Published in final edited form as:

Biochem J. 2008 August 1; 413(3): 405–416. doi:10.1042/BJ20080018.

BILIVERDIN REDUCTASE IS A TRANSPORTER OF HEME INTO THE NUCLEUS AND IS ESSENTIAL TO REGULATION OF *HO-1* GENE EXPRESSION BY HEMATIN

Cicerone Tudor^{*}, Nicole Lerner-Marmarosh[†], Yves Engelborghs^{*,‡}, Peter E. M Gibbs[†], and Mahin D. Maines^{†,‡}

^{*}Laboratory of Biomolecular Dynamics, Katholieke Universiteit Leuven, Leuven, Belgium.

[†]Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, NY.

Synopsis

Human biliverdin reductase (hBVR) is an enzyme that reduces biliverdin (the product of heme oxygenase HO-1 and HO-2 activity) to the antioxidant bilirubin. It also functions as a kinase and as a transcription factor in the MAPK signaling cascade. Fluorescence correlation spectroscopy was used to investigate the mobility of hBVR in living cells and its function in the nuclear transport of hematin for induction of *HO-1*. In transiently transfected HeLa cells only kinase-competent hBVR translocates to the nucleus. A reduced mobility in the nucleus of hematin-treated cells suggests formation of an hBVR:hematin complex and its further association with large nuclear components. The binding of hematin is specific, with the formation of a 1:1 molar complex and the C-terminal 7-residue fragment (KYCCSRK²⁹⁶) of hBVR contributes to the binding. The following data suggest formation of dynamic complexes of hBVR:hematin with chromatin: a) the reduction of hBVR mobility in the presence of hematin is greater in heterochromatic regions than in euchromatic domains and, b) hBVR mobility is not retarded by hematin in nuclear lysates that contain only soluble factors. Moreover, hBVR kinase activity is stimulated in the presence of double-stranded DNA fragments corresponding to *HO-1* antioxidant and hypoxia response elements and by hematin. Experiments with nuclear localization and export signal mutants, and si-hBVR indicate that nuclear localization of hBVR is required for induction of *HO-1* by hematin. Because gene regulation is energy-dependent and hematin regulates gene expression, our data suggest that hBVR functions as an essential component of the regulatory mechanisms for heme-responsive transcriptional activation.

Keywords

biliverdin reductase; heme oxygenase; live cell imaging; hemoproteins; antioxidants

INTRODUCTION

Biliverdin reductase A is an evolutionarily conserved enzyme [1] that catalyzes the reduction of biliverdin IXa, the product of the catalytic activity of the heme oxygenase (HO) enzymes: HO-1 & HO-2, to the quencher of free radicals, bilirubin [2–5]. Free radical-mediated oxidative stress is implicated in the etiology of a wide spectrum of pathophysiological conditions [6,7]. The second form, BVR-B is predominantly expressed in fetal tissue [8]. The reductase is unique

[‡]Corresponding author BVR: mahin_maines@urmc.rochester.edu, Tel: 585-275-5383, Fax: 585-275-6007. [‡]Corresponding author FCS: Yves.Engelborghs@fys.kuleuven.be, Tel: + 32 16 32 71 60, Fax: + 32 16 32 79 71 { add 4}.

among biological catalysts in having a dual pH/cofactor dependent activity profile [9]; either NADPH or NADH are used, with pH optima of 8.7 and 7.0, respectively. BVR is present across metazoan species and its homologue is found in unicellular cyanobacteria [1].

The human protein is a 296-residue, soluble protein (295 residues in the rat protein) that in the past was known to be localized only in the cytoplasm and recognized only for its reductase capacity. More recently, however, the protein was detected in the nucleus [10] in the fixed tissue of animals exposed to cytokines and free radical inducers of *HO-1*, and the enzyme was characterized as a transcription factor and a dual-specificity (serine/threonine/tyrosine) kinase. The human enzyme was shown, under strictly Mn^{2+} dependent conditions, to autophosphorylate several serine, at least one threonine and two of its six tyrosine residues [11]. IRS-1, PKC- β II and HO-2 were identified among the substrates for its S/T kinase activity [11,12], while PKC- ζ was found to be activated by the enzyme [13]. The kinase activity of hBVR was demonstrated to be stimulated by H_2O_2 , insulin, sodium arsenite and the metalloporphyrin, Co-PP [11,14]. Activation by hBVR of oxidative stress-responsive transcription factors in the MAPK pathways that are involved in the regulation of *HO-1*, including c-Jun, c-Fos, ATF2 and CREB, as well as NF- κ B, were uncovered [14–20].

The multiplicity of functions ascribed to hBVR reflects its primary and predicted secondary structural features. The predicted primary sequence of rat and human BVR and the crystal structures of the rat BVR-cofactor complex [21–23] permit dividing hBVR into two domains: the N-terminus catalytic /nucleotide-binding domain with a preponderance of hydrophobic and charged residues, and the carboxyl regulatory domain [24,25]. This latter domain, as predicted by the crystal structures of rat BVR, contains six strands that form a large β sheet, an ideal interface for protein: protein interaction [22,23]. The C-terminus of the protein is cysteine-rich and contains a potential dimerization motif, HCX₁₀CC [21]. Cysteine and histidine are ligands for the heme (Fe protoporphyrin IX) molecule in hemoprotein complexes, such as NOS, cytochrome P450 enzymes, haemoglobin and HO-2 [26,27]. The predicted consensus sequences of NLS, NES, S/T kinase and dimerization bZip motifs are found within the regulatory domain. Although the protein via its bZip motif has been shown to bind to DNA *in vitro* [14,16,28], it is not known whether the protein interacts with DNA in the cell, whether the predicted NLS and NES are functional in the nuclear transport of hBVR or whether the transport is an energy dependent process.

Developments in live cell imaging have allowed investigations into the localization and mobility of different intracellular proteins to reveal their specific molecular mechanisms. The discovery and development of genetically encoded fluorescent proteins (FPs) allows direct monitoring of the behaviour of proteins in their physiological context in living cells [29]. Among the available techniques, FCS, which is non-invasive, is considered best suited for the study of molecular mobility, transport and diffusion in living cells [29,30]. In FCS, low probe concentrations and laser powers are required, thus minimizing the perturbation of the biological system. FCS has been used to determine the diffusion coefficients of different sized DNA molecules in living cells, crowded solutions, cytosol extracts, and artificial actin networks [31]. Moreover, FCS offers the opportunity to investigate different aspects of intra- and intercellular communication *in vivo* and in real time. Among the multitude of applications of FCS are investigations on DNA structure and interactions [32], protein structure, interaction and function [33–35], ligand-receptor binding and diffusion [36].

In the current study, FCS was used to assess the mobility of hBVR in living HeLa cells under treated and control conditions and to determine whether kinase activity of hBVR plays a central role in its nuclear trafficking. Nuclear transport of hematin was used to gain insight into hBVR function in the nucleus. The noted features and current gathered data are consistent with the potential function of hBVR to serve as a conveyor of heme from the cytoplasm to the nucleus

for regulation of gene expression. The heme molecule is a regulator of gene expression, including that of *HO-1* and *ALA* synthase I, the rate limiting enzymes in heme degradation and biosynthesis, respectively. In turn, heme is essential for the synthesis and function of hemoproteins, including NOS, soluble guanylate cyclase, cytP450 enzymes and respiratory cytochromes [37–39]. Data presented in this study identify hBVR as a heme-binding and heme transport kinase, and suggest its role in the modulation of heme-regulated gene expression.

