New Insights into Biliverdin Reductase Functions: Linking Heme Metabolism to Cell Signaling

Biliverdin reductase (BVR) functions in cell signaling through three distinct tracks: a dual-specificity kinase that functions in the insulin receptor/MAPK pathways (25, 29, 51); a bzip-type transcription factor for ATF-2/CREB and HO-1 regulation (1, 25); and a reductase that catalyzes the conversion of biliverdin to bilirubin (27). These, together with the protein’s primary and secondary features, intimately link BVR to the entire spectrum of cell-signaling cascades.

Nearly four decades ago, an NADH-dependent enzyme that converts biliverdin to bilirubin was described (55). Later this enzyme was defined as an NADPH-dependent reductase (58). A decade later, the enzyme, known as “biliverdin reductase” (BVR) was obtained in homogeneous form and its unique dual pH/cofactor activity profile was revealed (27). The reductase activity is NADPH dependent at acidic pH, whereas NADPH is used in the basic range. Searching for the molecular basis for this feature of BVR has recently culminated in unraveling other fascinating secrets of a protein with an uncanny spectrum of potential functions in cell-signaling pathways. Those functions, together with its unique structural features, underscore the central role of this unusual protein in cell signaling.

BVR: Structure, Regulation, and Reductase Activity

BVR is not exclusive to mammals, contrary to the general perception. The protein is evolutionarily conserved, and not only is it present across metazoa, but a homolog of mammalian reductase is also found in red algae (3, 53). Comparison of mammalian BVR protein sequences with those of chick, Xenopus, dog, and puffer fish reveals a high degree of conservation (13, 24, 36). The average sequence identity between mammalian species is >80%, with conservation of certain key features (13, 24, 36, 83). The first two residues of BVR are deleted during the maturation process (13). The addition of an extra phosphoacceptor residue, threonine, could potentially affect structure, function, or stability of the protein.

BVR reduces C10 (γ-bridge) of biliverdin IXα, a product of heme (Fe-protoporphyrin IX) degradation by heme oxygenase (HO) isozymes HO-1 and HO-2, which catalyze the isomer-specific cleavage of heme at the α-methylene carbon bridge. Plants use biliverdin IXα, produced by ferredoxin-dependent HO (3), to synthesize phytochromes, the sensory photoreceptors, to modulate their growth. The reaction, as characterized for Arabidopsis, depends on a ferredoxin-dependent BVR (23).

Although the structural basis for the unique dual pH/cofactor-dependence activity profile of BVR remains unsolved, substituting serine residues with alanine and solving the secondary structure of the rat BVR-NADPH complex have offered some clues and have identified key residues in reductase activity. The primary structure of the rat BVR (13) and crystal structure of the rat BVR-enzyme-cofactor complex (22, 60) have implicated the NH2-terminal domain (Rossman fold) in dinucleotide binding. There is an extensive interaction between the two domains of BVR, the NH2-terminal (the cofactor-binding domain) and the COOH-terminal β-sheet of BVR (22, 60) (FIGURE 2). Point mutation inactivates the NH2-terminal domain, changing C73 to alanine, and substituting serine residues S21, S44, S149, S41, and S51 (FIGURE 2). Point mutation changing C73 to alanine inactivates the NH2-terminal domain, changing C73 to alanine (23). The S21, S44, S149, S41, and S51 residues are conserved in all vertebrates. The conserved S21 (FIGURE 2; S21, S44, S149, S41, S51) inactivates the reductase. Among these, S44 has proven to be essential for activity (38, 41, 51). Additionally,
Mes HO-1 and OOH-terminal bridge. Plants onine kinase modulate their addition of an point mutation ker homology exception of reductase enzyme cofactor. Hytchromes, unique dual offered some stability of verdin IX, a IX degrada-

reduced hindrance to NADH binding and NADH release. A noteworthy finding is that in human renal carcinoma BVR activity is increased, but only in the Vmax of the mutant BVR, due to reduced hindrance to NADH binding and NAD release. A noteworthy finding is that in human renal carcinoma BVR activity is increased, but only with NADH (35). The significance and cause of this increase in activity are not clear, but it would not be advantageous for the host; rather, it would advance the growth of the malignant cells.

