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THE PAS SUPERFAMILY: Sensors of Environmental and Developmental Signals

Yi-Zhong Gu, John B. Hogenesch, and Christopher A. Bradfield

McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine, Madison, Wisconsin 53706; e-mail: ygu@oncology.wisc.edu, johnh@scripps.edu, bradfield@oncology.wisc.edu

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■ **Abstract** Over the past decade, PAS domains have been identified in dozens of signal transduction molecules and various forms have been found in animals, plants, and prokaryotes. In this review, we summarize this rapidly expanding research area by providing a detailed description of three signal transduction pathways that utilize PAS protein heterodimers to drive their transcriptional output. It is hoped that these model pathways can provide a framework for use in understanding the biology of the less well-understood members of this emerging superfamily, as well as of those to be characterized in the days to come. We use this review to develop the idea that most eukaryotic PAS proteins can be classified by functional similarities, as well as by predicted phylogenetic relationships. We focus on the α-class proteins, which often act as sensors of environmental signals, and the β-class proteins, which typically act as broad-spectrum partners that target these heterodimers to their genomic targets.

INTRODUCTION

A major thesis of this review is that the PAS domain is a signature of proteins that play roles in the detection of and adaptation to environmental change. For all eukaryotic PAS proteins to be included under this rubric, we must put forth a fairly broad definition of environment. Put another way, we accept the idea that through evolution, mechanisms of environmental adaptation have also been put to use in a number of developmentally important processes. Results from *Hif, Arnt,* and *Ahr* null alleles support this idea and demonstrate that in many cases, environmental stresses and developmental signals may be functionally similar. Although this relationship may not turn out to be absolute, we have found it useful to view the developing embryo as an organism adapting to the environmental challenges imposed by multicellularity.

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To make the above points, we first provide a description of three of the most well-understood PAS-dependent pathways found in higher eukaryotes. Given historical precedent and our own scientific interests, we will begin with a discussion of the aryl hydrocarbon (Ah) receptor (AHR) pathway that allows animals to adapt to environments contaminated with planar aromatic compounds. We follow this with the description of signal transduction pathways that allow organisms to adapt to changes in atmospheric and cellular oxygen [the hypoxia inducible factor (HIF) system], as well as the pathway that entrains an animal's activity to its illuminated environment (the circadian response pathway). Finally, we provide support for the environmental sensor thesis by describing more recent observations that demonstrate that rudimentary PAS domains are found in a number of light and oxygen sensors of prokaryotes and plants. For additional viewpoints on these various pathways, the reader is also referred to a number of excellent reviews (1–4).

BACKGROUND

The PAS domain is found in a rapidly growing number of proteins. The term PAS comes from the first letter of each of the three founding members of the family: PER, ARNT, and SIM (Figure 1). The PER protein, the product of the *Drosophila* (d) *Period* (*per*) gene, was discovered as a result of its involvement in the regulation of circadian rhythms (5, 6). ARNT, the AHR nuclear translocator, was originally identified as a protein that was essential for normal signal transduction by the AHR (7). SIM, the product of the *Drosophila Single-minded* locus, was

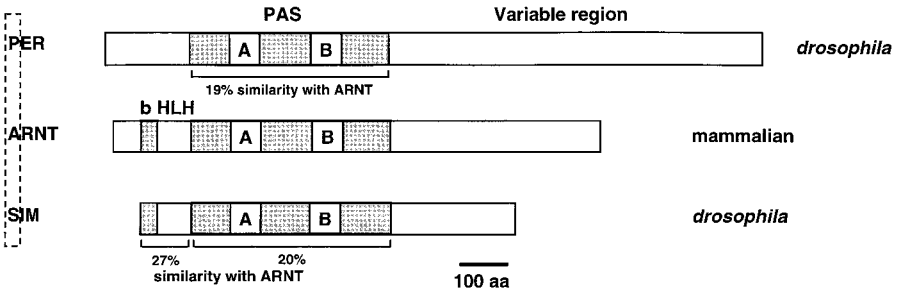

Figure 1 The founding PAS family members. Domain structures of the founding PAS proteins PER, ARNT, and SIM are shown. The name PAS stems from the first letters of PER, ARNT, and SIM (*boxed*). The basic region (b), helix-loop-helix (HLH), PAS, and C-terminal variable domains are labeled on *top*. The A and B repeat regions are shown within the PAS domain as *white boxes*. The percentage amino acid similarities of SIM and PER, as compared with ARNT, are labeled *beneath* their respective domains. See text for details.

identified through its role as a regulator of midline cell lineage (8, 9). The PAS domain is best described as a region of homology to these three founding members. It typically encompasses 250–300 amino acids and contains a pair of highly degenerate 50 amino acid subdomains termed the A and B repeats (7–9).

In higher eukaryotes, the PAS domain functions as a surface for both homotypic interactions with other PAS proteins and heterotypic interactions with cellular chaperones, such as the 90-kDa heat shock protein (Hsp90) (10, 11). In the case of the AHR, the PAS domain can also function as a binding surface for small-molecule ligands, such as the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) (12–14). Most of the PAS proteins that have been cloned to date also contain basic-helix-loop-helix (bHLH) motifs immediately N-terminal to their PAS domain. The HLH domains participate in homotypic dimerization between two bHLH-PAS proteins, and they position the basic regions to allow specific contacts within the major groove of target regulatory elements found in DNA (15, 16). Consistent with their activities as signal transduction molecules, most PAS proteins have transcriptionally active domains within their C-terminal ends. It is interesting to note that despite the relative conservation of the bHLH and PAS domains and their apparent functional similarities, most PAS proteins show little sequence homology in their C-terminal sequences.

THE AH RECEPTOR PATHWAY

One way in which vertebrates adapt to adverse chemical environments is by up-regulating batteries of xenobiotic metabolizing enzymes (XMEs), thus decreasing the biological half-life of the insulting chemical. It has long been observed that a number of XMEs are up-regulated upon exposure to their substrates (17–19). The most well-understood example is the induction of the microsomal cytochrome P450–dependent monooxygenases, such as CYP1A1 and CYP1A2, that occurs in response to exposure to planar aromatic hydrocarbons (PAHs) or dioxins (1, 20, 21). Compounds like benzo(a)pyrene and 3-methylcholanthrene are examples of a large number of highly toxic PAHs that are widely distributed in the environment. These compounds are byproducts of industrial processes, can be produced naturally, and are most commonly generated from the incomplete combustion of organic material (22). Prior exposure to many PAHs will decrease the biological half-life of structurally related compounds upon subsequent exposures. This adaptive response is a direct result of the up-regulation of XMEs.

A protein known as the AHR mediates the adaptive response to PAHs. This receptor was identified using both genetic and pharmacological approaches. Early on it was observed that the inductive response to PAHs was polymorphic among strains of mice. For example, it was observed that some murine strains were highly responsive to PAH induction of XMEs whereas others were relatively nonresponsive (23). Crosses, backcrosses, and intercrosses of these mouse lines dem-

onstrated that the difference in response was mediated by a single autosomal locus, termed *Ah* (for aryl hydrocarbon responsiveness) (24, 25). Segregation with the responsive and nonresponsive alleles is still an important method by which a role for the *Ah* locus is proven. It was later learned that halogenated aromatic compounds such as dioxin were much more potent agonists of this signal transduction system than were the PAHs (26). This greater potency was predicted to be related to their greater binding affinity for an *Ah*-encoded receptor, AHR (26). The increased binding affinity of halogenated agonists provided direction for the synthesis of high-affinity radioligands and supported a pharmacological approach to the study of this receptor (27–32).

The development of radioligands, purified receptor preparations, and AHR-specific antibodies provided initial insights into the mechanism of PAH/dioxin signal transduction. Reversible radiolabeled ligands allowed the biochemical demonstration of a saturable, high-affinity receptor present in the target cells (33, 34). Competitive binding studies allowed the correlation of congener binding affinity to biological response (27, 35, 36). Structure-activity relationships are still a central aspect of any proof that the AHR mediates a given biological response (37, 38). Radiolabeled ligands were also used to demonstrate that agonist exposure induced a change in the oligomeric state of AHR (10, 39–41). This change was coincident with a receptor species that gained a higher affinity for the nuclear compartment and specific sequences of the target DNA (30, 39, 42, 43). More recent saturation binding isotherms with ^{125}I-labeled congeners demonstrated that the AHR had a remarkably high binding affinity for halogenated agonists (e.g. the K_D of dioxin is approximately 1×10^{-12} M) (31). Related studies with these ligands demonstrated that the nonresponsive *Ah* alleles (Ah^d) encoded a receptor with a 2- to 10-fold lower binding affinity for agonists compared with AHRs encoded by responsive alleles (e.g. $Ah^{b\text{-}1}$ and $Ah^{b\text{-}2}$) (14, 44–49). The development of the photoaffinity label, [^{125}I]2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin provided the tool that led to the biochemical purification of the AHR and the generation of the first receptor-specific antibodies (31, 50, 51). These reagents also revealed the fact that size of the AHR can differ dramatically between species and strains of mice (51–53).