MATERIALS AND METHODS

Materials

8Br-cGMP and salmon sperm (SS) DNA were purchased from Sigma (St Louis, Mo). Hemin chloride (Fe-protoporphyrin IX) was a product of Porphyrin Products Inc., (Logan, Utah). [γ - 32 P]-ATP was purchased from PerkinElmer (Wellesley, MA). Myelin basic protein (MBP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). hBVR-based peptides: KKRILHC²⁸¹ and KYCCSRK²⁹⁶ were synthesized by Synpep (Dublin, CA). Oligonucleotides were obtained from IDT (Coralville, IA). Taq polymerase was obtained from New England Biolabs (Beverly, MA). TRIzol reagent and Superscript II were purchased from Invitrogen (Carlsbad, CA).

Expression vectors

hBVR and its hBVR mutants Gly¹⁷→Ala and Cys^{281,292,293}→Ala were expressed in *E. coli*, using the pGEX 4T-2 plasmid as described previously [11] and the fusion protein was collected on the GSH beads. To cleave the tag, the preparation was treated with thrombin (Amersham, UK). Depending on the experiment, the cleaved preparation or GST-tagged protein was used. Mutations in the EGFP-flag-hBVR fusion construct were introduced by site-directed mutagenesis. The candidate nuclear localization signal, GLKRRR (residues 222–227), was mutated to AIGSTG, while the candidate nuclear export signal, LVSLFGELSL (residues 176–185), was changed to AVSVFGEVSA.

Cell culture and transient transfection assays

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO₂ in a humidified atmosphere. Penicillin and streptomycin were included at 100 units/ml and 100 µg/ml, respectively. Cells were seeded one day prior to transfection in 8-well LabTek chambered coverglass cuvettes. Transfection of HeLa cells was performed at ~80% confluency using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. The cells were washed with OptiMem (Invitrogen) before measurement to eliminate background fluorescence due to phenol red in the medium that would interfere with FCS. Ligands were added 1 h before starting the FCS measurements. Hematin and 8Br-cGMP were used at final concentrations of 10 µM and 50 µM, respectively.

FCS experiments

For FCS experiments, transfections were performed in 8-well chambered cover glasses with 100 ng of vector per cm² of cell culture plate. Theoretical and mathematical details of the FCS method are provided in the supplement. Measurements were performed on a commercial LSM510 ConfoCor2 combination system at room temperature (~21°C). The images were collected using a LSM 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) with a 20 mW argon ion laser and a 40x Plan-Apochromat water immersion objective. Briefly, the 488 nm line of an argon-ion laser was focused through a Zeiss C-Apochromat 40X, NA 1.2 water immersion objective, and the fluorescence emission was recorded between 505 and 550 nm using an avalanche photodiode. Fluorescence intensity fluctuations were recorded at different spots in each cell (cytoplasm and nucleus). At each spot, measurements were

performed over 20 s, and repeated 10 times. On average, 10 cells per condition were analyzed. Using the Origin software, the autocorrelation curves from the living cells measurements were fitted to an anomalous diffusion model to derive the translational diffusion time through the confocal volume. The apparent diffusion coefficients obtained for each curve were then averaged. For the experiments performed in nuclear extracts where the nucleoplasm is highly diluted, the anomalous diffusion model gave an anomalous parameter $\alpha=1$, indicating free diffusion. FCS measurements in these conditions were best fitted with a single component model.

Nuclear extracts

HeLa cells were transfected in 6-well plates. At 36 h post-transfection, cells were washed with PBS, harvested, and centrifuged. The pellet was lysed in 400 μ l of hypo-osmotic buffer (10 mM Tris, 10 mM NaCl, 0.5 mM DTT, Protease Inhibitor Cocktail (PIC) 1X, 0.2 mM PMSF) with a Dounce homogenizer. Nuclei were then isolated by centrifugation at 12,000 rpm for 5 min and lysed in 50 μ l of hyper-osmotic buffer (50 mM Tris, 400 mM KCl, 1 mM EDTA, 0.1% Triton, 10% glycerol, PIC1X, 0.2 mM PMSF). After centrifugation, the supernatant was diluted with 3 volumes of 50 mM Tris, 1 mM EDTA, 10% glycerol, PIC1X, 0.2 mM PMSF to bring the salt concentration to 100 mM. Nuclear extracts were stored at -20°C and incubated with hematin for 1 h at 4°C before FCS

Spectroscopy methods

Spectra were recorded using an Aminco DW2 spectrophotometer with OLIS upgrade. 50 mM hematin solution was freshly prepared by dissolving in a 1:1 mixture of 1 M NaOH and methanol, and further diluted to a 500 μ M concentration using 100 mM tris-HCl, pH 7.5, 0.01% Tween 20 [26]. For further dilution, as required, the same buffer was used. Binding of hematin to hBVR was assessed using a procedure similar to that used previously [27]; difference spectra for GST-BVR plus hematin, compared to hematin alone, at molar ratios of hematin:hBVR as indicated in the appropriate figures were recorded between 350 and 650 nm. The difference in absorbance between 414 nm and 606 nm was measured and used to titrate binding. The data were fitted by regression analysis using Graphpad Prism 3.0 software. Hematin: hBVR sequence-based peptide (KKRILHC²⁸¹ and KYCCSRK²⁹⁶) interaction was assessed by a shift in the Soret absorbance band of hematin in the presence of the peptide [26]. Peptides were added incrementally to a 5 μ M heme solution, beginning with a 1:1 molar ratio, and the spectrum was recorded between 350 and 650 nm. Peptides were also added to the reference cuvette, although neither peptide showed any absorbance in this range. In the experiment testing competition between peptide and KCN for hematin binding, KCN (from a freshly prepared 500 mM stock solution) was added in increments to a solution of 20 μ M peptide KYCCSRK²⁹⁶ and 5 μ M hematin, with the spectrum being recorded after each addition.

hBVR kinase activity

Oligonucleotides (20 μ M) containing either an antioxidant response element (ARE) [40]: 5'-AGCTGCATTTCTGCTGCGTCATGTTTGGGAGG-3' or hypoxia response element (HRE) [41]: 5'-GATCGCCCTACGTGCTGTCTCAGATC-3', and their complements were annealed in 100 mM NaCl by slow cooling from 85° .

hBVR autophosphorylation was assayed using the kinase assay described previously [11], in the presence of nucleic acids or hematin, prepared as above. Purified recombinant hBVR (1.6 μ M) was incubated for 10 min in 50 μ l kinase buffer (50 mM Hepes pH 8.4, 30 mM MnCl_2 , 0.2 mM DTT) with 2 μ M oligonucleotide (ARE or HRE), or 100 μ g/ml SS DNA. The reaction was started by the addition of 10 μ M ATP plus 10 μ Ci [γ -³²P] ATP, incubated for 1 h at 30°C , and terminated with the addition of Laemmli sample buffer. The samples were run on 8% gels, transferred to PVDF and autoradiographed. hBVR kinase activity was assessed in kinase assay

buffer using 10 μ M MBP as the substrate. The reaction was initiated as above. Hematin (10 or 50 μ M) was added 10 min prior to the addition of ATP. The reaction was carried out in the dark, and was terminated with the addition of an equal volume of 10% phosphoric acid and an aliquot was directly transferred to P81 Whatman filters, washed in 0.75% phosphoric acid (5x) and counted in a Beckman liquid scintillation counter.

RT-PCR experiments

Cells in 6-well plates were transfected with pcDNA3-hBVR, mutants or an empty pcDNA3 vector. 4 h after DNA addition, the cells were allowed to recover in DMEM containing 10% FBS for 2 h. Cells were serum-starved for 1 h, then treated with 10 μ M hematin for 30 min. The medium was replaced with DMEM containing 10% FBS and incubation was continued. Cells were harvested 2 and 6 h after the last medium change. Untreated cells were used as a control – these were subjected to all medium changes, except for the addition of hematin. Cells were also treated with siBVR [14], or its scrambled form [13], 7 h and 3.5 h prior to hematin treatment for 30 min. The medium was replaced with DMEM containing 10% FBS and including si-BVR (or sc-BVR), if appropriate. Cells were harvested 2 and 6 h after the last medium change; a further addition of si-BVR or sc-BVR was made after 3 h.

