanes S), or control pre-immune serum (lane C). After extensive washing, immunoprecipitated proteins were separated by SDS–PAGE and visualized by fluorography. (**B**) Co-immunoprecipitation of PerPAS with Arnt. A cytosolic extract of RK13 cells containing high levels of vaccinia virus-expressed Arnt was incubated with *in vitro* translated, [^{35}S]methionine-labelled PerPAS as described in (A). Immunoprecipitation was performed with either control pre-immune serum (lane C) or antiserum directed against an N-terminal epitope of Arnt (lane S). After extensive washing, immunoprecipitated proteins were separated by SDS–PAGE and visualized by fluorography.

presence than in the absence of ligand (Figure 8A, compare lanes 1 and 2), indicating the possibility that a ligand-induced release of hsp90, or a conformational change, may enhance interaction between the dioxin receptor and PerPAS domains. In a similar experiment where cytosolic extracts containing high levels of vaccinia-expressed Arnt were incubated with *in vitro* translated, [^{35}S]methionine-labelled PerPAS, co-immunoprecipitation of PerPAS with Arnt antiserum was significantly greater than the background levels obtained with control pre-immune serum (Figure 8B, compare lanes 1 and 2). Thus both PAS domain-containing subunits of the dioxin receptor–Arnt heterodimer are capable of interaction with the PerPAS domain, emphasizing the ability of the dioxin receptor and Arnt PAS domains to undergo heterodimeric PAS–PAS interactions, and revealing the PerPAS domain to be capable of multiple heterodimeric PAS interactions as well as homodimerization (Huang *et al.*, 1993).

Discussion

Role of the PAS domains in formation of an activated dioxin receptor–Arnt heterodimer

Intracellular ligand signalling initiates a multistep process whereby the latent, apparently cytoplasmic dioxin receptor is transformed into the nuclear, DNA binding dioxin receptor–Arnt heterodimeric species. Mutant hepatoma cell lines deficient in Arnt show the absence of dioxin-induced phenotypes, but can be subsequently rescued in Arnt complementation experiments, thus proving that formation of this heterodimer is essential for dioxin-induced gene regulation (Reyes *et al.*, 1992; Whitelaw *et al.*, 1993b). *In vitro* studies have further shown that while neither the dioxin receptor nor Arnt alone is capable of recognizing the target DNA response element, the combination of these factors provides a dioxin-induced complex capable of binding the XRE enhancer (Dolwick *et al.*, 1993; Matsushita *et al.*, 1993; Probst *et al.*, 1993; Whitelaw *et al.*, 1993b). Purification of the XRE-bound dioxin-induced complex has recently revealed that the dioxin receptor and Arnt apparently are the only proteins present in this DNA-bound form of the receptor (Henry *et al.*, 1994).

Previous work has provided a preliminary mapping of the functional architecture within the dioxin receptor and Arnt proteins. The HLH domains in the N-termini of each protein form the primary dimerization interface, as a number of internal and C-terminal deletions in either the dioxin receptor or Arnt have failed to abrogate dimerization totally whenever the HLH domains have remained intact (Dolwick *et al.*, 1993; Reisz-Porszasz *et al.*, 1994; Antonsson *et al.*, 1995). Deletion of the Arnt basic region abrogates XRE binding without reducing dimerization (Reisz-Porszasz *et al.*, 1994; Figure 7C), thus indicating that these N-terminal bHLH domains perform the essential functions of providing dimerization interfaces and DNA contact points in analogy with other bHLH proteins. Powerful transactivation domains lie in the C-termini of both proteins (Jain *et al.*, 1994; Li *et al.*, 1994; Whitelaw *et al.*, 1994), although the relative activity of these domains may depend on cell type and promoter context (Whitelaw *et al.*, 1994). The dioxin receptor and Arnt therefore share a similar rudimentary design of dimerization/DNA binding domains in the N-terminus and transactivation domains in the C-terminus. Despite this similar design of N-terminal bHLH/PAS domains, the dioxin receptor is distinguished from Arnt by its central ligand and hsp90 binding region, an entity which encompasses PAS B. While the dioxin receptor PAS domain has been shown to confer conditional repression on the strong C-terminal transactivation domain in dioxin receptor chimeras (Whitelaw *et al.*, 1994), Arnt does not interact with hsp90 (Probst *et al.*, 1993; McGuire *et al.*, 1994), and Arnt chimeras form unconditional transactivators (Li *et al.*, 1994; Whitelaw *et al.*, 1994), suggesting no obvious mode of regulation is inherent in the Arnt PAS domain. As the entire PAS domains of the dioxin receptor and Arnt share only 19% identity, it is perhaps not surprising that some regions within these domains should have quite separate functions.