Protein sequence information from the purified protein lead to the molecular cloning of the receptor's cDNA and revealed that it was a member of the PAS superfamily (Figure 2) (12, 54). It is interesting to note that the ARNT protein was cloned about a year before the AHR (Figure 2). Its cDNA was cloned as the result of a genetic screen designed to identify gene products that played roles in AHR signal transduction in mouse hepatoma cells (7). In one class of signaling mutants identified in this screen, the AHR was present and bound ligand normally but did not attain an increased affinity for the nuclear compartment. A human gene fragment encoding the ARNT protein rescued this loss-of-function mutation. Further experiments demonstrated that the ARNT protein was required to direct the ligand-activated AHR to enhancer elements upstream of genomic targets similar to those found upstream of the *Cyp1a1* gene (7, 55). The realization that the

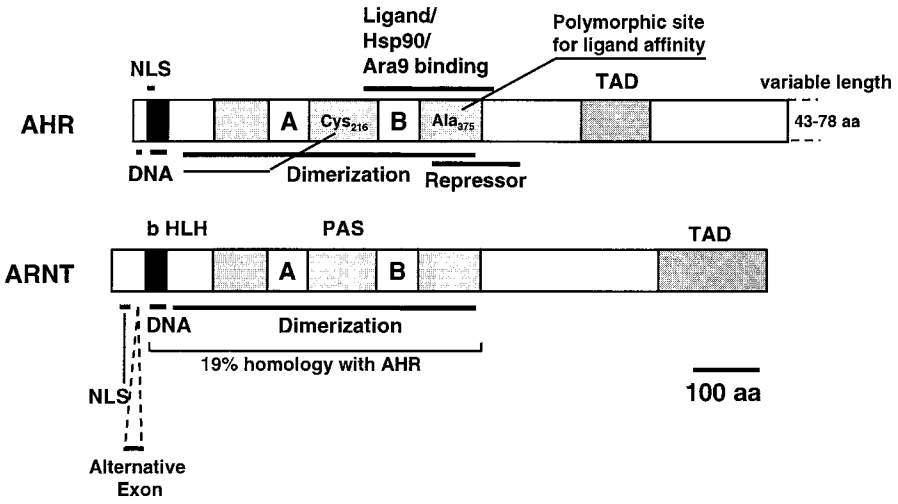

Figure 2 The molecular structures of Ah receptor (AHR) and ARNT. The basic region (b), helix-loop-helix (HLH), PAS, and transactivation (TAD) domains are labeled. The regions that have been shown to play a role in nuclear translocation (NLS), DNA binding (DNA), PAS protein dimerization, ligand/Hsp90/Ara9 binding, TAD, and repression of AHR activity are marked with *thick lines* and labeled. For AHR, Cys_{216} is marked for its role in DNA binding (264). Ala_{375} is marked for its importance for high-affinity ligand binding (14). The C-terminal end variable length represents the length of different AHR alleles (ahr^{b-1}, ahr^{b-2}, ahr^{b-3}, and ahr^{d}) in various mouse strains (14). For ARNT, the location of the alternative exon, the amino acid sequences that are involved in nuclear translocation (NLS), DNA binding, PAS protein dimerization, TAD, and its percentage similarity with AHR are marked. See text for details.

AHR and ARNT were structurally related bHLH-PAS proteins shed light on the model of AHR signal transduction and provided the first example of a bHLH-PAS heterodimer.

The current model of the adaptive response pathway to PAHs is the result of research from a number of laboratories (Figure 3). A widely held model is that the unliganded AHR is maintained in a complex with a dimer of HSP90 and additional cellular chaperones such as ARA9 (also known as AIP1 or XAP2) and p23 (56–59). The interaction of an HSP90 dimer with the AHR is essential and is believed to help fold the C-terminal half of the PAS domain in a conformation that can bind ligand (60–63). Our understanding of the roles of ARA9 or p23 is more nascent. Although the ARA9-AHR interaction was originally identified in two-hybrid screens, copurification and coimmunoprecipitation experiments have confirmed the biological relevance of this association (57, 58, 64). Recent evidence suggests that the ARA9 protein enhances signal transduction in mammalian cells by increasing the functional receptor number in the cytosolic compartment (65, 66). Presumably, the ARA9 protein acts by stabilizing the AHR-chaperone

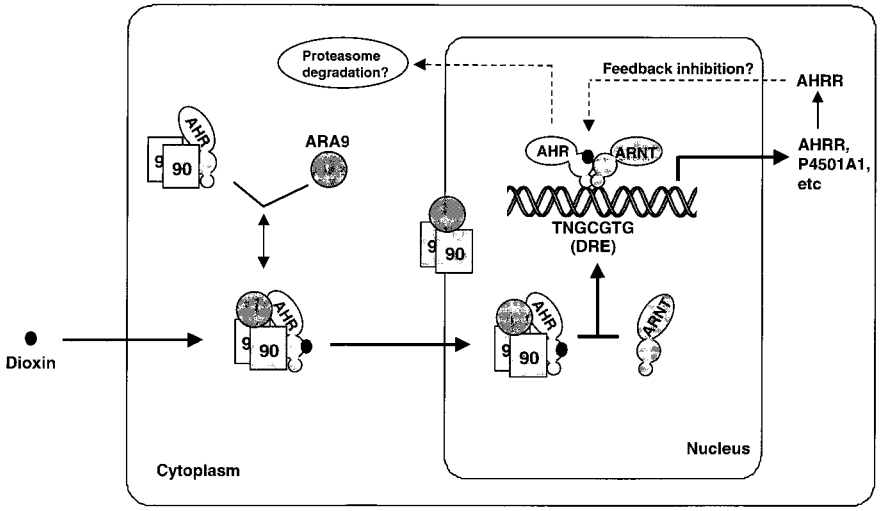

Figure 3 Model of dioxin signaling pathways. The Ah receptor (AHR) normally resides in cytoplasm with a dimer of Hsp90 holding it in a ligand binding form. ARA9 also stabilizes the ligand binding form of AHR and increases the number of functional receptors through this stabilization. On activation by its ligand, AHR translocates from cytoplasm into the nucleus and exchanges its chaperones for ARNT. The AHR-ARNT heterodimer then binds to the dioxin responsive element (DRE) with the core sequence of TNGCGTG and activates transcription of downstream target genes. Among the activated target genes, the cytochrome p450 isozymes are involved in the adaptive response, and the AHR repressor (AHRR) may form a feedback inhibition loop by competing for the binding of ARNT with AHR and by actively repressing transcription from DRE-driven promoters. AHR may finally be degraded through a proteasome pathway. See text for details.

complex. An argument against an absolute requirement for ARA9 comes from the observation that AHR functions normally in the yeast *Saccharomyces cerevisiae*, an organism that has no clear structural homologues of ARA9 (67). Recent data also suggest that the AHR is associated with the same p23 protein that is associated with the glucocorticoid receptor-HSP90 complex (59). Although this physical association is intriguing, it has not been proven that p23 has functional significance in the AHR signal transduction pathway.

The unliganded AHR complex appears to be primarily cytosolic, although in some cell types it may also be nuclear (43, 68, 69). The ligand-induced translocation from the cytosolic to nuclear compartments is associated with a reduction in the size of the receptor's oligomeric state that appears to result from a shedding of cellular chaperones and/or their exchange for the nuclear ARNT protein (39, 41, 70, 71). The nuclear localization sequences for both AHR and ARNT have recently been identified (Figure 2) (68, 72). Within the nucleus, the AHR-ARNT heterodimer is formed and becomes competent to bind specific dioxin response

elements (DREs) and drive transcription from adjacent target promoters (13, 42, 71, 73–78). Molecular analysis, coupled with the identification of consensus DREs from known target genes, has defined the core DRE as TNGCGTG (73, 75, 79–82). Evidence generated in vitro indicates that the AHR binds to the TNGC half site, whereas ARNT binds to the GTG half site (79, 80). The target genes of this adaptive response include a battery of XMEs such as CYP1A1, CYP1B1, CYP1A2, the glutathione S transferase Ya subunit and quinone oxidoreductase (1). The protein sequences required to drive transcription appear to reside in the C-terminal halves of both the AHR and ARNT (83–86). Some laboratories have proposed that these two proteins contribute differently to transcriptional activation (87, 88). For a recent review on AHR-mediated gene transcription, see Whitlock (1).

In addition to the induction of XMEs, exposure of most vertebrates to halogenated aromatic hydrocarbons, like dioxin, can lead to epithelial changes, porphyria, liver damage, thymic involution, cancer, teratogenicity, a severe wasting syndrome, and death (89, 90). Application of the pharmacological and genetic proofs outlined above indicates that the AHR is directly involved in mediating many, if not all, of these toxic endpoints. Although it should be emphasized that there is no proof that alterations in gene transcription lie at the root of receptor-mediated toxicity, there is an understandable expectation that the toxic pathway will in some way be a reflection of the adaptive pathway as defined in Figure 3. Although this may ultimately be proven true, it is important to emphasize that the pharmacological and genetic proofs for AHR involvement do not necessarily implicate the ARNT protein or DRE-mediated gene expression in any toxic mechanism. Thus, although the mechanism of XME induction is well characterized, the molecular mechanism underlying most aspects of the AHR-mediated toxic response is currently unknown.

Other than a role for the AHR, little is understood about the mechanisms that underlie most of dioxin's toxic effects. Two important exceptions are that tumor necrosis factor α has been implicated in dioxin-induced hyperinflammation, and there is recent evidence to indicate that thymic involution may be the result of dioxin signaling in bone marrow stromal cells (91–94). In a search to explain this broad spectrum of toxic effects, links have been proposed between the AHR and the regulation of genes involved in epithelial cell growth and differentiation (95, 96) as well as in dioxin-induced alterations in the levels of various cytokines (91, 97–99). Potential mechanisms for toxicity include the existence of low-affinity DRE sites upstream of genes involved in the above processes, the potential for the AHR to signal through nontranscriptional pathways, and the possibility that the AHR competes for and sequesters ARNT or other limiting factors from parallel cellular pathways (see cross talk below) (90, 100–102). In our view, each of these potential mechanisms is plausible, yet none has particularly compelling experimental support.