Cells were lysed by the addition of TRIzol reagent. RNA was purified from lysates as described by the manufacturer. Total RNA (2.5 μ g) was used as a template for cDNA synthesis, using Superscript II. The cDNA was used as a template for PCR, using gene-specific primers noted below and Taq polymerase. Reactions included two sets of primers – *HO-1* (HO-1f - CCCAGCACCGGCCGATG; HO-1r - GCCAGAGCTGGGCAGGTCCAG) was amplified together with β -actin (actin-f - GTGCTACGTCGCCCTGGAC; actin-r - CTAGAAGCATTTGCGGTGGAC), and *hBVR* (hBVRf - CTGTGGGAGCTGGCTGAGCAG; hBVR - TTACTTCCTTGAACAGCAATATTTCTG) was amplified together with *GAPDH* (GAPDH-f – AAGGTCGGAGTCAACGGATTTGGT; GAPDH-r TGCAGGAGGCATTGCTGATGATCT). Reaction conditions – annealing temperature and number of cycles – were adjusted for the sequences being amplified. Reaction products were resolved by gel electrophoresis, photographed on an imaging system, and signals integrated. Specific signals were normalized on β -actin or *GAPDH* as appropriate.

RESULTS

EGFP-BVR is localized in both the cytoplasm and nucleus and exhibits a diffuse distribution

HeLa cells were transiently transfected with expression vectors for EGFP-hBVR. This allowed for examination of the pattern of distribution of the green fluorescent proteins by confocal imaging (Figure 1). As shown in the figure, the wild-type protein is present in both the cytoplasmic and nuclear compartments, while it is excluded from the nucleoli (panel a). To further investigate a possible change in cellular localization and the pattern of expression of hBVR in response to stimulation with ligands, HeLa cells were transfected with EGFP-wt hBVR, treated with hematin (10 μ M) or 8 Br-cGMP (50 μ M), and used for image collection (panels b, c). Hematin and 8 Br-cGMP were added 1 h before the start of the FCS experiments. The images obtained showed that as with wt hBVR, in the presence of the ligands the protein was distributed between the nucleus and cytoplasm and excluded from the nucleolus. In all cases, the protein in both compartments displayed a diffuse staining pattern.

FCS Study of hBVR Mobility in Diffuse Stained Cells

FCS was used to determine the mobility of hBVR, and the effect of ligands on mobility of the protein in transiently transfected HeLa cells. The experiments were performed in areas with a diffuse expression pattern, meaning that only cells with low expression levels (nanomolar

range, as can be estimated from the number of molecules in the $\sim 1 \mu\text{m}^3$, i.e. 1 fL, spot) were selected for the FCS experiments.

The FCS measurements were performed over 20 sec in each spot selected in the cell and the autocorrelation curves obtained from the analysis of fluctuations of fluorescence intensity due to the diffusion of fluorescent molecules through the confocal volume were fitted with an anomalous diffusion model (Figure 1, Supplementary Data). Applying this model to hBVR diffusion in the cytoplasm and the nucleus showed high mobility of the protein in both compartments (Table I). As a control for the hBVR FCS experiments, the mobility of EGFP alone was studied in the cytoplasm and nucleus of HeLa cells.

The diffusion coefficients calculated from the resulting average diffusion times show that the diffusion of EGFP-hBVR is rapid in both compartments, and that the protein is not immobilized (Table I). The addition of 8Br-cGMP did not influence the mobility of the protein in either the cytoplasm or the nucleus. Interestingly, while hematin addition to wt hBVR did not influence the protein mobility in the cytoplasm, it significantly reduced the mobility in the nucleus from $12 \pm 0.6 \mu\text{m}^2/\text{s}$ to $4.5 \pm 0.2 \mu\text{m}^2/\text{s}$ (Table I). {When assessed quantitatively, the treatment with either hematin or 8Br-cGMP resulted in a preferential distribution of hBVR into the nucleus (Figure 2) as evidenced by the ratio of the number of hBVR particles in the nucleus to the number in the cytosol.} It is noteworthy that although in the presence of 8Br-cGMP the mobility of nuclear EGFP-hBVR was not reduced, there is still a significant increase of EGFP-hBVR in the nucleus when this ligand is added. However, this effect is less pronounced in comparison with hematin.

***In vitro* evidence of hematin interaction with hBVR**

The results of the hBVR mobility analysis in the nuclei of living cells (Figure 2) revealed an apparent reduction of mobility in the presence of hematin, suggesting hematin-hBVR interactions. In order to characterize this phenomenon further, additional *in vitro* experiments to examine hBVR-hematin interactions were performed.

In preliminary experiments, it was noted that addition of an equimolar amount of hBVR to a solution of hematin resulted in an altered hematin absorbance spectrum. Because hBVR purified from *E. coli* does display absorbance in the visible spectrum in the range of 350 and 650 nm, the change in spectrum in the presence of hematin was surmised to be indicative of hematin binding to hBVR. Those observations were further extended and the results are shown (Figure 3). Addition of a GST-hBVR fusion protein to a solution of hematin resulted in a shift in the absorbance spectrum. The difference spectra of hematin in the presence or absence of GST-hBVR are presented in panel a. Hematin solutions of different concentrations were examined in the presence of a constant concentration of GST-hBVR. Incremental increases in absorbance were observed up to a 1:1 ratio of hematin to protein, after which there was an apparent discontinuity, such that further addition was ineffective, until ratios exceeding 1.5:1 were achieved. While the data shown are for GST-hBVR, it was noted that identical results were seen with purified hBVR, lacking GST. These data were used to titrate hematin binding to hBVR [27]. At each hematin:protein ratio, the absorbance at 414 nm was determined from difference spectra as in panel a. The data for two independent experiments are plotted in panel b – linear regression of the data for hematin to protein ratios $\geq 1:1$ yield a slope that is not statistically different from zero, whereas there is a clear increase up to an approximately 1:1 ratio. The lines intersect at a ratio of 0.93:1, suggesting that each molecule of hBVR is able to bind one molecule of heme.

An hBVR peptide binds hematin

We had reason to suspect the involvement of the C-terminal cysteine-rich domain of hBVR in hematin binding. Cysteine and histidine are candidates for heme iron binding and two 7-residue peptides from this region of the protein have been shown to modulate the activity of PKC- ζ [13]. The peptide KKRILHC (residues 275–281) is a potent inhibitor of the activity of this kinase, while the peptide KYCCSRK (residues 290–296) activates the enzyme. We have previously demonstrated that short peptides containing a heme regulatory domain (HRM) motif with the cysteine-proline core dipeptide of HO-2 bind hematin, and the cysteine residue is the heme iron ligand [26]. The same approach was applied here. The peptides were added to a hematin solution at pH 9, in a 4-fold molar excess. There was no discernable change in color for KKRILHC, whereas the addition of KYCCSRK resulted in a change to a deep reddish brown (Figure 4a). The apparent inability of the KKRILHC peptide to alter the hematin color may be a consequence of the position of the cysteine residue at the end of the peptide, where it may be a less effective ligand for heme iron, as opposed to the more central location(s) in KYCCSRK. The binding affinities of two CPF sequences in the HO-2 HRM are dependent on the nature of their flanking residues [26]. For comparison, a solution of GST-hBVR fusion with hematin is also shown in the panel. These observations were demonstrated more rigorously by examination of the hematin spectrum in the presence of peptides. A 4-fold molar excess of KYCCSRK produced changes in the visible spectrum – the Soret band shifted to a shorter wavelength, and this peak also became broader (Figure 4b). The α and β bands at longer wavelengths were lost (inset, Figure 4b). Taken together, the data of Figure 4a and Figure 4b indicate that hematin is bound by the peptide. On the other hand, the addition of KKRILHC did not affect the spectrum (Figure 4c) which is consistent with the data shown in panel a. Two other peptides, KYCCSRA and KFCCSRK, related to the hematin-binding hBVR peptide, were also examined (Figure 4d, e). Both peptides produced the same spectrum changes as seen for KYCCSRK, suggesting that both bind hematin, and further, that neither the tyrosine nor C-terminal lysine residues are involved in binding. In this respect, the KYCCSRA peptide behaves differently in hematin binding than it does with respect to PKC- ζ activity, where, unlike its parent molecule, it is ineffective in activating the kinase.