While the concept of bHLH motifs being essential for dimerization and DNA binding is general for all bHLH proteins, further motifs have been identified as being necessary to form functional dimeric complexes. A subset of bHLH proteins (e.g. Myc, Max, USF) contain leucine zippers contiguous with the HLH region, and in the case of Myc/Max, mutations in these leucines destroy formation of an active complex (Reddy *et al.*, 1992; Davis and Halazonetis, 1993). The newly defined bHLH/PAS family forms another subset of bHLH proteins, where the PAS regions are critical for function. *In vitro* experiments have shown that deletion of the Arnt PAS domain depletes, but does not abrogate, dimerization with the dioxin receptor (Reisz-Porszasz *et al.*, 1994), consistent with a role for the PAS domain in stabilizing HLH-mediated dimerization. Our studies reveal that the dioxin receptor and Arnt PAS domains are not only competent to consolidate the HLH

dimerization process, but are capable of forming an independent dimerization interface. Use of a highly sensitive mammalian hybrid interaction system has allowed us to detect protein–protein interactions which would be difficult to detect under the stringent conditions of most *in vitro* protocols. Indeed, previous *in vitro* experiments investigating dimerization between the dioxin receptor and deletion mutants of Arnt which lack residues from the bHLH region have failed to detect any interaction (Mason *et al.*, 1994; Reisz-Porszasz *et al.*, 1994). Optimizing low stringency *in vitro* conditions has now allowed detection of a primary interaction between the dioxin receptor and Arnt PAS domains, demonstrated by the ability of a co-immunoprecipitation assay to reveal binding between native Arnt, but not ArntΔPAS, and GRDBD/DR83–421, a chimera spanning the entire PAS domain of the dioxin receptor but lacking its bHLH motif. In further attempts to delineate the dimerizing interface within the PAS domains, we deleted the N-terminal half of the dioxin receptor PAS domain, leaving the core ligand binding domain between amino acids 230 and 421. This deletion did not greatly decrease the interaction given by the full PAS region between amino acids 83 and 421. The PAS A segment of the dioxin receptor seems therefore to be of only minor relevance for PAS–PAS interaction between the dioxin receptor and Arnt. We have detected no evidence for dioxin receptor regions C-terminal of the core ligand binding domain being able to interact with Arnt, although it must be noted that, since the receptor C-terminus contains transactivation functions, the mammalian hybrid interaction system employed here is not well suited to detect such an interaction.

A further functional role for the dioxin receptor PAS domain in establishing a high affinity of the dioxin receptor–Arnt heterodimer for the target XRE sequence has been suggested (Antonsson *et al.*, 1995). Consistent with this hypothesis, a dioxin receptor C-terminal deletion mutant, leaving residues only N-terminal to PAS B, is capable of Arnt dimerization and constitutive XRE binding at levels equivalent to those obtained by the ligand-activated native dioxin receptor (Dolwick *et al.*, 1993; McGuire *et al.*, unpublished results). In contrast, however, further truncation of the dioxin receptor, to leave only the bHLH and PAS A regions, produces a fragment that is unable to provide a DNA binding species when incubated with Arnt (Dolwick *et al.*, 1993). It would therefore seem that the intervening amino acids between PAS A and PAS B are necessary for the ability of the PAS domain to affect DNA binding. Whether these amino acids are alone sufficient, or the context of the contiguous PAS A region is also necessary, is unclear at present. The PAS region of the dioxin receptor may thus be considered as comprised of two elements with dramatically opposing functions. The PAS residues N-terminal of PAS B can be viewed as a positively acting portion of the receptor, providing a competence to bind the specific XRE DNA sequence. In strict contrast, the ligand and hsp90 binding PAS B region performs a repressive function, inhibiting dimerization with Arnt, and therefore DNA binding, in the unliganded state. It will now be important to test this demarcation of functional domains further by creating more precise deletions and mutations in the PAS A and B repeats, perhaps enabling critical individual amino acids for dioxin receptor activation and repression to be revealed.