BVR is activated by phosphorylation, and this is increased by LPS oxidative stress and free radicals. All tyrosine residues of hBVR, at least four serines changing C73 in the rat BVR (C74 in hBVR) to alanine can accept a phosphate group (29). As will be discussed below, reduction of biliverdin, a kinase inhibitor, is an important aspect of regulatory activity of BVR.

BVR is the product of a single-copy gene that, in the rat, is 17,025 bp in length and consists of seven exons and six introns (38) (GenBank). The human BVR gene has eight exons, with the initiation codon in exon 2. Exon 5 includes the most strongly conserved motif, which is found in every oxidoreductase (30). In the rat, the ~1.6-kb transcript is expressed transcriptional activity, embryonic gene expression, elements (38) associated with regulation of tran-
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sequences were obtained from GenBank entries and were aligned using the MultAlin program (8a). The Alignment of biliverdin reductase genes from nine species FIGURE 1. Alignment of biliverdin reductase genes from nine species Sequences were obtained from GenBank entries and were aligned using the MultAlin program (8a). The Xenopus sequence is from X. tropicalis; the puffer fish is Tetraodon nigroviridis and is apparently truncated at the 3’ end, possibly as a consequence of inadequate sequence assignment during annotation. The chimpanzee data are from the genomic sequence and were obtained by searching using the human sequence. Sequences equivalent to exons 2 and 4 were not found; these probably lie in gaps within the sequence assembly. Shaded residues indicate regions of amino acid conserva-
tion in mammals. Residues are either identical or conservative substitutions (S-T, D-E, K-R, Q-N, V-L-I-M, V-A) in at least 4 of 5 or 5 of 6 mammalian proteins. Such residues are also highlighted if they appear in the nonmammal species. Because of the potential for phosphorylation of tyrosine, the substitution of F for Y, or vice versa, was not considered conservative in this context. Green highlighting marks residues of particular functional inter-

alignment gaps are indicated by “-”. Domains/motifs/residues that are known or are predicted to be involved in cell signaling are highlighted and characterized for particular functional inter-

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to hyperthermia, cytokines and LPS induce BVR transport (33).
hBVR expression is downregulated by the zinc- finger hematopoietic transcription factor GATA1 (7, 15) and upregulated by heme (Gibbs PEM and Maines MD, unpublished observation). GATA1 is a key factor in lineage-specific development of stem cells and may be indicative of a function for BVR in the establishment of gene-expression profiles in developing stem cells. GATA2, a marker of skeletal muscle hypertrophy (44), does not affect BVR tran- scription.

**Biliverdin Reductase: An Intracellular Transporter?**

There is the likelihood that in higher forms of life the conserved COOH-terminal-domain cysteines within the HXX_CXXH motif and the extensive 3-sheet in this domain are involved in interactions with other proteins, sulfhydryl reagents, in Zn/metal binding, and in dimerization; native hBVR is a Zn metalloprotein (27, 36). Functions that are assigned to this cysteine-rich domain include: 1) being the interaction site for hetro-dimerization with kinases/signaling molecules and homodimerization (1); 2) its function as a "molecular switch" in cell signaling through its S-S-interconversion; and, 3) being the site of interaction with substrate/cofactor and heme (40). The involvement of BVR in intracellular trafficking of signaling factors is consistent with the observation that BVR interacts with the insulin receptor kinase (IRK) domain (29) and localizes into the nucleus upon activation by cGMP (33).