Binding of hematin to the peptide KYCCSRK is dose-dependent (Figure 5a). The shift in the Soret peak is first seen after the addition of an equimolar amount of peptide. Continued addition leads to a complete change after addition of a 4-fold excess – further addition did not alter the spectrum. Similar changes were seen for the α and β bands (Figure 5a inset). The affinity of binding was examined by conversion to the cyanide hemochromogen (Figure 5b). Incremental addition of KCN to the KYCCSRK-hematin complex resulted in a shift in the Soret peak from the short-wavelength characteristic of the complex to that of the cyanide hemochromogen. These data enabled determination of the equilibrium constant for the reaction heme:peptide to heme:CN, yielding a value of $K = 2.4 \times 10^{-7}$, which suggests that the BVR peptide binds hematin less strongly than cysteine-containing peptides from HO-2, which yielded values for $K < 10^{-10}$ [26].

Impaired nuclear mobility in hematin treated cells requires kinase competent hBVR

To further investigate the mobility of hBVR, two hBVR mutants, defective either for ATP binding (Gly¹⁷ mutant) or for protein:protein dependent interaction and/or hematin binding were generated. Specifically, mutations were introduced in the N-terminal ATP binding/kinase domain (GlyxGly¹⁷xxGly) and the C-terminal cysteine-rich motif (HC²⁸¹X₁₀C²⁹²C²⁹³). HeLa cells were transfected with expression vectors for EGFP-wt hBVR, Gly¹⁷, or Cys^{281,292,293} mutant in the absence or presence of ligands, hematin, and 8Br-cGMP and the mobility was studied by FCS in living HeLa cells (Figure 6 and Table II). The FCS data reveal that the EGFP-BVR Gly¹⁷ mutant exhibits nearly similar diffusion coefficients to the wild type protein, and that treatment with 8Br-cGMP did not affect the mobility of the mutant protein in either

cytoplasm or nucleus (Table II). Interestingly, the effect of hematin on the mobility of the wild type protein in the nucleus was abolished for this mutant. Instead, a remarkable change in the pattern of hBVR distribution was observed after hematin stimulation, with a punctate appearance in the cytoplasm and a decreased concentration in the nucleus compared with 8Br-cGMP stimulated cells. Because protein mobility in a cellular compartment can be an energy dependent process, the fact that the mobility of the EGFP-BVR Gly¹⁷ mutant is not altered upon adding hematin is most likely due to the inability of this mutant to bind ATP. In the case of the EGFP-hBVR Cys^{281,292,293} mutant, the pattern of distribution of the protein and its mobility were similar to those of the wild type protein in the absence of ligand, and are not affected by 8Br-cGMP stimulation (Figure 6 and Table II). However, as noted, although there is a significant decrease in mobility of the hematin-bound mutant hBVR in the nucleus, the diffusion constant is 50% higher compared to that of wt hBVR after hematin treatment. This observation is consistent with the data shown in Figure 5 that suggest involvement of cysteine residues in the KYCCSRK peptide in hematin binding. However, the fact that in the presence hematin, diffusion in the nucleus was nonetheless slowed down evoked the possibility that other potential heme ligands including His²⁸⁰, that flanks Cys²⁸¹, Cys⁷⁴ or Cys²⁰⁴ might also participate in heme binding.

BVR diffusion in heterochromatic regions and in nuclear lysates reveals an interaction with DNA in presence of hematin

The reduced mobility of hBVR in the nucleus upon hematin treatment suggested an interaction with large complexes of proteins or transient interactions with rather immobile components of the nucleus, such as chromatin. This possibility of an hBVR-chromatin interaction was examined by applying a model of 3D-genome scanning that was recently proposed for several proteins that act as transcription factors [42] and depicts non-specific and transient interactions with chromatin. We reasoned that if hBVR is interacting with chromatin in the presence of hematin, this would imply that hBVR can scan chromatin, transiently interacting with non-specific binding sites through its DNA binding domain. In this case hBVR mobility in the presence of hematin should be affected mainly in regions where chromatin is dense and hence where the concentration of potential binding sites is high. To visualize chromatin density in living cells, HeLa cells were cotransfected with EGFP-hBVR and H2B-mRFP (Figure 7); mRFP intensity is directly linked to chromatin concentration, enabling discrimination between hetero- and euchromatin [43]. Although both in the absence and presence of hematin, EGFP-BVR diffuses on average more slowly in regions of higher chromatin density (Figure 7b), a dramatic reduction of hBVR mobility was observed in heterochromatic regions in the presence of hematin, suggesting an interaction of hBVR with chromatin. Also, the anomalous parameter was decreased in these areas, reflecting the change in interactions with the environment (data not shown). To test whether chromatin plays a role in the lower mobility of hBVR in the nuclei in the presence of hematin, the mobility of wt hBVR in nuclear extracts, where only soluble factors are present, was measured. Interestingly, the effect of hematin observed in living cells is abolished for the nuclear extracts (Table III), suggesting that the lower mobility of hBVR in the living cells upon adding hematin is due mainly to interactions with chromatin. Taken together, these findings suggest that the binding of hBVR to selective regions of chromatin causes structural/functional changes that affect its functions in the nucleus including transcriptional and kinase activities.

Inhibition of hBVR nuclear transport inhibits heme-dependent *HO-1* gene transcription

If, as we postulated, hematin is binding to hBVR and being transported to the nucleus, with a subsequent reduction in mobility of the protein, it might be expected that interfering with the transport of hBVR into the nucleus after treatment of cells with hematin would inhibit the ability of hematin to activate gene expression. For this purpose, transcription of the *HO-1* gene in cells transfected with a wt hBVR expression construct, and in parallel cultures transfected

with NLS or NES hBVR mutants was examined. Transport of hematin to the nucleus by hBVR would enable its delivery to the transcriptional repressor Bach1, which, on binding hematin dissociates from the DNA, and is replaced by the Nrf2 transcription factor [44,45]. While hematin treatment of cells transfected with wt hBVR plasmid activated *HO-1*, the hematin effect was significantly reduced when cells transfected with NLS or NES mutants or an empty plasmid (Figure 8a). Mutation of the NLS signal in hBVR would be expected to impair the delivery of the protein-bound hematin to the nucleus, whereas the NES mutant would accumulate in the nucleus.

To further examine the role of hBVR in induction of *HO-1*, we reasoned that reduction in the total hBVR content of the cell using siRNA should impair *HO-1* gene expression in response to hematin treatment. In comparison to cells treated with a control si-RNA, in which the hBVR-specific sequence was scrambled (sc-hBVR), or to uninfected cells, cells pretreated with si-hBVR [14] for 7 h prior to addition of hematin showed a significant reduction of *HO-1* induction (Figure 8b).

Collectively, the data shown in panels a and b reveal that when normal nuclear trafficking of hBVR is prevented, or the overall level of protein in the cell is reduced, induction of *HO-1* by hematin is attenuated.