Recent data suggest that other PAS proteins may be involved in dioxin signaling. A novel PAS protein, an AHR repressor (AHRR), containing only one of

the conserved 50 amino acid PAS repeats, was found to inhibit AHR signal transduction (103). This repression appears to be the result of two events. First, when expressed, the AHRR is a constitutively active protein that competes with AHR for ARNT dimerization and DRE binding. Second, the AHRR protein has activity as a transcriptional repressor and may directly inhibit gene expression from DRE-linked promoters. It has been suggested that AHRR may be a part of a negative feedback loop to down-regulate or attenuate an activated AHR pathway. The feedback inhibition idea is based upon the observations that the AHRR promoter is driven by a functional DRE and that the level of the AHRR mRNA is up-regulated by agonists of the AHR (103). It is interesting to note that the AHRR is now one of two mechanisms by which the AHR signal can be down-regulated. It has also been demonstrated that the ligand-activated AHR is rapidly proteolyzed, leading to a decreased receptor number immediately following agonist exposure of many cell types (104–106). This agonist-dependent degradation may provide an additional mechanism to attenuate this response and to protect cells from the consequences of prolonged exposure to high concentrations of agonists.

A close structural homologue of the ARNT molecule (ARNT2) has also been described (107, 108). The ARNT2 protein has been proposed to play a role in PAH/dioxin signal transduction by acting as an alternate partner for the AHR (107). This assertion is based upon the observations that ARNT2 dimerizes with the AHR in vitro and that the resultant complex is capable of driving transcription from a DRE-linked promoter in a heterologous expression system. Developmental profiling suggests that if ARNT2 is active in AHR signal transduction, it may only be playing a role in a small subset of cells. Side-by-side analysis of the AHR, ARNT, and ARNT2 expression in the developing mouse embryo has been performed by in situ hybridization (109, 110). These results suggest that although the AHR and ARNT are coexpressed in a variety of cell types, ARNT2 is expressed in the central nervous system, primarily in areas where the AHR expression is low (107, 109, 110). This result suggests that at most cellular locations, ARNT is the more common partner of the AHR in vivo and that ARNT2 has other important biological roles (see below). Another ARNT homologue, MOP3 (member of PAS 3) [also called BMAL1 (brain and muscle ARNT-like protein 1)] (111–113) is coexpressed with the AHR in a number of cell types (111–113). Yet, initial studies indicate that the dimerization affinity between MOP3 and AHR may be too low to have consequences in vivo (111, 114). Thus, ARNT may be the most prominent bHLH-PAS partner of the AHR that has been cloned to date, with the roles of ARNT2 and MOP3 in this pathway still to be understood.

It is hard to imagine that the AHR and ARNT evolved solely as a defense against PAHs or related environmental toxicants. If this idea is correct, then the characterization of functional AHRs and ARNTs in marine, aquatic, avian, and mammalian species is an indication that there has been significant evolutionary pressure for conservation of this adaptive system due to a common chemical stress (7, 12, 115–117). A second explanation for the conservation of this system is that the AHR-ARNT dimer has a physiological purpose in addition to its role in

xenobiotic adaptation. Evidence to support an important physiological role has come from gene targeting experiments in mice, where the AHR has been shown to have an important role in mouse development. Mouse strains deficient in the AHR protein have recently been developed by three independent laboratories (118–121). Although there are some phenotypic differences, these mouse lines commonly show defects in liver development, decreased animal weights, and poor fecundity (118, 119). As predicted, AHR null mice fail to show up-regulation of XMEs in response to agonists or the classical toxic endpoints on exposure to dioxins (119, 122, 123). Taken together, these data suggest that the developing organism has a developmental requirement for this adaptive pathway, possibly due to some unavoidable or endogenous toxicant, and/or that the receptor system plays other roles in addition to its known adaptive functions.

HYPOXIA RESPONSE PATHWAY

Hypoxia can stimulate a variety of systemic, local, and cellular responses (124). In mammalian systems, the systemic response includes the transcriptional up-regulation of the gene encoding the peptide hormone erythropoietin (EPO). This cytokine increases the red blood cell count by stimulating erythropoiesis, thus increasing the efficiency of O_2 transport throughout the body (125). A second aspect of the systemic response to low oxygen tension is the increase in respiration rate that occurs through dopaminergic input to the carotid body. The up-regulation of dopamine is due to the hypoxia-induced transcriptional activation of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis (126). Local areas of hypoxia can arise during embryogenesis, wound healing, and tumor growth. In these processes, hypoxic tissues up-regulate the transcription of genes encoding various angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (127–129), as well as vasodilators produced by enzymes, such as inducible nitric oxide synthase and heme oxygenase-1 (130, 131). The up-regulation of these factors results in an increased vascular bed density, vascular permeability, and oxygen availability to the starved tissues. At the cellular level, hypoxia can limit oxidative metabolism and thus decrease energy production. To adapt to a low-oxygen environment, many cell-types convert to glycolysis for energy. This cellular response is mounted through the transcriptional activation of genes encoding glycolytic enzymes such as aldolase A, phosphoglycerate kinase 1, lactate dehydrogenase A, and phosphofructokinase L and glucose transporters such as GLUT-1 (132–135).

The connection between oxygen homeostasis and PAS proteins was revealed through studies designed to understand the regulation of hypoxia-induced genes, such as *Epo*. Early on, the up-regulation of EPO production by hypoxia was shown to be due in large part to increased transcription of the *Epo* gene (136, 137). This regulation was shown to be mediated by a hypoxia-inducible factor

(HIF1), which bound to a hypoxia responsive element (HRE) found in a region of the *Epo* gene that corresponded to the 3' untranslated portion of its mRNA (136, 138, 139). Purification of the HIF1 protein from induced HeLa cells revealed that this transcription factor was composed of two subunits, HIF1α and HIF1β (140, 141). Amino acid sequence analysis demonstrated that HIF1β was identical to the ARNT protein previously shown to be required for AHR signal transduction (140, 141). Protein sequencing and cDNA cloning experiments also demonstrated that the HIF1α subunit was a novel member of the PAS superfamily. The characterization of the HIF1α-ARNT dimer provided the second example of a bHLH-PAS heterodimer that played an important role in sensing and adapting to environmental change.

The observation that HIF1 was a heterodimer of two bHLH-PAS proteins suggested that the mechanism underlying the hypoxia response would share certain features with that of PAH/dioxin signal transduction pathway (Figure 4). In this regard, the core sequences found in the HRE and the DRE share a number of similarities. Based upon functionally active HREs identified in known target genes, the core consensus sequence for the binding of HIF1α-ARNT dimer has been defined as either 5'-TACGTG-3' or 5'-RCGTG-3' (133, 142). Methylation interference assays support this core element and indicate contact between the HIF1 dimer and all four guanine residues found in both strands of the *Epo* HRE, i.e. 5'-TA*CG*T*GC*T-3' (143). This result is consistent with the consensus data and indicates that contacts between HIF1α and its response element extend beyond the minimal core sequence. The length of the core sequence and the idea that recognition extends beyond the core element is similar to that seen for the AHR-ARNT heterodimer (79, 80, 144, 145). Based upon these similarities, one can predict that the ARNT protein maintains the same half-site specificity within the HRE as it does within the DRE (i.e. the 3' GTG half-site). This prediction is based upon the observations that the AHR has been definitively shown to bind to the 5' TNGC half-site and ARNT to the 3'GTG half-site of the core DRE sequence, TNGCGTG (79, 80). It follows that the HIF1α subunit would bind to the 5' TAC half-site of the HRE whereas ARNT would bind to the 3' GTG. This latter prediction has not been formally tested.

The mechanism by which HIF1α transduces the hypoxia signal is an area of active investigation. Early studies of hypoxia-driven gene expression by pO_2, iron chelators, and divalent metal ions provided evidence that a heme protein was involved in this process (125). Experiments using antibodies against HIF1α indicated that the levels of the HIF1α protein rise dramatically in response to hypoxia, desferoxamine, or $CoCl_2$, whereas levels of the ARNT protein are relatively nonresponsive to these treatments (100, 140, 146). This observation is consistent with the idea that HIF1α functions directly in the hypoxia sensor pathway and that ARNT is a constitutively active factor required for the hypoxia signal to reach its nuclear targets. Thus, it seems fair to describe HIF1α as a sensor and ARNT as a broad-spectrum partner. In keeping with the terminology of the hypoxia field,