While separate functions seem to be encoded in the dioxin receptor PAS A and PAS B regions, deletion of either PAS A or PAS B from Arnt gives a similar attenuated function of Arnt in terms of impaired dimerization with the ligand-activated dioxin receptor and diminished XRE binding. As the loss of XRE binding seen in the Arnt PAS deletions approximately correlated to the degree of loss in dimerization (Reisz-Porszasz *et al.*, 1994), the PAS A and PAS B regions of Arnt seem to function primarily as dimerization motifs. However, the observation that none of the Arnt PAS deletion mutants could rescue a dioxin-responsive phenotype in Arnt-deficient C4 hepatoma cells has implicated additional cryptic functions for these domains to be essential for full biological activity (Reisz-Porszasz *et al.*, 1994).

The PAS domains of the dioxin receptor and Arnt can generate heterodimeric, but not homodimeric, complexes

Our mammalian hybrid interaction system was able to efficiently detect heterodimeric PAS–PAS interactions between the dioxin receptor and Arnt, but we detected no evidence for homodimeric PAS–PAS interactions. As the 'baited' dioxin receptor or Arnt fusion proteins used in these experiments, GRDBD/DR83–421 and GRDBD/Arnt128–603 respectively, lack the C-terminal transactivation domains of their corresponding native proteins, any homodimeric PAS interactions with the full length dioxin receptor or Arnt should have been detected. In the case of the dioxin receptor, where the presence of Arnt in addition to ligand may play a role in release of hsp90 and activation of the receptor (McGuire *et al.*, 1994), the attempted coupling of GRDBD/DR83–421 and native receptor may have been flawed by an inability to activate the native receptor efficiently by ligand alone. As no such activation is needed for Arnt, however, Arnt PAS homodimers should have been detected in this system if they were capable of forming. The inability of the subunits of the dioxin receptor complex to form PAS-mediated homodimers is in direct contrast to the PerPAS domain, which has shown an ability to form homodimeric complexes *in vitro* (Huang *et al.*, 1993).

The observation that the dioxin receptor and Arnt PAS domains are particularly adept at heterodimerizing with each other but show no tendency to homodimerize is consistent with the hypothesis that a critical function of these domains is to aid in specificity of bHLH partner selection. Thus the PAS domains may be critical in preventing inaccurate HLH partnership with other members of the bHLH family, which could lead to complexes of altered DNA binding specificity or formation of abortive non-functional complexes.

Exogenous PAS domains—potential negative regulators of dioxin signalling and bHLH/PAS factor function

Of the four proteins currently known to harbour PAS domains, Per is alone in lacking a bHLH domain. Sim has been shown recently to be a transcription factor with an organization similar to the dioxin receptor and Arnt, with the bHLH/PAS domain in the N-terminus and trans-