Previous studies had suggested that phosphorylation of hBVR is essential for its DNA binding [14]. Therefore we examined further whether addition of DNA to purified hBVR might affect its kinase activity. As shown in panel c, incubation of hBVR with a double-stranded oligonucleotide containing either an ARE or HRE consensus sequence resulted in enhanced autophosphorylation of the protein when compared to the protein alone. Denatured DNA from salmon sperm, on the other hand, appears to inhibit the activity, suggesting that hBVR may interact with single-stranded DNA differently.

In the subsequent experiment, we examined the effect of hematin on the kinase activity of hBVR. Preincubation of hBVR with hematin resulted in up to 2-fold activation of kinase activity, at a concentration of 50 μ M (panel d) – lower concentrations also enhance activity, albeit to a lesser extent. Thus, conditions that result in the reduced mobility of hBVR in the nucleus also result in increased kinase activity of the protein, suggesting that this function of the protein is of paramount importance in the nucleus, in the context of its mobility and chromatin binding.

DISCUSSION

To test the possibility of hBVR functioning as a heme transporter, the present study involving imaging hBVR in the living cell was undertaken. Trafficking of distinct proteins among intracellular compartments and to and from the cell membrane mediates cell signalling and the transport of gene regulatory stimuli. However, understanding protein behaviour, localization, trafficking and participation in complexes in living cells are among the challenges in cell biology. In this study, advantage was taken of advanced confocal microscopy combined with the use of fluorescent fusion proteins to investigate the localization and mobility of hBVR in the absence and presence of hematin and cGMP, and to explore its potential functions in the transport of hematin and the regulation of gene expression. The present investigation has identified the function of hBVR as a cytoplasmic-nuclear transporter of hematin. And expression of *HO-1*, which was used as the prototype for heme-regulated genes was shown to be influenced by nuclear transport of hBVR. Although the metalloporphyrin complex is a commonly known regulator of genes that control cell respiration, energy production and respond to changes in the oxidation status of the cellular milieu, prior to this study its cellular transporter was not identified. In the past, it was generally assumed that hBVR is localized

exclusively in the cytosol. The images of living cells obtained here show that in cells that have recently undergone mitosis the protein is, for the most part, nuclear-localized allowing the evaluation of its mobility in the nucleus of hematin-treated cells.

FCS, a technique with microsecond resolution that has proven highly satisfactory in measuring the diffusion of different proteins in living cells [33], proved useful in the elucidation of nuclear mobility and its potential function in the nucleus. The environment of living cells is crowded, especially in the nucleus, which contains structural networks such as chromatin and the nuclear matrix, and elevated protein concentrations [46]. The model of anomalous diffusion and application of correction factors compensate for steric hindrance or binding events that can alter the free diffusion process [47]. When the anomalous diffusion model was applied to the movement of hBVR in living cells, the result was an improvement in accuracy of the autocorrelation fits. The validity of this approach was confirmed by the observation that the anomaly parameter was always smaller than one, reflecting the complexity of the cellular environment. An interesting result from the FCS data is that un-liganded (free) wt hBVR has a diffusion coefficient that reflects a high mobility of the protein in both the cytoplasm and nucleus (Table I). Assessment of hBVR mobility in living cells in the presence of 8Br-cGMP revealed that mobility of hBVR was not reduced in the cytoplasm or nucleus upon addition of the ligand. However, although stimulation with hematin did not affect mobility of the protein in the cytoplasm it caused a remarkable decrease in its nuclear mobility (Table I).

The reduced mobility of the protein in the nucleus is likely due to its interaction with slow moving nuclear components, with chromatin being the most likely candidate. The suggested interaction of hBVR with chromatin is consistent with its demonstrated binding in a dimeric complex to a DNA fragment from the *HO-1* promoter containing 2 AP-1 sites *in vitro* [14, 28]; and, induction of *HO-1* mRNA and ATF-2/CREB mRNA in cells infected with Ad-hBVR [16]. Based on reported quantitative FRAP studies, a high proportion of transcription factors transiently interact with specific and non-specific sites on chromatin [42,48,49] and the interaction greatly affects the diffusion of the proteins; the proteins are essentially performing a “3D genome scanning” until they reach binding sites at their target genes [42].

By using H2B-mRFP to visualize the state of chromatin, a markedly altered mobility of hBVR in heterochromatic regions in the presence of hematin was observed, which suggests an enhanced hBVR-chromatin interaction in the dense chromatin regions. This interpretation is consistent with the FCS results that show that the hematin-mediated reduction in hBVR mobility observed in the living cells was abolished in nuclear extracts devoid of chromatin material. The fact that the measured diffusion coefficient of EGFP-hBVR ($19.2 - 22.2 \mu\text{m}^2/\text{s}$) in lysates of known viscosity was substantially lower than the value of $38 \mu\text{m}^2/\text{s}$ calculated for the monomer in the same medium suggests interaction with other soluble proteins.

Accordingly, the possibility that hBVR interaction with chromatin is an event mediated and/or enhanced by the soluble cofactors is rather likely. Both types of interactions — hBVR: chromatin and protein: protein — are likely to be involved in the function of hBVR in the regulation of oxidative stress-inducible gene expression and presently observed induction of *HO-1* by hematin. Binding of hBVR to regulatory elements would orchestrate recruitment of transcriptional machinery to the promoter region of oxidation stress responsive genes. In this context, the present findings that ARE- or HRE-containing oligonucleotides stimulate hBVR kinase activity may suggest participation by hBVR in the recruitment of the HIF-1 transcription factor complex, which is central to cellular oxygen sensing. In the same context, change in the structure of a protein is a known mechanism of stimulation of kinases, and regulatory proteins can be induced by protein:protein interaction.

In the context of gene regulation the present findings, demonstrating hBVR:hematin complex formation, and requirement for intact nuclear export/import signals for response of *HO-1* to

the metalloporphyrin inducer, define a previously unknown dimension of gene regulation by hBVR as an intracellular transporter of hematin; notably, heme is synthesized in the cytoplasm. It is also noteworthy that hematin activates the kinase activity of hBVR, whereas similar high concentrations of hematin inhibit the reductase activity [9], perhaps by competing with biliverdin binding to the protein.

The availability of an hBVR mutant with impaired ATP binding (Gly¹⁷ mutant) afforded the opportunity to further explore the existence of a link between hBVR kinase activity and its function in hematin-transport. The finding that in untreated living cells, the mobility of the mutant hBVR, as assessed by FCS in both cytoplasm and nucleus (Table II), was rather similar to that exhibited by the wild-type protein, while the hematin-mediated slowing of nuclear mobility was abolished for the Gly¹⁷ mutant, is indicative of an active (ATP-dependent) process of hematin transport into the nucleus; thus it is not a diffusion process. This interpretation is not unprecedented and is consistent with reported findings that the phosphorylation status of β -catenin may influence its nuclear localization [50]. This interpretation is reinforced by the image shown in Figure 6 that displays a perinuclear accumulation of the kinase-defective hBVR upon treatment with hematin. Furthermore, the availability of the disrupted potential heme interacting domain mutant (Cys^{281,292,293}), and the use of peptides derived from the C-terminal domain, allowed for identification of an hBVR region that is involved in hematin binding. The mutation of the cysteine residues likely would not interfere with hBVR kinase activity. It is plausible that the inability of the cysteine mutant protein to form an S-S bond with a potential partner protein could disrupt the nuclear function of hBVR, as suggested by the observation (Table II) that, while the diffusion of the cysteine mutant is slowed in the nuclei of hematin-treated cells, it has a diffusion coefficient 50 % greater than that of wt hBVR.