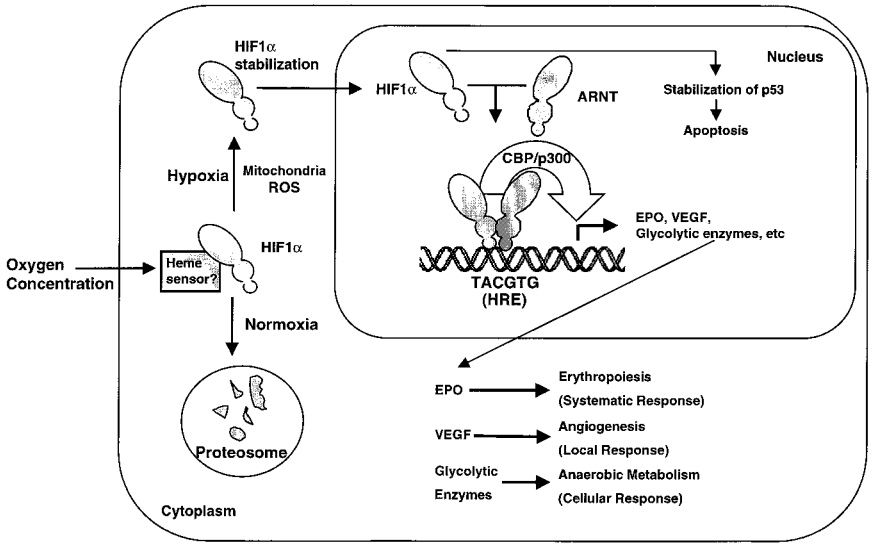

Figure 4 Model of hypoxia signaling pathways. Under normoxic conditions, HIF1α protein is degraded through the ubiquitin-proteasome pathway. When a cell is exposed to a hypoxic environment, the HIF1α protein is stabilized. A heme protein and/or the generation of mitochondrially generated reactive oxygen species (ROS) may be necessary for hypoxia induction of HIF1α protein levels. HIF1α protein is translocated from cytoplasm to nucleus, where it dimerizes with ARNT. The HIF1α-ARNT heterodimer then binds to the hypoxia responsive element (HRE) and activates the transcription of downstream target genes. The transcription coactivator CBP/p300 is involved in this transcription activation event. The target genes include erythropoietin (EPO), vascular endothelial growth factor (VEGF), and a series of glycolytic enzymes involved in the systematic, local, and cellular hypoxic responses. HIF1α also is involved in the stabilization of p53 protein and may play a role in the hypoxia-induced apoptosis. See text for details.

as well as of that proposed at the outset of this review, we propose to classify these proteins as α and β class, respectively.

The primary mechanism by which hypoxia regulates HIF1α appears to reside mainly at the level of the protein. In the majority of published reports, the levels of HIF1α protein rise in response to hypoxia, $CoCl_2$, etc, whereas its mRNA level remains stable (146–149). Moreover, it has been observed that under normoxic conditions, the HIF1α protein undergoes rapid degradation via the ubiquitin-proteasome pathway, whereas under hypoxic conditions the protein is relatively stable (138, 143, 150–152). The mechanism by which the HIF1α protein's stability is achieved is poorly understood, although a dependence on the ARNT protein has been proposed (147). Metabolic inhibitors, such as cycloheximide, actinomycin D, and 2-aminopurine, have been used to demonstrate a requirement for cellular translation, transcription, and protein phosphorylation in the hypoxia

response (138, 143, 153). Furthermore, results from a number of laboratories have indicated that HIF1α activation is dependent on the red-ox state of the cell (148, 150, 154). Recently, it has been shown that hypoxia- but not Co^{2+}-induced HIF1α activation requires the production of reactive oxygen species (ROS) that is dependent on the mitochondria. This observation suggests that hypoxia and Co^{2+} induction of HIF1α may employ different pathways (154). Taken in sum, these results suggest an extremely complicated regulatory mechanism for the stabilization of the HIF1α protein. To add to this complexity, results from a number of labs have indicated that under certain circumstances, the level of HIF1α mRNA can also be up-regulated (143, 155, 156). Although this has not been a widely reproduced finding, it should not be discounted and may be an indication that alternative methods of regulation are at play. In this regard, recent reports have demonstrated that IFN-β, but not IFN-α or IFN-γ, can up-regulate HIF1α mRNA by about sevenfold in a human fibrosarcoma cell line (157).

The bHLH and PAS domains of HIF1α and ARNT domains confer both DNA binding and dimerization specificity, as would be predicted based upon data from the AHR and ARNT studies (158). Using GAL4 chimeras and a GAL-UAS–driven reporter system, two hypoxia responsive domains (HRDs) have been mapped to the C-terminal half of HIF1α (134, 159, 160). The exact boundaries and identifiers for these domains vary between labs. For simplicity, we use the definitions of Jiang et al (160). That is, HRD1 lies between residues 531 and 575 and HRD2 lies between residues 786 and 826 of the human HIF1α protein (160). It is interesting to note that these two HRDs appear to influence HIF1α activity by different mechanisms. The HRD1 domain responds to low O_2 tension by stabilizing the HIF1α protein (159, 160), whereas HRD2 appears to act as a hypoxia-activated transcriptionally active domain (134, 159, 160). The transactivational activity of HIF1α is found to be potentiated by the general coactivator CBP/p300 (161, 162).

Just as in the AHR system, screens of expressed sequence tag (EST) databases have added to the number of PAS proteins at play in the hypoxia pathway. In addition to HIF1α, two α-class homologues have the capacity to sense low-oxygen tension, HIF2α (also called EPAS1 or MOP2) and HIF3α (107, 111, 112, 163–166). HIF2α was identified by a number of laboratories and is highly homologous to HIF1α in the bHLH-PAS domains (111, 164–166). In addition, HIF2α contains structural and functional similarity to the HRD1 and HRD2 domains found in HIF1α (167, 168). In contrast to the widespread expression of HIF1α, HIF2α is expressed mainly in endothelial cells and in certain nonendothelial tissues such as the olfactory epithelium and the adrenal gland (109, 165). HIF3α is a newly identified hypoxia-inducible factor that shares considerable sequence homology with HIF1α and HIF2α in the basic region, the HLH and PAS domain. It dimerizes with ARNT, and this complex activates transcription of reporter genes driven by HRE elements in a heterologous expression system (163). Less is known about the expression of the 3α homologue, although preliminary evidence from our laboratory suggests high-level expression in the developing trachea and

olfactory epithelium (Y-Z Gu, SM Moran & CA Bradfield, unpublished observation). It is interesting to note that both sequence analysis and functional analysis suggest that HIF3α contains an HRD1 domain but does not harbor a domain equivalent to HRD2 (163).

The ARNT2 and MOP3 homologues of ARNT (described above) may also play roles as β-class partners of the α-class HIF sensor subunits. The ARNT2 protein shares 81% identity with ARNT in the bHLH-PAS domains (57% overall sequence identity) and thus was predicted to be a second partner of AHR (107, 108). In DNA binding assays, the ARNT2 protein is able to substitute for ARNT, directing HIF1α, HIF2α, and HIF3α to HREs (JB Hogenesch, Y-Z Gu & CA Bradfield, unpublished results). Coupled with these in vitro results and the overlapping developmental profiles of ARNT2 and the α-class HIF proteins, it seems highly likely that these proteins are biologically relevant partners in vivo (109). The third ARNT homologue MOP3 was originally cloned in EST screens for novel PAS-encoding cDNAs (111–113). It has homology with the ARNT protein in both its bHLH and PAS domains (66% and 40% identity, respectively). Although MOP3 and HIF1α are coexpressed in a number of tissues, MOP3 is a fairly weak dimerization partner of the α-class HIFs (109, 114). Thus, it is unclear if MOP3 plays a significant role in hypoxia signal transduction.

To understand the biological roles of these PAS proteins in hypoxia signaling, a number of gene inactivation models have been exploited. Murine strains lacking the ARNT protein were the first to be developed (169, 170). These mice displayed embryonic lethality between 9.5 and 10.5 days of gestation. Although some controversy remains as to whether the yolk sac circulation was affected, both ARNT knockout mouse strains display major blocks in developmental angiogenesis, which suggests that this failure is the primary cause of embryonic lethality (169, 170). The phenotype of the ARNT null mice provided important genetic support for the idea that hypoxia is an important signal in normal development. Null alleles at loci encoding either of the other putative β-class partners, i.e. ARNT2 or MOP3, have not been reported.

In keeping with the idea that hypoxia is an important signal for normal development, mice homozygous for disruption at the *Hif1*α locus (*Hif1*α*-/-*) display embryonic lethality at day 11 of gestation with neural tube defects, cardiovascular malformation, and lack of cephalic vascularization (171, 172). In addition, these mice display a lack of the classic hypoxia responses, such as the up-regulation of VEGF, glycolytic enzymes, and glucose transporters (171–173). This phenotype is similar to the ARNT null mice discussed earlier and is consistent with the role of HIF1 heterodimer in the hypoxia-driven transcriptional activation of a number of genes involved in developmental angiogenesis, such as VEGF, FGF, and PDGF. Like many null alleles, the phenotype of *Hif2*α null mice provided a surprising result. The *Hif2*α*-/-* embryos die at day 12.5 of gestation because of pronounced bradycardia related to substantially decreased catecholamine levels (174). This phenotype is consistent with the high level of expression of HIF2α in the organ of Zuckerkandl (OZ), the principal source of catecholamine production (174).

Because of the early death of HIF2α homozygous embryo, it is unclear whether HIF2α plays a role in hypoxia responses in later stages of life or whether this protein plays a role in angiogenesis, as might have been predicted based upon its expression in the vascular endothelium. Conditional knockout of this gene may shed more light on such physiological functions.

The above discussion should also highlight the fact that hypoxia signal transduction is a complex pathway and that this pathway is likely to take multiple forms in different cell types and under different physiological conditions. Moreover, attempts to model this biology must explain how a similar response can be elicited from low O_2 tension, ferric ions, cobalt, and oxidative stress. In addition, it must explain how some responses to hypoxia can be evoked at moderately low levels (8% O_2) (175, 176), whereas other responses require more extreme hypoxia (close to 1% O_2) (177). It must also take into account the apparent redundancy of both the α- and β-class partners (e.g. HIF1α, HIF2α, HIF3α, ARNT, and ARNT2), as well as the multiple levels of control that appear to be exerted over this system (i.e. evidence for the importance of translation, transcription, phosphorylation, ROS, heme, and protein stability).