activation domains residing in the C-terminus (Franks and Crews, 1994). Although Per is a large protein of a calculated 127 kDa, no functional domains apart from the PAS dimerization region have been described. The observation that both *per* mRNA and Per protein fluctuate out of phase in a circadian manner, with the decrease in *per* mRNA being influenced by the Per protein, is suggestive of a negative feedback loop controlling *per* gene expression (Hardin *et al.*, 1992; Zeng *et al.*, 1994). Such a feedback mechanism may involve some direct activity of Per itself, or perhaps activity of Per on some intermediary protein, such as a bHLH/PAS transcription factor. The lack of a bHLH domain within Per would seem to render it well suited as a negative regulator for bHLH/PAS proteins and, although dimerization *in vitro* between the bHLH/PAS factor Sim and the PerPAS domain has been reported (Huang *et al.*, 1993), no functional consequence has been demonstrated for the Sim–Per heterodimer. It would be reasonable to predict, however, that the interaction of Per with Sim would preclude Sim from either homodimerizing, or dimerizing with another bHLH partner, thereby modulating the Sim transactivation pathway. It is worthy of note that the PAS domain of Sim seems crucial for Sim activity, as deletion of this domain produces a non-functional protein (Franks and Crews, 1994). The potential for Per to be a negative regulator is demonstrated by our experiments where ectopic Per expression was able to disrupt the dioxin signalling pathway in a transient transfection assay, and addition of Per was able to abort *in vitro* formation of a DNA binding dioxin receptor–Arnt heterodimer. The ability of the isolated PerPAS domain to also abrogate dioxin receptor–Arnt DNA binding suggests that the inhibitory mechanism is via interference of critical dioxin receptor–Arnt PAS interaction. Consistent with such a mechanism, our immunoprecipitation assays reveal an ability of both the dioxin receptor and Arnt to interact with PerPAS, establishing the PerPAS domain to be a particularly active dimerization interface, capable of both homodimerizing and heterodimerizing with all known members of the bHLH/PAS family. Whether the PerPAS domain prevents formation of the dioxin receptor–Arnt heterodimer, or merely disrupts the ability of the heterodimer to bind the DNA target sequence, remains to be elucidated.

Materials and methods

Plasmid constructions

Plasmids pMMTV-AF, pXRE-Oct-AF, pCMV4/DR, pArnt/GEM7, pCMV4/Arnt, pMT-GRDBD, pMT-GRDBD/DR83–805, pMT-GRDBD/DR340–805, pMT-GRDBD/DR422–805, pGRDBD/Arnt128–774/CMV4, pGRDBD/Arnt128–603/CMV4 and pDR593/CMV4 have been described previously (Mason *et al.*, 1994; Whitelaw *et al.*, 1994). Deletion of a *Bcl*I fragment containing codons 66–160 from pArnt/GEM7 provided pArntΔbHLH/GEM7. Codons 1–128 of pBM5Neo/M1-1 (Hoffman *et al.*, 1991) were amplified by PCR with primers containing upstream *Cla*I and downstream *Xho*I sites. *Cla*I–*Xho*I digestion and subcloning of the fragment into *Cla*I–*Xho*I-digested pGEM7 provided pArntbHLH/GEM7. The majority of PCR-derived residues were replaced with a *Nco*I–*Kpn*I fragment from pBM5Neo/M1-1. Similar amplification and digestion of codons 407–774 of Arnt using *Xho*I restriction sites allowed subcloning into *Xho*I-digested pArntbHLH/GEM7 to give pArntΔPAS/GEM7. The majority of C-terminal PCR-derived residues were replaced by a *Kpn*I–*Xba*I fragment from pBM5Neo/M1-1. Dideoxy sequencing verified the fidelity of the remaining PCR-derived residues. Codons 88–774 of pBM5Neo/M1-1 were amplified by PCR using an upstream primer containing a *Bam*HI site and a downstream C-terminal primer. Subcloning of the *Bam*HI–*Cel*II-digested amplified fragment into *Bcl*I–*Cel*II-digested pArnt/GEM7 gave pArntΔb/GEM7, thus deleting codons 66–87 from native Arnt. Plasmids pArntΔPAS/CMV4, pArntΔbHLH/CMV4 and pArntΔb/CMV4 were constructed by subcloning *Bam*HI fragments from the relative GEM vectors into *Bam*HI-digested pCMV4. Plasmids pGRDBD/DR83–421/GEM, pMT-GRDBD/DR83–421, pMT-GRDBD/DR83–337 and pMT-GRDBD/DR230–421 were constructed by subcloning *Cla*I–*Xba*I fragments from the relevant τDBD/DR/CMV4 vectors (Whitelaw *et al.*, 1993a, 1994) into *Cla*I–*Xba*I-digested pGRDBD/GEM7 or pMT-GRDBD (Whitelaw *et al.*, 1994). Plasmid pGRDBD/DR422–593/CMV4 was constructed by subcloning a *Not*I–*Xba*I fragment from pDR593/CMV4 into *Not*I–*Xba*I-digested pGRDBD/DR422–805/CMV4. Plasmids encoding full length Per (pSP65ATper) and PerPAS (pBSC2H) have been described previously (Huang *et al.*, 1993). Plasmid pCMV/Per was created by excision of the complete Per coding sequence from pSP65ATper with *Hin*dIII–*Xba*I and ligating into *Hin*dIII–*Xba*I-digested pCMV4. Reporter gene pCMV5-AF was constructed by digestion of pMMTV-AF with *Xho*I, blunt ending with Klenow enzyme, then excision of the alkaline phosphatase gene and secretion signal sequence with *Nhe*I, and subcloning the fragment into *Xba*I–*Sma*I-digested pCMV5 (Andersson *et al.*, 1989).