Collectively, the FCS data support the likelihood of chromatin/DNA:hBVR interaction and possibly hBVR's involvement in the formation of protein:protein complexes in the nucleus. In addition, the data are consistent with the possibility that hBVR is an active, ATP-dependent, transporter of hematin within the cell, and therefore, is a key component of the mechanism for heme-regulated gene expression. Furthermore, this study has further proven the usefulness of the powerful FCS technique in finding new molecular mechanisms underlying hBVR mobility in living cells; and a reliable examination of its behaviour in the presence and absence of ligands, and thereby has revealed new insights into functions of hBVR in the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ALA synthase, aminolevulinate synthase
 ARE, antioxidant response element
 bZip, basic leucine zipper protein
 DTT, Dithiothreitol
 Co-PP, cobalt protoporphyrin
 EGFP, enhanced green fluorescent protein
 FCS, fluorescent correlation spectroscopy
 FP, fluorescent protein
 hBVR, human biliverdin reductase
 hematin, Fe-protoporphyrinIX
 HO-1, heme oxygenase-1
 HRE, hypoxia response element

MBP, myelin basic protein
 NES, nuclear export signal
 NLS, nuclear localization signal
 NOS, nitric oxide synthase

REFERENCES

1. Maines MD. New insights into biliverdin reductase functions: linking heme metabolism to cell signaling. *Physiology (Bethesda)* 2005;20:382–389. [PubMed: 16287987]
2. Maines MD, Gibbs PE. 30 some years of heme oxygenase: from a "molecular wrecking ball" to a "mesmerizing" trigger of cellular events. *Biochem. Biophys. Res. Commun* 2005;338:568–577. [PubMed: 16183036]
3. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043–1046. [PubMed: 3029864]
4. Mancuso C, Pani G, Calabrese V. Bilirubin: an endogenous scavenger of nitric oxide and reactive nitrogen species. *Redox Rep* 2006;11:207–213. [PubMed: 17132269]
5. Ryter SW, Morse D, Choi AM. Carbon monoxide and bilirubin: potential therapies for pulmonary/vascular injury and disease. *Am. J. Respir. Cell. Mol. Biol* 2007;36:175–182. [PubMed: 16980550]
6. Halliwell B. Biochemistry of oxidative stress. *Biochem. Soc. Trans* 2007;35:1147–1150. [PubMed: 17956298]
7. Mantena SK, King AL, Andringa KK, Landar A, Darley-Usmar V, Bailey SM. Novel interactions of mitochondria and reactive oxygen/nitrogen species in alcohol mediated liver disease. *World J. Gastroenterol* 2007;13:4967–4973. [PubMed: 17854139]
8. Mantle TJ. Haem degradation in animals and plants. *Biochem. Soc. Trans* 2002;30:630–633. [PubMed: 12196151]
9. Kutty RK, Maines MD. Purification and characterization of biliverdin reductase from rat liver. *J. Biol. Chem* 1981;256:3956–3962. [PubMed: 7217067]
10. Maines MD, Ewing JF, Huang TJ, Panahian N. Nuclear localization of biliverdin reductase in the rat kidney: response to nephrotoxins that induce heme oxygenase-1. *J. Pharmacol. Exp. Ther* 2001;296:1091–1097. [PubMed: 11181945]
11. Lerner-Marmarosh N, Shen J, Torno MD, Kravets A, Hu Z, Maines MD. Human biliverdin reductase: a member of the insulin receptor substrate family with serine/threonine/tyrosine kinase activity. *Proc. Natl. Acad. Sci. U S A* 2005;102:7109–7114. [PubMed: 15870194]
12. Maines MD, Miralem T, Lerner-Marmarosh N, Shen J, Gibbs PE. Human biliverdin reductase, a previously unknown activator of protein kinase C β II. *J. Biol. Chem* 2007;282:8110–8122. [PubMed: 17227757]
13. Lerner-Marmarosh N, Miralem T, Gibbs PE, Maines MD. Regulation of TNF- α -activated PKC- ζ signaling by the human biliverdin reductase: Identification of activating and inhibitory domains of the reductase. *FASEB J* 2007;21:3949–3362
14. Miralem T, Hu Z, Torno MD, Lelli KM, Maines MD. Small interference RNA-mediated gene silencing of human biliverdin reductase, but not that of heme oxygenase-1, attenuates arsenite-mediated induction of the oxygenase and increases apoptosis in 293A kidney cells. *J. Biol. Chem* 2005;280:17084–17092. [PubMed: 15741166]
15. Gibbs PE, Maines MD. Biliverdin inhibits activation of NF- κ B: reversal of inhibition by human biliverdin reductase. *Int. J. Cancer* 2007;121:2567–2574. [PubMed: 17683071]
16. Kravets A, Hu Z, Miralem T, Torno MD, Maines MD. Biliverdin reductase: A novel regulator for Induction of activating transcription factor-2 and heme oxygenase-1. *J. Biol. Chem* 2004;279:19916–19923. [PubMed: 14988408]
17. Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, Choi AM. Mechanisms of cell death in oxidative stress. *Antioxid. Redox. Signal* 2007;9:49–89. [PubMed: 17115887]
18. Fujiwara T, Harigae H, Takahashi S, Furuyama K, Nakajima O, Sun J, Igarashi K, Yamamoto M, Sassa S, Kaku M, Sasaki T. Differential gene expression profiling between wild-type and ALAS2-null erythroblasts: identification of novel heme-regulated genes. *Biochem. Biophys. Res. Commun* 2006;340:105–110. [PubMed: 16356476]