This complexity leads us to apply Occam's razor and state the simplest possibility, that the α-class HIFs are in fact the heme sensors themselves or that they directly interact with an upstream PAS-containing heme sensor. If we use the similarity to the AHR system (above), as well as the prokaryotic systems (below), we might also predict that the PAS domain is the region of the α-class HIFs or the upstream protein that senses oxygen through a bound heme group. One version of this model would be that the α-class HIF senses the oxygen environment as it comes off of the ribosome. This oxygen sensing could occur through the integration of a heme moiety or through a PAS-PAS interaction with an upstream heme-containing PAS protein. Under low oxygen, the α-class HIF conformation could be such that it avoids the ubiquitination, whereas in normoxia, the folded state leads the protein down a rapid degradative pathway. Such a model is based more upon our belief that the AHR is a prototype of signaling through PAS proteins rather than an exception.

Experiments from a number of laboratories have demonstrated the importance of heterodimerization of the α- and β-class subunits in the regulation of the systematic, local, and cellular responses to hypoxia (2, 127, 132, 133, 136, 139). Despite the importance of heterodimerization, recent evidence also suggests that HIFs may signal through heterologous interactions with non-PAS containing proteins. In this regard, HIF1α has also been shown to be involved in the stabilization of p53 protein and may play a role in hypoxia-induced apoptosis (178) (Figure 4). This stabilization appears to be directly related to protein-protein interactions between HIF1α and p53 protein. Such a mechanism of protein stabilization may be a common activity of HIF1α. More recent evidence has suggested that the interaction between HIF1α and the VHL protein, the product of the von Hippel-Lindau (VHL) tumor suppressor gene, is necessary for the oxygen-dependent degradation of HIF α subunits (179). Such a relationship may explain the highly

vascularized tumors of VHL patients because α-class HIF subunits would be constitutively up-regulated in the absence of the VHL protein (180).

THE CIRCADIAN RESPONSE PATHWAY

Biological clocks help entrain an organism's activity to changes in daily and seasonal environment. In keeping with our thesis that PAS is a signature of proteins involved in environmental adaptation, we view diurnal changes in light and temperature as some of the most fundamental environmental variables that challenge terrestrial species. To meet this environmental challenge, circadian rhythms of various biological activities are maintained through both an internal clock and responsiveness to environmental cues that keep that clock in tune (3).

Nowhere is the PAS domain more prominent and nowhere are the PAS proteins more structurally diverse than in pathways regulating circadian rhythmicity. Vertebrates and invertebrates employ orthologues of a number of PAS proteins, including PER, CLOCK, and MOP3, to control this important biological process (3). Even simple eukaryotes, such as the slime mold *Neurospora crassa*, control circadian rhythmicity through the gene products of the *white collar* loci. The WHITE COLLAR (WC) proteins, WC-1 and WC-2, display a single PAS repeat motif instead of containing the signature A and B domains found in the PAS domains of *Drosophila* and mammals (8, 181). WC proteins have been shown to be required for transcriptional activation of *Neurospora* circadian responsive gene *FRQ* and are essential for maintaining circadian rhythms (181). Like the PAS domains of their mammalian counterparts, the PAS domains of the WC proteins appear to serve as dimerization surfaces. This is demonstrated by the fact that these domains mediate the formation of WC homodimers and heterodimers, as well as the formation of WC-heterodimers with mammalian PAS proteins such as the AHR (182, 183).

The first PAS factor ever to be characterized was the product of the *Drosophila period* (*per*) locus, a regulator of the circadian rhythms of locomotor activity (5, 6, 8). Proof for the involvement of this locus in rhythmicity was provided through genetic screens for mutants with an aberrant circadian free-running time (184, 185). The three original mutant alleles at this locus were designated per_s (*short*), per_l (*long*), and per_0 (*null*). The *per* locus encodes a 1218–amino acid protein generated from eight exons (8). When SIM and ARNT were later cloned, the consensus PAS domain emerged from sequence comparisons (186). Compared with the wild type, the per_s mutants shorten whereas the per_l mutants lengthen the free-running locomotor rhythms. The per_0 mutants are arrhythmic under free-running conditions. It is interesting to note that the per_l mutation (valine 243 to aspartic acid) resides within the PAS domain whereas the other two mutations reside immediately C-terminal to the PAS domain (i.e. per_s mutation, serine 589 to asparagine; per_0 mutation, glutamine 464 to stop). A second observation that supports a role for this locus in rhythmicity is that the PER protein and mRNA

levels oscillate in a circadian manner (187, 188). Not only does the PER protein level display a rhythm, its localization into the nucleus also appears to be regulated in a similar fashion (189–191).

A number of early observations provided important insights into how the PER protein might work at a molecular level. The cloning of the dSIM and the mammalian ARNT and AHR cDNAs revealed that the dPER protein was a unique member of this superfamily in that it did not harbor a bHLH domain. It was also shown that the PAS domain of PER could act as a dimerization surface to support interactions with other PAS domain-containing proteins, such as SIM and ARNT (192). Coupling these observations to the evidence that constitutive overexpression of PER inhibited the cycling of its own mRNA (193) led to the suspicion that PER was a dominant negative inhibitor of its own transcription (possibly acting by inhibiting another bHLH-PAS pair) (114). Such a model had precedence from a similar mechanism in the myoD field (194). This model has now gained support from experiments in both mice and flies (see below) (195–198).

The fact that PAS proteins were found to play important roles in the circadian rhythm pathways of organisms as diverse as arthropods and mammals has allowed investigators to apply what is learned from one model system to another. This convergence of ideas has led to a rapid advancement in the field and the development of a model that describes the core working of the circadian clock in a wide variety of species. The first mammalian gene shown to play a central role in maintenance of rhythmicity was encoded by the murine *Clock* locus (199). Because there was precedence for autosomal dominant and semidominant mutations in *Drosophila* and *Neurospora* (6, 181), a genetic screen was employed to identify mutations in mice that would alter their normal free-running period of 24 h (199). In an ethylnitrosourea (ENU) mutagenesis screen of mice, a mutant locus, called *Clock*, was identified that displayed a free-running period of slightly longer than 25 h. Mice homozygous for the *Clock* mutant allele had an even longer period, and this period degraded more quickly while in free-running conditions (200). The positional cloning of the murine *Clock* gene was undertaken by a number of parallel approaches and was aided by the identification of exons encoding a bHLH-PAS domain within the target genomic region (199). Complementation with the corresponding bacterial artificial chromosome confirmed the biological importance of the PAS gene product (201). The ENU-induced mutation in *Clock* was found to generate the deletion of a single exon encoding a region within the C terminus of the CLOCK protein (199). Corresponding regions in other bHLH-PAS members have been shown to harbor transactivation activity (83, 86, 160, 167).

Following the cloning of murine *Clock*, three mammalian homologues of *Drosophila* PER were identified by genomic sequencing, searches of ESTs, and degenerate polymerase chain reaction (202–205). Like their *Drosophila* homologue, mRNA levels of the mammalian PERs responded to light and phase shifted in a circadian manner in the suprachiasmatic nucleus, the site of the master cir-

cadian oscillator in mammals. Another observation that allowed the decoding of the circadian clock pathway was the demonstration that a 69-bp region upstream of the *Drosophila per* promoter was sufficient to drive in vivo cycling of synthetic reporter genes in flies (206). Further analysis revealed that an element harboring an E-box core sequence, *CACGTG*AGC (the E-box is underlined), was necessary for the robust amplitude of this cycling (206). These results coupled with the observation that β-class PAS proteins such as ARNT recognize the 5'GTG of an E-box prompted the idea that this element was bound by an α- and β-class bHLH-PAS heterodimer. These observations were also consistent with the idea that the PER protein acted as an inhibitor of a CLOCK-bHLH-PAS heterodimer in both *Drosophila* and mammals.

The characterization of this E-box–bound heterodimer came in a flurry of papers shortly after the cloning of CLOCK. It is interesting to note that the partner of CLOCK was identified as the putative β-class bHLH-PAS protein, known as MOP3/BMAL1, that was pulled out in earlier EST screens (111, 112). Using yeast two-hybrid screens, it was determined that a partnership was formed between the mammalian MOP3 and CLOCK proteins in vivo (114, 195, 207). In parallel experiments, *Drosophila* homologues of these proteins were identified as the result of screens for circadian rhythm mutants (208, 209). Mutations in the *Drosophila cycle* and *clock* loci were shown to result in a significant decrease in the expression of both PER and TIM, and both mutations resulted in arrhythmia as homozygous alleles (208, 209). Sequence analysis of the gene products of these loci indicated that they were orthologues of the mammalian MOP3 and CLOCK, respectively. These experiments provided both the biochemical and genetic proofs that MOP3 (CYCLE) and CLOCK were components of the circadian clock in various species and that the mammalian and *Drosophila* circadian pathways would be functionally similar.

Proof that the MOP3-CLOCK heterodimer bound the circadian response element came from three lines of evidence. First, our own lab was aided by the fact that a CLOCK homologue, MOP4/NPAS2, was identified from earlier EST screens like those that revealed MOP3 (111, 210). The observation that the CLOCK and MOP4 proteins share 84% and 73% identity in their bHLH and PAS domains, respectively, indicated that MOP3-MOP4 interactions provided a model system for MOP3-CLOCK interactions. In a randomized screen for the response element recognized by the human MOP3-MOP4 heterodimer, the sequence *CACGTG*ACC was identified and named the M34 responsive element (M34RE) (114). Heterologous expression experiments demonstrated that MOP3-MOP4 and MOP3-CLOCK combinations were capable of driving transcription from this M34 element (114). It is important to note that this sequence differs at only a single nucleotide (*CACGTG*AGC) from the circadian enhancer found upstream of the *Drosophila per* gene (206). In addition, this sequence appears three times in the structural gene of the hPER3 gene. Second, in parallel experiments, other laboratories had also demonstrated that a similar circadian element was harbored

in the mammalian PER1 promoter, and that the CLOCK-MOP3 heterodimer was capable of driving transcription of synthetic promoters harboring this element (207). Third, concurrent experiments from *Drosophila* also supported this model. These experiments demonstrated that the *Drosophila* CLOCK activates *per* transcription through the previously described 69-bp 5' flanking sequence containing the E-box element (195). It was also shown that PER and TIM could inhibit CLOCK/CYCLE-induced transcription of their own messages (195), which supports the feedback inhibition hypothesis for PER and provides a role for two additional bHLH-PAS protein in circadian rhythms of flies, mice, and humans.