Cell culture and transfections

Chinese hamster ovary (CHO) and mutant C4 murine hepatoma cells were grown in Ham's F12 and minimal essential medium supplemented with 2 mM L-glutamine, respectively. All media were supplemented with 10% fetal calf serum, 100 U of penicillin and 100 μg of streptomycin/ml (Gibco/BRL). Cells were seeded at a density of 10^5/35×10 mm dish and left for 24 h for recuperation. Reporter plasmids and either metallothionein (MT) or cytomegalovirus (CMV) promoter-driven expression plasmids (1.5 μg of reporter and 0.5 μg of each expression vector, with a total DNA content made up to 2.5 μg with empty expression vector when needed) were transfected with 7.5 μl DOTAP according to the manufacturer's instructions (Boehringer). For transfections with Per, cells were transfected with 1.5 μg of either pXRE-Oct-AF or pCMV5/AF reporter plasmids and a combination of CMV4 expression vectors for dioxin receptor (50 ng), Arnt (50 ng), increasing amounts of Per (0.3, 0.5 or 0.7 μg) and empty CMV4 vector to a total quantity of 3 μg transfected DNA. After a 12 h transfection period, cells were induced with ligand (10 nM TCDD) or vehicle alone (0.1% DMSO) for periods of up to 72 h. Secreted alkaline phosphatase was assayed by colorimetry as previously described (Whitelaw *et al.*, 1993a). All transfections were performed in duplicate and the average values were recorded. Duplicate transfections were repeated between three and six times, and figures report representative data from these independent transfection experiments.