The proposed function of BVR in protein-protein interaction and intracellular transport of signaling factors is based on its kinase activity, its primary structural features, and the secondary structure of the COOH-terminal domain as a large 3-sheet made of six strands; such a structure characterizes a monomer-monomer interface site, similar to those found in intracellular-trafficking "scaffold" (or signalosome) proteins. Notably, two copies of the CX CXC motif are present in the C domain of PKCs (42, 46). Oxidation of cysteine residues to the disulfide form (or covalently bonding with -SH-reactive compounds) prevents BVR dimerization and reductase activity (27). Homodimeric BVR is incapable of docking with other proteins, and this could arise from exposure to factors that affect the oxidation state of the cell and/or have affinity for sulfhydryls. Protein sulfhydryls are a target for the NO radical (4), for example. Over two decades ago, when little was known about the primary and secondary structure of BVR, observations were made that were unexplainable at the time but when revisited in light of the current information offer support for the function of cysteine residues in BVR protein-protein interactions. In one study rat BVR was characterized as "extremely sensitive" to -SH-reactive reagents, although biliverdin was found to fully protect the enzyme (27). Another observation (16) was the "interconversion" of two molecular forms of rat liver BVR, a ~34-kDa form was converted to a larger molecular form (~68 kDa) when rats were treated with -SH-reactive agents. Reduced thioredoxin, an agent that can reduce disulfide bonds to cysteines, was able to reverse the conversion. The larger form was found to lack sensitivity to sulfhydryl reagents. These observations would suggest occurrence of dynamic interchange between mono- and dimeric forms of BVR as the consequence of sulfhydryl disulfide interconversion in vivo.

**Regulation of Oxidative Response and HO-1 Expression**

Regulation of HO-1 by means of reductase and transcriptional activities may serve as a paradigm for gene regulation of oxidative-response gene expression by BVR and HO-1. The ability of BVR and HO-1 to interact requires an [FIGURE 1] that demonstrates nuclear localization of BVR and HO-1 on the basis of the nuclear localization signal (NLS). The ability of BVR to interact with xenobiotic-inducible transcription factors and/or with other BVR-related proteins to form homo- or heterodimers in the presence of heme and/or substrates for reductase activity (27) is consistent with the putative role for BVR in intracellular trafficking of signaling factors of the cGMP-I RK domain (29) and localizes into the nucleus upon activation by cGMP (33).

FIGURE 2: Ribbon diagram of the rat BVR-NADH enzyme cofactor complex

Adapted from Whisby et al. (58).
expression by BVR. The first direct link between BVR and HO-1 response was provided by a study that demonstrated nuclear localization of BVR in rat kidneys in response to inducers of HO-1, such as bromobenzene and bacterial LPS (33). BVR nuclear localization is an active process and requires an intact nuclear localization signal (FIGURE 1) (23). Gene-array analysis suggests that BVR activates a large number of genes in cell-signaling pathways, immune, and stress-response, and further analysis showed that, indeed, ho-1 and another gene expression (25) are controlled by BVR. The ability of BVR to activate expression of these genes was established in 293A cells from human embryonic kidney transfected with an adenoviral construct containing BVR. BVR is a bzip-type transcription factor that binds in dimeric form to AP-1 sites. Activation of AP-1 is the key event in oxidative-stress response of ho-1 and other stress proteins (54).

BVR regulates cellular levels of biliverdin, a potent gene regulator as illustrated by its being the determinant factor for dorsal axis development in Xenopus larva, by the suppression of PKC isozymes, and by activation of PKC by inhibition of BVR binding to AP-1 regulatory elements in the promoter of ho-1, and by activation of Ah receptor (1, 14, 41). On an equimolar basis, and by activation of c-Jun, c-Fos, ATF-2, CREB, Myc, and Bach-1, all are able to form heterodimeric complexes. The bzip motif of BVR is involved in DNA binding (1). BVR is a regulator of c-Jun and atf-2/creb gene expression (25). In the case of ATF-2, when its levels are increased, it effectively competes with c-Fos, the usual dimer partner of c-Jun. The ATF-2/c-Jun heterodimerpreferentially binds to the seven-base AP-1 sites (TGGTCA) rather than the usual site of ATF-2, CRE (TGACNTCA). The ATF-2/c-Jun dimer DNA complex is more stable than the c-Fos/c-Jun DNA complex. Moreover, heterodimerization not only alters ATF-2 binding with remarkable variation in affinity for different AP-1/CRE sites but also alters gene-regulatory activity of the dimeric partner. The identity of the association partner likely will result in a wide spectrum of changes in the cell.