19. Immenschuh S, Fahimi HD, Baumgart-Vogt E. Complementary regulation of heme oxygenase-1 and peroxiredoxin I gene expression by oxidative stress in the liver. *Cell Mol. Biol* 2005;51:471–477. [PubMed: 16309569](Noisy-le-grand)
20. Thompson D, Basu-Modak S, Gordon M, Poore S, Markovitch D, Tyrrell RM. Exercise-induced expression of heme oxygenase-1 in human lymphocytes. *Free Radic. Res* 2005;39:63–69. [PubMed: 15875813]
21. Maines MD, Plevoda BV, Huang TJ, McCoubrey WK Jr. Human biliverdin IXalpha reductase is a zinc-metalloprotein. Characterization of purified and *Escherichia coli* expressed enzymes. *Eur. J. Biochem* 1996;235:372–381. [PubMed: 8631357]
22. Whitby FG, Phillips JD, Hill CP, McCoubrey W, Maines MD. Crystal structure of a biliverdin IXalpha reductase enzyme-cofactor complex. *J. Mol. Biol* 2002;319:1199–1210. [PubMed: 12079357]
23. Kikuchi A, Park SY, Miyatake H, Sun D, Sato M, Yoshida T, Shiro Y. Crystal structure of rat biliverdin reductase. *Nat. Struct. Biol* 2001;8:221–225. [PubMed: 11224565]
24. Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *Faseb J* 1995;9:576–596. [PubMed: 7768349]
25. Hunter T, Cooper JA. Protein-tyrosine kinases. *Annu. Rev. Biochem* 1985;54:897–930. [PubMed: 2992362]
26. Huang TJ, McCoubrey WK Jr, Maines MD. Heme oxygenase-2 interaction with metalloporphyrins: function of heme regulatory motifs. *Antioxid. Redox. Signal* 2001;3:685–696. [PubMed: 11554454]
27. Rublevskaya I, Maines MD. Interaction of Fe-protoporphyrin IX and heme analogues with purified recombinant heme oxygenase-2, the constitutive isozyme of the brain and testes. *J. Biol. Chem* 1994;269:26390–26395. [PubMed: 7929359]
28. Ahmad Z, Salim M, Maines MD. Human biliverdin reductase is a leucine zipper-like DNA-binding protein and functions in transcriptional activation of heme oxygenase-1 by oxidative stress. *J. Biol. Chem* 2002;277:9226–9232. [PubMed: 11773068]
29. Lippincott-Schwartz J, Patterson GH. Development and use of fluorescent protein markers in living cells. *Science* 2003;300:87–91. [PubMed: 12677058]
30. Brock R, Hink MA, Jovin TM. Fluorescence correlation microscopy of cells in the presence of autofluorescence. *Biophys. J* 1998;75:2547–2557. [PubMed: 9788950]
31. Dauty E, Verkman AS. Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: a new barrier for non-viral gene delivery. *J. Biol. Chem* 2005;280:7823–7828. [PubMed: 15632160]
32. Katiliene Z, Katilius E, Woodbury NW. Single molecule detection of DNA looping by NgoMIV restriction endonuclease. *Biophys. J* 2003;84:4053–4061. [PubMed: 12770909]
33. Feige JN, Gelman L, Tudor C, Engelborghs Y, Wahli W, Desvergne B. Fluorescence imaging reveals the nuclear behavior of peroxisome proliferator-activated receptor/retinoid X receptor heterodimers in the absence and presence of ligand. *J. Biol. Chem* 2005;280:17880–17890. [PubMed: 15731109]
34. Tudor C, Feige JN, Pingali H, Lohray VB, Wahli W, Desvergne B, Engelborghs Y, Gelman L. Association with coregulators is the major determinant governing peroxisome proliferator-activated receptor mobility in living cells. *J. Biol. Chem* 2007;282:4417–4426. [PubMed: 17164241]
35. Krouglova T, Vercammen J, Engelborghs Y. Correct diffusion coefficients of proteins in fluorescence correlation spectroscopy. Application to tubulin oligomers induced by Mg²⁺ and Paclitaxel. *Biophys. J* 2004;87:2635–2646. [PubMed: 15454458]
36. Jermutus L, Kolly R, Foldes-Papp Z, Hanes J, Rigler R, Pluckthun A. Ligand binding of a ribosome-displayed protein detected in solution at the single molecule level by fluorescence correlation spectroscopy. *Eur. Biophys. J* 2002;31:179–184. [PubMed: 12029330]
37. Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. *Handb. Exp. Pharmacol* 2006:213–254. [PubMed: 16999221]
38. Gonzalez FJ, Yu AM. Cytochrome P450 and xenobiotic receptor humanized mice. *Annu. Rev. Pharmacol. Toxicol* 2006;46:41–64. [PubMed: 16402898]
39. Landar A, Darley-Usmar VM. Evidence for oxygen as the master regulator of the responsiveness of soluble guanylate cyclase and cytochrome c oxidase to nitric oxide. *Biochem. J* 2007;405:e3–e4. [PubMed: 17590153]

40. Alam J, Den Z. Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J. Biol. Chem* 1992;267:21894–21900. [PubMed: 1400499]
41. Jozkowicz A, Nigisch A, Wegrzyn J, Weigel G, Huk I, Dulak J. Opposite effects of prostaglandin-J2 on VEGF in normoxia and hypoxia: role of HIF-1. *Biochem. Biophys. Res. Commun* 2004;314:31–38. [PubMed: 14715242]
42. Phair RD, Scaffidi P, Elbi C, Vecerova J, Dey A, Ozato K, Brown DT, Hager G, Bustin M, Misteli T. Global nature of dynamic protein-chromatin interactions in vivo: three-dimensional genome scanning and dynamic interaction networks of chromatin proteins. *Mol. Cell. Biol* 2004;24:6393–6402. [PubMed: 15226439]
43. Keppler A, Arrivoli C, Sironi L, Ellenberg J. Fluorophores for live cell imaging of AGT fusion proteins across the visible spectrum. *Biotechniques* 2006;41:167–170. [PubMed: 16925018]172,174–175
44. Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H, Igarashi K. Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. *Embo. J* 2001;20:2835–2843. [PubMed: 11387216]
45. Dhakshinamoorthy S, Jain AK, Bloom DA, Jaiswal AK. Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J. Biol. Chem* 2005;280:16891–16900. [PubMed: 15734732]
46. Minton AP. Implications of macromolecular crowding for protein assembly. *Curr. Opin. Struct. Biol* 2000;10:34–39. [PubMed: 10679465]
47. Wachsmuth M, Waldeck W, Langowski J. Anomalous diffusion of fluorescent probes inside living cell nuclei investigated by spatially-resolved fluorescence correlation spectroscopy. *J. Mol. Biol* 2000;298:677–689. [PubMed: 10788329]
48. Karpova TS, Chen TY, Sprague BL, McNally JG. Dynamic interactions of a transcription factor with DNA are accelerated by a chromatin remodeller. *EMBO Rep* 2004;5:1064–1070. [PubMed: 15514679]
49. Farla P, Hersmus R, Geverts B, Mari PO, Nigg AL, Dubbink HJ, Trapman J, Houtsmuller AB. The androgen receptor ligand-binding domain stabilizes DNA binding in living cells. *J. Struct. Biol* 2004;147:50–61. [PubMed: 15109605]
50. Staal FJ, Noort Mv M, Strous GJ, Clevers HC. Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. *EMBO Rep* 2002;3:63–68. [PubMed: 11751573]

Acknowledgements

This work was supported by NIH grants ES04066 and ES012187 (MDM), by the Research Council of the Katholieke Universiteit Leuven (fellowship awarded to C.T.) and the grant GOA 2006/09 (Y.E.) and the project G.0584.06 of the Fund for Scientific Research Flanders (FWO). We thank Dr. J. Ellenberg (EMBL, Heidelberg, Germany) for the H2B-mRFP construct. We also thank Jenny Shen for technical assistance.

Figure 1. The dispersion pattern of the EGFP-wt hBVR in HeLa cells in the presence or absence of hematin or 8 Br-cGMP

HeLa cells were transfected with pEGFP-hBVR as described in the text, in 8-well chambered coverglasses. The ligands – hematin (10 μ M) or 8Br-cGMP (50 μ M) – were dissolved in phenol red free OptiMEM and were added 1 h before initiating FCS measurements. Acquisitions for live-cell imaging were performed as described in the text, using excitation at 488 nm and detection between 505 and 550 nm. Images obtained with untreated cells are shown in panel **a**. Panel **b** images are those of cells treated with hematin, and panel **c** shows images of cells treated with 8Br-cGMP.

Figure 2. Treatment with hematin and 8Br-cGMP results in an increase in the number of discrete hBVR particles in the nucleus

HeLa cells were transiently transfected with the expression vector for EGFP-wt hBVR. The mobility of the fluorescent protein in the cytoplasm and nucleus was analyzed using FCS, in the absence or presence of hematin or 8Br-cGMP. Autocorrelation curves were fitted with the Origin software to an anomalous diffusion model to derive the number of particles diffusing through the confocal volume and a graph representing the ratio number of particles in the nucleus relative to cytoplasm is shown.

Figure 3. *In vitro* evidence of hematin binding to hBVR

(a) Hematin solution, yielding the final concentrations of 0.2 (spectrum a), 0.4 (b), 0.6 (c), 0.8 (d), 1.0 (e), 1.25 (f) and 1.5 (g) μM shown, was added to both sample and reference cuvettes; the sample cuvette contained 1 μM GST-hBVR while the reference cuvette contained an equal volume of the GST-hBVR vehicle. Difference spectra were recorded for each hematin concentration. **(b)** The absorbance at 414 nm was determined from spectra as in a, from two independent experiments. The lines were fitted by linear regression of the data between 0 and a 1:1 molar ratio, and for data at ratios $\geq 1:1$.