The circadian rhythm pathway also depends on a number of heterotypic interactions between bHLH-PAS proteins and non-PAS proteins. The *Drosophila* protein timeless (TIM) was the first such non-PAS protein to be identified (190, 191). The cyclic expression of TIM appears to dictate the timing of PER protein accumulation and nuclear localization, and it is hypothesized that PER and TIM translocate to the nucleus as a complex (191, 211, 212). In addition, both the TIM message and protein levels have been found to cycle in a circadian manner (211, 213). The recently cloned *Drosophila* gene *double-time* (DBT) encodes a protein closely related to human casein kinase Iε and fine-tunes the length of circadian rhythm by promoting PER phosphorylation and subsequent degradation (214, 215). In *double-time* mutants, the PER protein accumulates in the nucleus in a noncircadian fashion, leading to abnormal locomotor rhythms.

Besides TIM and DBT, other heterotypic non-PAS interactors are involved in circadian regulation. Although the PER protein oscillates and phase shifts in response to light, PER may not be the first molecule that senses light. In fact, evidences suggest that there is an upstream mediator of the light-sensing process. Recently, the *Drosophila* cryptochrome (CRY) and mouse CRY1 and CRY2 cDNAs were cloned and were found to be members of the plant blue-light photoreceptor and photolyase family (216, 217). Mutational analysis proved that these proteins were essential for maintenance of circadian rhythms (216, 218–220). Both the *Drosophila* CRY and mammalian CRYs are shown to interact with the core components of the circadian clock, PER and TIM (196, 221). The functional consequences of this interaction are the relief of the repression by PER and TIM in flies, thereby allowing for light input into the circadian clock (196, 221).

The research of the past few years has led to a plausible mechanism for cellular mRNA oscillations (Figure 5): Two bHLH-PAS transcription factors, CLOCK and MOP3, form a heterodimer and bind to response element sequences termed M34RE (or a circadian responsive E-box). These elements are present in the enhancer/promoter regions of circadian-regulated genes such as *per.* As a result, The MOP3-CLOCK heterodimer positively regulates the levels of circadian responsive gene products (191). In return, PER and TIM negatively regulate the CLOCK/MOP3 complex, either by binding to one member of the complex and disrupting its function or by indirectly influencing the signaling of the MOP3/CLOCK complex through interactions with the basal transcriptional machinery (195–198). These mechanisms are in agreement with the observation of feedback

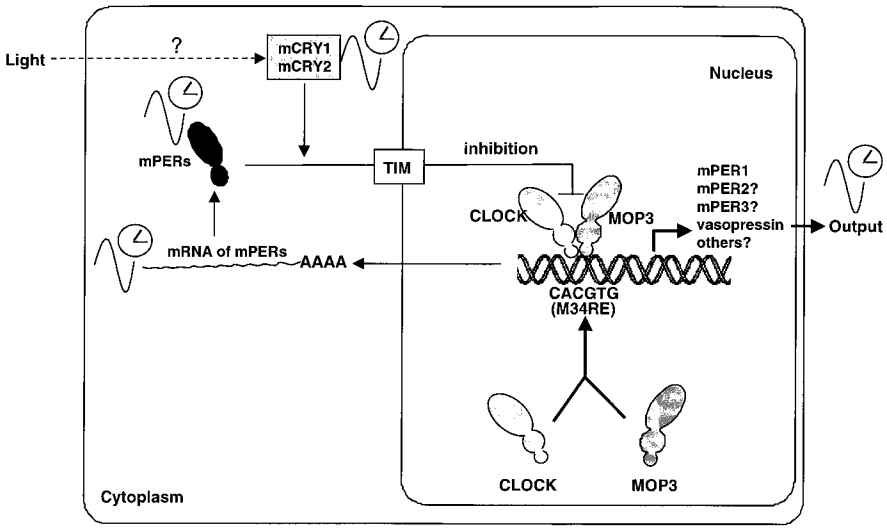

Figure 5 Model of the mammalian circadian response pathways. The mammalian and fruit fly circadian response pathways may be slightly different; thus, the model of the mammalian pathway is shown here. The expression of PERs and CRYs are circadian regulated (*clock icon*). CLOCK and MOP3 form a heterodimer. The heterodimer binds to the responsive element M34RE (the MOP3 and MOP4 responsive element) containing the core sequence of CACGTG and activates the transcription of downstream target genes such as mPER1 and vasopressin. It is not known whether or how CRYs respond to light, but mCRYs interact with mPERs and help in translocating mPERs from cytoplasm into nucleus. PER, TIM, and CRY can block the CLOCK-MOP3–dependent transcriptional activation and, therefore, complete the feedback inhibition loop of PER. See text for details.

inhibition by PER and TIM. Because these genes negatively regulate their own transcription, and because there is a delay between their translation and functional interference in the CLOCK-MOP3/CYCLE complex, an oscillation occurs and is maintained (Figure 5).

PAS PROTEINS IN OTHER RESPONSE PATHWAYS

Our choice to focus on AHR, hypoxia, and circadian biology was the result of our interest in emphasizing pathways where bHLH-PAS heterodimers played important roles. A number of excellent reviews are recommended to learn more about these pathways (4, 222–224).

Among the important signal transduction molecules not discussed here is one of the founding members of this superfamily, the product of the *single-minded* locus (SIM) (225). This protein is a regulator of midline development in flies. The recent cloning of two mammalian SIM homologues has also been reported

(SIM1 and SIM2) (226–230). Although it has not been clearly shown that either of the mammalian SIMs is a bona fide orthologue of the *Drosophila* SIM, gene-targeting experiments have demonstrated that homozygous *Sim1* mutant mice die shortly after birth. The death is due to developmental defect of the hypothalamic-pituitary axis marked by a lack of secretory neurons (231).

The product of the *Drosophila trachealess* locus (TRH) is another interesting bHLH-PAS protein that is essential for the development of tracheal pits in *Drosophila* (232, 233). Given the role hypoxia has as a developmental signal in the development of vascular tubes (234), it is tempting to speculate that trachealogenesis may also respond directly to low oxygen and that TRH is a sensor in that pathway (232, 233). It is interesting to note that in vitro experiments indicate that the mammalian SIMs dimerize with ARNT and that both the dSIM and dTRH appear to dimerize with TANGO, the putative *Drosophila* orthologue of ARNT (235, 236). Using our simplified classification scheme, this would lead us to predict that TRH and the SIMs are sensors of some input signal (α-class PAS proteins). Another *Drosophila* PAS protein that has been identified is Similar (dSIMA) (237). dSIMA contains both bHLH and PAS domains and is inducible by hypoxia, cobaltous ions, and desferrioxamine in transient transfection experiments (238), which suggests that dSIMA functions as a hypoxia sensor in *Drosophila* and that the hypoxia signaling pathway is also conserved between flies and humans.

Not all proteins fit neatly under the title of sensor or broad-spectrum partner or have been shown to participate in heterodimeric interactions to bind DNA. Most notably, three bHLH-PAS proteins have been shown to act as coactivators for members of the nuclear receptor superfamily (239). This surprising result came from a number of two-hybrid screens using steroid receptors as the baits. The cDNA identified in these screens were a unique γ-class of PAS proteins, commonly referred to as coactivators (240–244) (Figure 6). These coactivators mediate the interaction of the nuclear receptors and the transcriptional activator/integrators such as CBP/p300 and are required for the full transcriptional activity of the nuclear receptors (241, 242, 245). It is interesting to note that it appears that neither the bHLH nor PAS domains of these proteins are required for coactivator activity (246). This suggests that this class of proteins may have more than one cellular role and that bHLH-PAS partners of this class of proteins may also exist.

SENSORS, PARTNERS, AND COACTIVATORS

An examination of the bHLH-PAS superfamily suggests that these proteins can be classified based upon functional similarities (sensors, partners, and coactivators) or evolutionary relatedness (α, β, and γ class) (Figure 6). In most cases, these two methods of classification overlap. Our understanding of the above signaling pathways provides evidence that many PAS proteins can act as either sensors of an input signal or as general partners required for the dimers to interact

with their nuclear targets. With respect to sensors, we have reviewed the considerable evidence that proteins such as the AHR, HIF1α, HIF2α, and HIF3α are directly sensing environmental signals. In the case of the AHR, direct binding of a ligand is the mechanism at play, whereas in the case of the HIFα subunits, they appear to be either directly sensing oxygen or being influenced by an oxygen-sensing protein. Similarly, the function of the ARNT, ARNT2, and MOP3 proteins appears to act as partners that target the multiple sensor PAS proteins to their cognate enhancer elements. The ARNT protein is the best characterized in vivo and clearly serves as a partner for both the AHR and some or all of the HIFαs. As mentioned above, the putative *Drosophila* orthologue of ARNT, the TANGO protein, has also shown to be a partner of SIM and TRH (235). The dimerization profile of ARNT2 strongly suggests its in vivo function will be similar to ARNT and that ARNT2 is a broad-spectrum partner for many of the same sensor proteins as ARNT (albeit in neuronal tissue). The published data also provide support for the classification of MOP3 as a general partner based upon the observation that it can dimerize with CLOCK and MOP4, and to a lesser degree with the HIFα subunits. To date, all biologically relevant PAS heterodimers are composed of one α-class and one β-class partner. The γ-class coactivators participate in transcriptional activation of steroid receptors, and they form a special subgroup of this superfamily, with the functions of their PAS domains unknown. To date, coactivators have not been shown to form heterodimers with other bHLH-PAS proteins. This class of transcriptional coactivators (γ-class) has received less attention from this chapter but has been the focus of a number of recent reviews (239, 247).