That BVR regulates ho-1 expression by controlling gene-repressor activity of the hypoxia-inducible factor Bach-1 must be considered. This home-regulated transcription-repressor factor is a bzip factor with demonstrated ability to form a heterodimer with a small Maf protein, an activator of gene expression from AP-1/CREB recognition sites (6). The heterodimer prevents Maf by recognizing the MARE sequence motif in DNA (47). Because BVR is also a bzip protein that binds to AP-1/CREB

“Perhaps the most unexpected and arguably important finding is the dual-specificity kinase character of BVR.”
Role in Cell Signaling: Kinase Activity

A select few protein kinases, known as dual-specificity kinases, are able to autophosphorylate on, or transfer phosphate to, serine/threonine and tyrosine residues (21). The protein we have been studying for the past 25 years, BVR, turns out to be one of these entities. Whereas tyrosine kinases are mostly membrane bound (e.g., IRK), a few, including BVR, are soluble. Protein phosphorylation and dephosphorylation are essential components of signal transduction in the cell in response to various intra- and extracellular stimuli: hormones, metal complexes, and others. FIGURE 3 shows the insulin- and MAPK-signaling pathways and the junctures already demonstrated to be influenced by BVR. Insulin/insulin-like growth factor (IGF) action is mediated through activation of the insulin receptor (IR/IGFR), which is a disulfide-linked heterotetrameric complex. Insulin binds to the -subunits, and tyrosine phosphorylation of the -subunit activates the receptor, which includes uptake and dephosphorylation of protein tyrosine kinases such as Abl, Src, and yes, synthesis, as the Abl pathway is regulated by PKC (coincides with mitogen-activated protein kinase [MAPK/p38 pathway]) (27). Tyrosine kinases located at the plasma membrane and downstream of growth factor receptors include the insulin receptor substrate (IRS-1 through 6 belonging to IRS family) (28, 29) and Shp-2 (30).

Two major pathways, insulin and MAPK, are delineated (12). The Jun amino-terminal kinase (JNK) pathway is activated by upstream kinase(s) in the cell in response to various stimuli, including cytokines, stress, and UV irradiation. The mitogen-activated protein kinase (MAPK) pathway is activated by upstream MAPK kinase (MEK) (31). At least 11 such proteins exist, and two MAPKs (ERK and JNK) are downstream of MEK. The MEK family of kinases is activated by upstream MAPK kinase kinase(s) (32). These pathways are not mutually exclusive; for example, MEK may be activated by both upstream MAPK kinase kinase(s). The cross-regulatory interactions between the two pathways are vital for maintaining cellular stress and growth control. The two pathways converge at the levels of IRS-1 and IRS-2 complexes, which recruit the Grb2/sos complex downstream of the MAPK pathway. IRS contains a YMXM motif of IRS phosphotyrosine binding protein (c-Cbl) binding, which leads to act with SH2 and SH3 domains of the SH2-containing tyrosine phosphatase (SHIP) and SH2-containing tyrosine phosphatase 2 (SHIP-2) to deactivate MEK (33).

FIGURE 3. Sites of BVR interaction with insulin/IGF-1 signaling cascade

ATF-2, activating transcription factor 2; BAD, Bcl-2 antagonist of cell death; Shp2, Src homology 2 protein tyrosine phosphatase; G6s, growth factor receptor-bound protein; mSOS, son of sevenless homolog; CREB, cAMP response-element binding; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; IRS, insulin receptor substrate; P38, phosphatidylinositol 3-kinase; PDK, 3-phosphoinositide-dependent kinase; FKHR, member of the forkhead family of transcription factors.