Figure 4. The C-terminal peptide of hBVR interacts with hematin

(a) In the left-hand panel, an equal volume of 2 mM peptide was added to 400 μ M hematin, in 100 mM tris-HCl pH 9, 75 mM NaCl, 0.0 % tween 20. For the sample labeled hematin, buffer only was added. For comparison, a sample containing 40 μ M hematin and 10 μ M GST-hBVR in the same buffer is also shown. (b) The spectrum of a 5 μ M hematin solution, in 100 mM trisHCl pH 7.5, 0.01 % tween20 was determined (with buffer alone in the reference cuvette, spectrum a), after which a 4-fold molar excess of the peptide KYCCSRK (hBVR aa290–296) was added to each cuvette, and the spectrum was determined again (spectrum b). (c–e) Spectra were measured as in b, except that the peptides – KKRILHC (hBVR aa275–281), KYCCSRA or KFCCSRK, respectively, were used.

Figure 5. Dose dependency of peptide hematin interaction

(a). The spectrum of a $5\ \mu\text{M}$ solution of hematin in 100 mM tris pH 7.5, 0.01 % tween20 was measured. The peptide – KYCCSRK – was incrementally added to the sample and reference cuvettes and the difference spectrum was recorded after each addition. The hematin spectrum is labeled a, while spectra b–g have 5, 10, 15, 20, 25 and $30\ \mu\text{M}$ peptide added. (b) 10 mM KYCCSRK was added incrementally to $5\ \mu\text{M}$ hematin until a complete shift in the Soret peak was achieved – the reference cuvette was adjusted to the same peptide concentration. Freshly prepared 0.5 mM KCN was then added incrementally, and the spectrum recorded after each addition. The insets show the spectra between 500 and 650 nm at an expanded scale. Spectrum

a is that of hematin with 30 μ M peptide; spectra b–l have 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5 and 25 mM KCN added, respectively.

Figure 6. Pattern of dispersion of EGFP- hBVR Gly¹⁷→Ala and Cys^{281,292,293}→Ala mutants in living cells in presence and absence of hematin and 8Br-cGMP

(a) HeLa cells, in 8-well chambered coverglasses, were transfected with expression vectors encoding the EGFP-hBVR Gly¹⁷→Ala or Cys^{281,292,293}→Ala mutants. Treatment of cells on coverglasses and addition of ligands were as described in the legend to Figure 1. Acquisitions for live-cell imaging were performed, and images of EGFP-hBVR were obtained, as described in the text. (b) The average diffusion coefficient of wt EGFP-hBVR or the Cys^{281,292,293}→Ala mutant in the absence or presence of hematin.

Figure 7. Diffusion of hBVR in euchromatin versus heterochromatin

HeLa cells were transfected with expression vectors for H2B-mRFP and EGFP-hBVR. **(a)** FCS in the absence or presence of hematin, in chromatin-rich (high mRFP signal) or chromatin-poor (weak mRFP signal) regions measured diffusion. **(b)** The average diffusion coefficient of EGFP-hBVR in the absence or presence of hematin as a function of histone concentration.

Figure 8. Regulating *HO-1* gene expression by hematin involves hBVR cytoplasm-nucleus translocation

(a) Cells were transfected with expression constructs encoding wt hBVR, its NLS and NES mutants, or with an empty vector. 6 h after transfection, cells were starved for 1 h, then treated with 10 μ M hematin for 30 min. Incubation continued in the absence of hematin; RNA was then isolated from the cells, and used as a template for RT-PCR. Cells at zero time were not treated with hematin. **(b)** Cells were infected with si-hBVR, or the scrambled form (sc-hBVR), at 7 h and 3.5 h prior to hematin treatment. Cells were starved and treated with hematin as in **a**. Incubation was continued in medium containing the appropriate si-hBVR construct, and an additional si-hBVR virus was added 3 h after medium replacement. RNA was prepared and used for RT-PCR as in **a**. **(c)** hBVR kinase activity was increased in the presence of DNA containing regulatory elements. Autophosphorylation of hBVR was measured as described in the text, in the presence of double-stranded oligonucleotides containing ARE or HRE consensus sequences, or single-stranded salmon sperm DNA. The phosphorylated protein was resolved by gel electrophoresis, transferred to PVDF membrane, and detected by autoradiography. **(d)** Hematin binding stimulates hBVR kinase activity. The kinase activity of hBVR, using MBP as a substrate, was measured as described in the text in the presence or absence of hematin.

Hematin reduces the mobility of the EGFP-hBVR in the nucleus of the living cell
HeLa cells were transiently transfected with the expression vector for EGFP-wt hBVR. The mobility of the fluorescent protein was analyzed in the cytoplasm and nucleus. Autocorrelation curves were fitted with the Origin software to an anomalous diffusion model to derive the translational diffusion time through the confocal volume.

EGFP Construct	Addition	Diffusion Coefficient ($\mu\text{m}^2/\text{s}$) $\pm$ SEM		anomaly parameter α	
		cytoplasm	nucleus	cytoplasm	nucleus
EGFP	-	33.4 $\pm$ 1.1	24.0 $\pm$ 1.3	$\alpha=0.86$	$\alpha=0.80$
hBVR	-	18.1 $\pm$ 0.7	12.0 $\pm$ 0.6	$\alpha=0.75$	$\alpha=0.70$
hBVR	hematin	17.6 $\pm$ 0.7	4.5 $\pm$ 0.2	$\alpha=0.80$	$\alpha=0.68$
hBVR	8 Br-cGMP	18.9 $\pm$ 0.6	13.1 $\pm$ 0.6	$\alpha=0.78$	$\alpha=0.79$

Table II
The diffusion coefficients of EGFP-hBVR Gly¹⁷→A and Cys^{281,292,293}→A mutants obtained in living cells
HeLa cells were transiently transfected with the expression vector for EGFP-hBVR mutant constructs. The mobility of the fluorescent protein was analyzed in the cytoplasm and nucleus. Autocorrelation curves were fitted with the Origin software as in Table I

EGFP-hBVR Mutant	Addition	Diffusion Coefficient (μm ² /s) ± SEM		anomaly parameter α	
		cytoplasm	nucleus	cytoplasm	nucleus
EGFP	-	33.4 +/- 1.1	24.0 +/- 1.3	α=0.86	α=0.80
Gly ¹⁷	-	15.2 +/- 0.6	10.6 +/- 0.7	α=0.83	α=0.79
Gly ¹⁷	hematin	19.6 +/- 0.7	12.6 +/- 0.3	α=0.76	α=0.70
Gly ¹⁷	8 Br-cGMP	16.3 +/- 0.6	11.6 +/- 0.6	α=0.78	α=0.74
Cys ^{281,292,293}	-	18.4 +/- 0.5	17.9 +/- 0.6	α=0.78	α=0.78
Cys ^{281,292,293}	hematin	15.6 +/- 0.7	6.8 +/- 0.1	α=0.76	α=0.66
Cys ^{281,292,293}	8 Br-cGMP	16.8 +/- 0.7	16.7 +/- 0.6	α=0.69	α=0.65

Table III**Hematin does not reduce the mobility of the EGFP-hBVR in the nuclear extract devoid of chromatin**

HeLa cells were transiently transfected with the expression vector for EGFP-wt hBVR, the chromatin-depleted nuclear extract was prepared as described in the text and the mobility of the fluorescent protein in the extract was analyzed. Autocorrelation curves were fitted with the Origin software to a free diffusion (one-component) model to derive the translational diffusion time through the confocal volume.

		Diffusion Coefficient ($\mu\text{m}^2/\text{s}$) $\pm$ SEM
EGFP Construct	Addition	Nuclear Lysates
EGFP	-	51.1 $\pm$ 1.2
hBVR	-	19.2 $\pm$ 0.8
hBVR	hematin	22.2 $\pm$ 0.9