An amino acid sequence comparison of the PAS domains can also be used to classify members of this superfamily. This phylogenetic analysis suggests that proteins classified as general partners are more closely related to each other than to other members of this superfamily (Figure 6). This can also be said for the γ-class coactivators and for the larger number of proteins that fall under the rubric of sensor (α-class). By extension, the above classifications allow us to make some predictions about many of the less well-understood PAS proteins. Based upon the above pairing rules and phylogenetic comparisons, we would place CLOCK, MOP4, and SIM in the α-sensor class. Calling these proteins sensors is a bit premature; nevertheless, it is provocative to think of the CLOCK protein as sensing photic input and playing a role in adjusting circadian rhythms to the environmental surroundings. Perhaps this sensing of environmental input comes from direct interactions with the PER protein that is also in this sensing pathway.

CROSS TALK BETWEEN PAS PROTEIN–MEDIATED SIGNALING PATHWAYS

The fact that bHLH-PAS proteins could be involved in more than one cellular pathway leads to the possibility that signaling through one pathway could influence the responsiveness of another (90). Such a situation could arise when parallel

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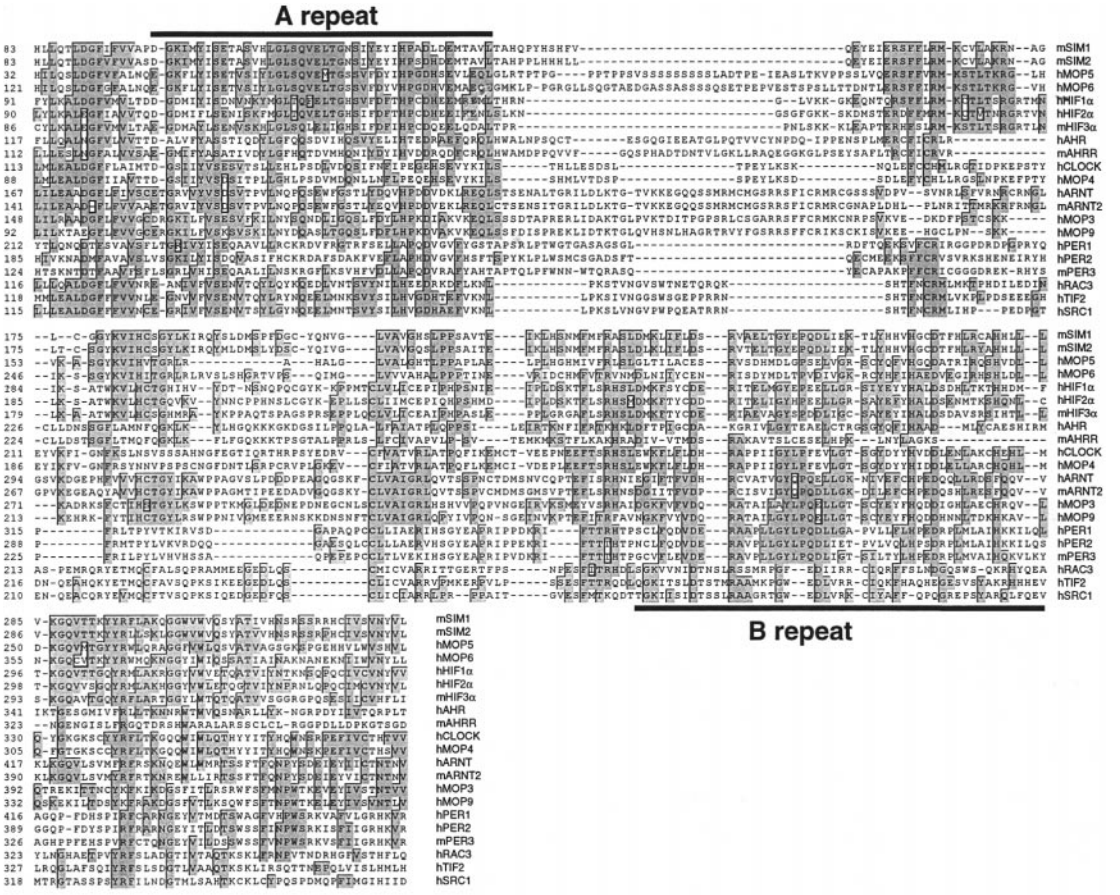

Figure 6a See Figure 6b for this caption.

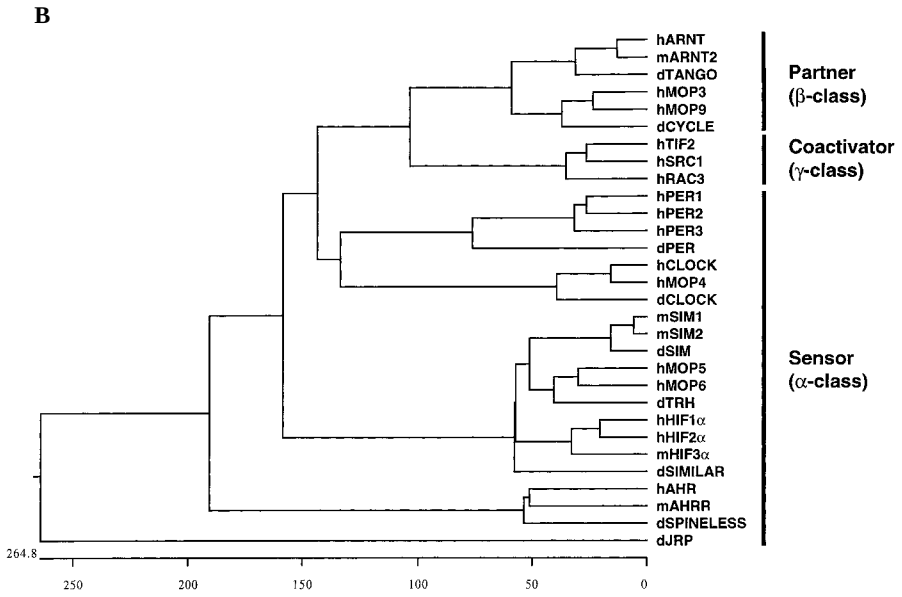

Figure 6 Selected PAS domain family members. (*A*) Clustal alignment for the sequences corresponding to the PAS domains of mammalian family members (265). Besides the published sequences, two newly identified PAS proteins in our lab, hMOP6 and hMOP9, are also included (RS Thomas, JB Hogenesch & CA Bradfield, unpublished observation). The consensus residues are *shaded.* The alignment conditions were as follows. Multiple: gap penalty, 10; gap length penalty, 10. Pairwise: ktuple, 1; gap penalty, 3. Species prefix h represents human and m represents mouse. (*Thick lines*) The PAS A and B repeats. For hARNT, the A repeat starts at ETGR and ends at REQL and the B repeat starts at EGIF and ends at QQVV. See text for details. (*B*). A phylogenetic tree constructed using clustal alignment. The *axis below* denotes sequence distance. JRP is the drosophila juvenile hormone-resistance protein (266). The PAS proteins are classified into sensors (α-class), partners (β-class), and coactivators (γ-class), as indicated on the *right.* Species prefix h represents human, m represents mouse, and d represents drosophila. See text for details.

pathways within the same cell share a limiting common partner, such as ARNT. In support of this idea, it has been shown that AHR and HIF1α compete for the binding of ARNT in vitro and that under certain conditions parallel signaling can be inhibitory (100, 146, 248). Although the simplest explanation of these data is that ARNT is a limiting factor, these experiments do not formally exclude other explanations, such as the possibility that other shared and limiting factors are important. In this regard, the significance of limiting heterologous factors is suggested by the recent demonstration of interference between the dioxin and the progesterone signaling pathway (249). Adding to the potential complexity of the cross-talk concept is the observation that certain responsive genes may be protected from such an event. For example, it has been shown that although there is

interference between the dioxin and the hypoxia pathways in vitro and in cell culture, the human *Epo* gene was protected from this interference in hepatoma cells. This protection appears to be due to the fact that the Epo promoter is influenced by both the classical HREs in its 3' regions, as well as a number of degenerate DREs immediately upstream of its promoter (100). Thus, for an EPO response, an additive effect of dioxin and hypoxia was observed instead of an inhibition, as might be predicted by the cross-talk model (100). It will be interesting to see whether this is a common mechanism of regulation for other hypoxia inducible genes.

THE PAS PROTEINS AS ENVIRONMENTAL SENSORS IN PROKARYOTES AND PLANTS

Any discussion of the mammalian PAS superfamily would be incomplete without mention of its more distant relatives in prokaryotes and plants. Although prokaryotes harbor no consensus bHLH proteins, they do express proteins that contain local homology to PAS domains (Figure 7) (250, 251). Members of this group of proteins are involved in oxygen regulation (FixL), sporulation (KinA), nitrogen fixation (NtrB), and negative phototropism [photoactive yellow protein (PYP)] (Figure 7) (252–254). Like their eukaryotic PAS relatives, these proteins often transduce signals in response to environmental change. Unlike their eukaryotic relatives, these prokaryotic proteins often harbor histidine kinase activity and transduce their signals via phosphorylation cascades that lead to activation of transcription factors (255).