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<th>BVR</th>
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BVR may also regulate ho-1 gene expression using a site on BVR for binding metalloporphyrins distinct from its biliverdin binding site (5). Depending on the chelated metal, metalloporphyrins can activate or inhibit BVR; iron hematoporphyrin was characterized as an inhibitor of the reductase (5), whereas cobalt protoporphyrin is an activator of the reductase. Metalloporphyrin complexes regulate expression of ho-1 as well as a vast number of genes including ALA synthase, the rate-limiting enzyme in its biosynthesis (17). A wide range of functions in the cell are subject to changes in ho-1 activity, including those controlled by nitric oxide radicals (8, 10, 11, 32, 34, 43, 48, 52).

Therefore, interplay between BVR, its substrate, and the product in the regulation of the stress response of ho-1 would have a major impact on the cell.
MAPK response-including BVR—has been shown to be one of the MAPK-signaling pathways. A change in conformation of a kinase can function both in directing proteins to subcellular targets and in modulating their activity. This suggests that BVR phosphorylates IRS on serine residues; and insulin-mediated glucose uptake is increased when BVR expression is knocked down using siBVR mRNA. Consistent with this concept, BVR phosphorylates IRS-1 peptides at sites known to negatively affect glucose uptake, and, under conditions unfavorable to its autophosphorylation, phosphorylation of BVR is increased when both BVR and IRS-1 are available to IRK, reflecting what is most likely direct interaction of BVR and IRS proteins. As the second major arm for insulin signaling, activation of the MAPK pathway primarily activates substrates that function in gene expression/mitogenesis; therefore, activation of BVR by IRK could affect a wide spectrum of functions in the cell, consistent with previous reports (25). Of the MAPK family (ERK, p38, JNK/SAPK), ERK is primarily activated by growth factors and phorbol esters and is associated with proliferation and differentiation of cells, whereas JNK/SAPK and p38 are activated by stress or growth factors.
extracellular stress and cytokines. BVR clearly has a regulatory role in the stress-response pathway of the MAPK cascade (1, 25, 41). Ablation of BVR by siRNA causes a four- to fivefold increase in the number of cells that undergo apoptosis concomitantly with an increase in the levels of factors associated with apoptosis after treatment with sodium arsenite (41).

Further evidence for involvement of BVR in both arms of HGF/growth factor signaling follows from our initial findings that BVR has PKB/Akt-like activity and modulates PKC enzymes as well (Miralem T, Lerner-Marmarosh N, and Maines MD, unpublished observations); PKB is a key mediator of signal-transduction processes, stimulates cell proliferation, and inhibits apoptosis (28). PKB-like activity of BVR is reflected by the finding that BVR transfection of the normally undifferentiated MCF7 breast cancer cells causes them to display morphological characteristics of differentiated cells (FIGURE 4). This figure also shows that it does not appear to be cell line specific as denoted by the profound change in morphology of HeLa cells transfected with BVR.

Conclusion

In the past, BVR was considered only in the context of converting biliverdin to bilirubin in the heme-degradation pathway. Studies in recent years have led to the identification of the human BVR along with its substrate and activity product as key players in the signal-transduction pathways, a regulator of cell proliferation, and a crucial component of cellular defense mechanisms and immune response. Perhaps the most unexpected and arguably important finding is the dual-specificity kinase character of BVR. Dual-specificity kinases control functions such as glucose metabolism, cell growth, and apoptosis, as well as development of human diseases such as cancer and diabetes. The new discoveries plus the recent description of biliverdin as a regulator of cell signaling pathways, a modulator of cell signaling and bilirubin as an antioxidant underscores relevance of this highly unusual molecule as a key factor in regulating cellular response to endogenous and exogenous factors and stimuli. The multidimensional input of BVR into insulin/IGF cascade of cell signaling and the regulation of oxidative stress-responsive genes may find a useful place in therapeutic settings to recast expression of the genes that function in cell growth, differentiation, and insulin resistance.

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References