In vitro translation and immunoprecipitation experiments

Arnt deletion mutant proteins, Per, PerPAS and GRDBD/DR83–421 were translated from the respective GEM plasmids in rabbit reticulocyte lysates (Promega), in the presence or absence of [^{35}S]methionine, as recommended by the manufacturer. Aliquots (2 μl) of each radiolabelled translation mixture were separated by 7.5% SDS–PAGE and visualized by fluorography. For immunoprecipitations using GRDBD/DR83–421, translation mixtures containing [^{35}S]methionine-labelled GRDBD/DR83–421 (5 μl) and either unlabelled Arnt or ArntΔPAS (10 μl) were incubated in TEG buffer [45 μl; 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10% (w/v) glycerol, 1 mM DTT] containing dioxin (20 nM) or vehicle alone (1% DMSO) and the protease inhibitors aprotinin and leupeptin (5 μg/ml) for 3 h at 25°C. The samples were then pre-cleared by addition of pre-immune serum (10 μl), incubation for 15 min on ice, addition of 50 μl of a 50% slurry of protein A-Sepharose (Pharmacia) in TEG buffer and further incubation on ice for 15 min. After centrifugation, equal volumes of supernatant were added to pre-immune serum (10 μl) or Arnt antiserum (10 μl) and incubated for 30 min on ice. Protein A-Sepharose [50 μl of a 50% slurry in TEG/wash buffer (TEG/150 mM NaCl/0.2% Triton X-100/1 mg/ml ovalbumin)] was added, incubation on ice was continued for 45 min, then the resin was washed three times with 500 μl TEG/wash buffer. The pelleted resin was boiled in SDS sample buffer and immunoprecipitated proteins were separated through a 7.5% SDS–polyacrylamide gel, then the radiolabelled precipitate was visualized by fluorography. For immunoprecipitations of PerPAS, cytosolic extracts (20 μl) of RK13 cells which had been programmed to overexpress either the dioxin receptor or Arnt with recombinant vaccinia virus (Whitelaw, unpublished data) were incubated with 2 μl or 5 μl of [^{35}S]methionine-labelled, *in vitro* translated PerPAS, respectively. For

dioxin receptor–PerPAS incubations, mixtures were diluted to 100 µl with TEG buffer containing TCDD (10 nM) or DMSO vehicle and protease inhibitors as described above. For Arnt–PerPAS incubations, TEG buffer with protease inhibitors was added to a final volume of 200 µl. Incubations were for 2 h at 25°C, followed by 16 h at 4°C. The mixtures were pre-cleared with pre-immune serum as described above, then incubated with 15 µl dioxin receptor or Arnt antiserum, or control pre-immune serum, for 2 h at 25°C. Precipitation with protein A–Sepharose, washing of the precipitates and detection of radiolabelled, co-immunoprecipitated PerPAS by fluorography was as described above.

Immunoblotting

CHO cells (60×10 mm dishes) were transiently transfected with CMV-driven expression vectors (6 µg) and DOTAP (25 µg, Boehringer) for a period of 12 h, followed by incubation in fresh media for 8 h. After a second identical transfection, cells were incubated in fresh media for a further 24 h, then whole cell extracts were obtained as described previously (Whitelaw *et al.*, 1993a). Samples (100 µg protein) were subjected to 7.5% SDS–PAGE and proteins transferred to nitrocellulose in a semi-dry blotter (Bio-Rad). Arnt and Arnt deletion mutant proteins were detected with previously described rabbit polyclonal antisera directed against an N-terminal peptide of human Arnt (Mason *et al.*, 1994).

Electrophoretic mobility shift assays

DNA binding assays were performed with crude cytosolic extract from Hepa1c1c7 murine hepatoma cells as described previously (Hapgood *et al.*, 1989). Briefly, mixtures of cytosolic extract (10 µl, ~5 mg/ml) and aliquots of *in vitro* translation mixtures containing Per, PerPAS or ArntΔb (5, 10 or 15 µl for each factor) were adjusted to a total input of 15 µl reticulocyte lysate with blank lysate reaction mixture, and were incubated with dioxin (10 nM) or vehicle alone (0.1% DMSO) for 2 h at 25°C. A further incubation with a 36 bp ^{32}P-3′-end labelled, double-stranded oligonucleotide XRE1 sequence from the rat cytochrome P4501A1 promoter (Fujisawa-Sahara *et al.*, 1987) was performed in a buffer of 10 mM HEPES (pH 7.9), 5% glycerol, 0.5 mM dithiothreitol, 2.5 mM $MgCl_2$, 1 mM EDTA, 0.08% Ficoll in a final volume of 50 µl containing 50 mM NaCl and 1 µg poly(dI.dC) (Pharmacia) non-specific competitor DNA. After 30 min at 25°C, protein–DNA complexes were analysed on a 4% low ionic strength native polyacrylamide gel in a Tris–glycine–EDTA buffer (Hapgood *et al.*, 1989).

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