The PYP protein, a bacterial blue-light photosensor that contains a rudimentary PAS repeat, was the first PAS-like molecule from which the three-dimensional structure was obtained (257, 258). This solution provided the first look into the structure of a simple PAS repeat (254). The idea that such prokaryotic domains were models of PAS was followed by the crystalization of the heme binding domain of the oxygen sensor FixL (259). The examination of these models shows that the structure of PAS repeat is highly conserved evolutionarily [Figure 8 (see

Figure 7 Alignment of selected prokaryotic and eucaryotic minimal PAS domains. (*Top*). A clustal alignment for mammalian PAS protein hARNT, hMOP3, hHIF1α, mCLOCK, and mAHR and bacterial PAS protein PYP and FixL. The consensus residues are *boxed.* The alignment conditions are as follows. Multiple: gap penalty, 10; gap length penalty, 20. Pairwise: ktuple, 1; gap penalty, 3. (*Bottom*). A phylogenetic tree was constructed based upon results from the above clustal alignment. The *axis below* denotes sequence distance. (*Thick line*) The PAS B repeat. For hARNT, the B repeat starts at EGIF and ends at QQVV. See text for details.

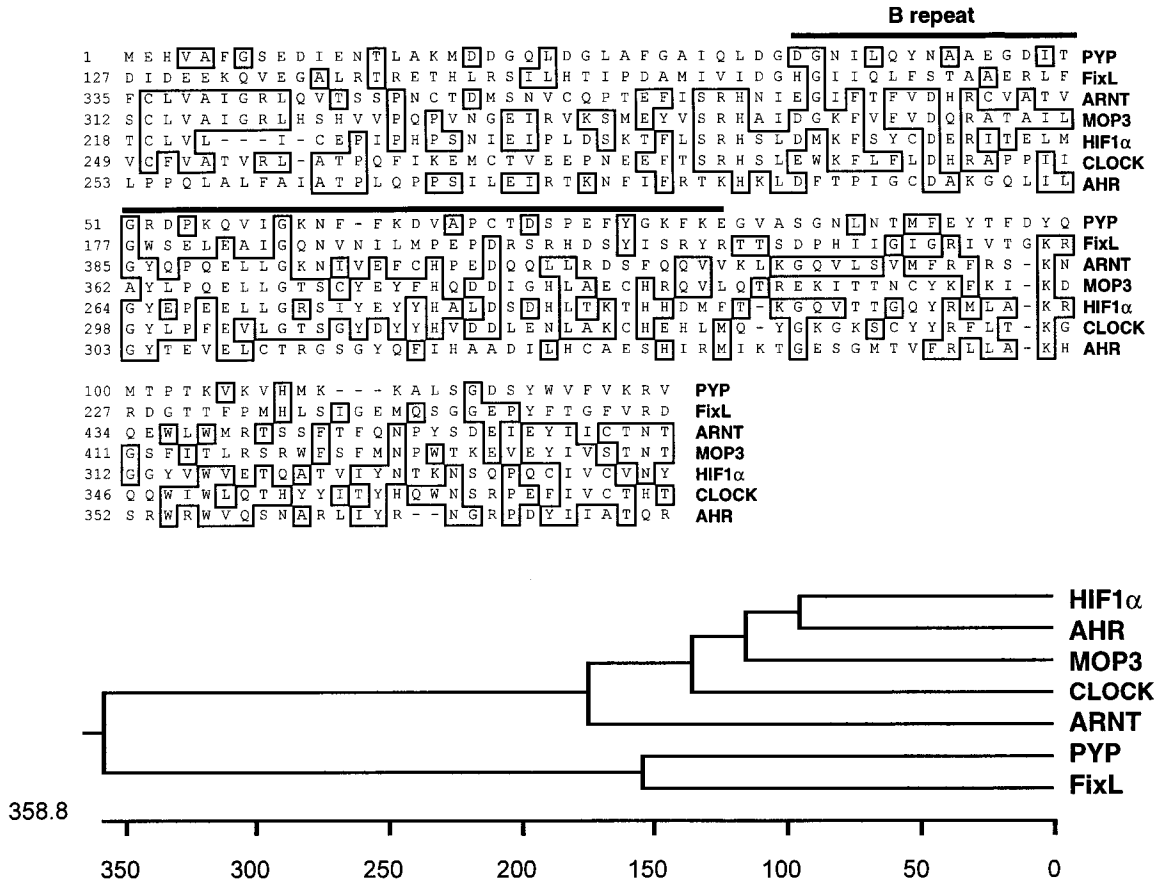

Figure 7 Figure caption on bottom of facing page.

color insert)] and indicates the functional importance of this structure as an interface for an environmental sensor. The cocrystalization of the chromophore 4-hydroxycinnamic acid with PYP (258) and heme bound with FixL (259) provided additional evidence that PAS domains can be directly involved in sensing environmental change. This observation is also consistent with the idea that this sensing process requires a bound low-molecular-weight ligand and that these prokaryotic domains are the forerunners of the AHR dioxin binding domain.

Structural homology with the PAS domain has also been identified in several photoreceptors in plants. In *Arabidopsis*, these proteins include NPH1, phytochromes from PhyA to PhyE, and the phytochrome interacting factor PIF3 (223, 224). The blue-light photoreceptor NPH1 is required for directional growth toward light (phototropism) (260). This protein has two repeats of approximately 110 amino acids, referred to as light, oxygen, voltage (LOV) sensor domains (261). The LOV domains share sequence homology to the PAS domains and have been shown to function as the binding sites for the chromophore flavin mononucleotide (261). Five types of phytochromes (from PhyA to PhyE) have been identified. They respond mainly to light at the red/far-red region of the spectrum and are involved in many aspects of plant development (224). These phytochromes contain two repeats homologous to the typical PAS domain and a histidine kinase–like domain at their C terminus (224). In keeping with the importance of homotypic interactions in PAS protein function, a yeast two-hybrid screen using the C-terminal region of PhyB as the bait lead to the identification of PIF3. This protein was shown to harbor the bHLH-PAS domain and to participate in the signaling pathways of both PhyA and PhyB (262). The presence of both bHLH and PAS domains as well as their involvement in PhyA and PhyB signaling suggest that PIF3 is a transcription factor (262).

SUMMARY

With the help of the rapidly expanding EST database and the fast pace of PAS protein research, more than 20 mammalian PAS cDNAs have been cloned to date, with related proteins being found in flies, plants, and prokaryotes (Figure 6). Many of these proteins function in heterodimeric pairs and can be classified as either sensors or broad-spectrum partners. The sensors, such as the AHR, the HIFαs, and possibly even CLOCK or PER, detect changes in the environment and regulate an adaptive response. The partners, such as the ARNTs and MOP3, dimerize with a broad spectrum of sensors and are essential for the transcriptional output of a number of these biologically important pathways. The sensors often act by directly detecting environmental change (AHR ligand binding) or by transducing a signal from an upstream sensor (HIF1α from low O_2 tension or PER from light). The mechanism by which the signal transduction occurs in response to environ-

mental cues are diverse and can involve ligand binding (AHR), protein stability (HIF1α), or subcellular localization (PER). Finally, cross talk between different bHLH-PAS pathways may be an important mechanism by which these proteins can signal through or attenuate parallel pathways.

Describing these models of signal transduction was performed as part of our interest in predicting how novel PAS proteins function and how their pathways mediate environmental adaptation in adults and developing embryos. Although we have focused on heterodimers of bHLH-PAS proteins with transcriptional outputs, it is not clear if this will prove to be the most common mechanism by which these proteins act. In this regard, bHLH-PAS coactivators have been shown to directly interact with and modify the transcriptional responses of nuclear receptors (241–245). Although these PAS proteins have bHLH and PAS domains, they have not yet been shown to heterodimerize with other bHLH-PAS proteins, nor have they been shown to directly contact specific DNA response elements (239). Thus, their participation in bHLH-PAS heterodimers might be predicted but has not been demonstrated. In addition, individual PAS proteins have recently emerged that are being shown to have a function apart from the nuclear compartment. An important example of this is the recent identification and crystalization of a PAS domain within the HERG K^+ channel (263). The PAS domain at the N terminus of this channel molecule may participate in regulating the rate of channel deactivation (263).

It is now easy to predict that the PAS domain will be found in one of the largest families of signal transduction molecules encoded by the mammalian genome, rivaling the size of the nuclear receptor superfamily. The elucidation of dioxin, hypoxia, and circadian signal transduction pathways has provided valuable information about this superfamily and has allowed us to make a number of generalizations about PAS protein function. The more recent realizations that rudimentary PAS domains are found in prokaryotic light and oxygen sensors, as well as in plant photoreceptors, adds strength to our assertion that these proteins represent a primary mechanism by which organisms adapt to environmental change. The idea that normal development would utilize many of these same pathways should not be a surprise and should serve as a reminder that ontogeny of complex organisms can also be viewed as a cellular struggle to adapt to environmental change.

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Figure 8 Structure of model prokaryotic PAS domains. Superimposed tertiary structure of FixL (*red*) and PYP (*yellow*) and their respective cofactors, heme and hydroxycinnamate (Credit: Weimin Gong and Michael K. Chan, Ohio State University) (259).