Hpr6 (Heme-1 Domain Protein) Regulates the Susceptibility of Cancer Cells to Chemotherapeutic Drugs

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ABSTRACT

Cancer cells have varying levels of susceptibility to chemotherapeutic agents, and the proteins that direct drug susceptibility are promising targets for intervention in cancer. Hpr6 (heme-1 domain protein)/PGRMC1 (progestosterone receptor membrane component 1) is overexpressed in tumors, and Hpr6 is the human homolog of a budding yeast damage resistance gene called Dap1p. Cells lacking Dap1p are damage-sensitive, and we have found that inhibition of Hpr6 expression by RNA inhibition (RNAi) increases sensitivity of breast cancer cells to chemotherapeutic drugs. Hpr6 is composed largely of a cytochrome b5-related heme-1 domain, and we have found that purified Hpr6 binds to heme, similar to its yeast and rodent homologues. We generated an aspartate 120-to-glycine (D120G) mutant of Hpr6 at a highly conserved site in the heme-1 domain and demonstrated that Hpr6-D120G cannot bind to heme. The Hpr6-D120G mutant was named Hpr6hbd for heme binding defective. We prepared an adenovirus encoding Hpr6hbd and found that adenovirus Hpr6hbd increases susceptibility of breast cancer cells to doxorubicin and camptothecin. Our findings support a model in which Hpr6, similar to its yeast homolog, binds to heme and regulates susceptibility to damaging agents.

Treatments for solid tumors usually include a combination of DNA-damaging agents. Doxorubicin or Adriamycin is an anthracycline antibiotic that is used in combination with other drugs for treating breast cancer (Chabner et al., 2001). Doxorubicin acts through multiple mechanisms that include intercalating within DNA, binding to topoisomerase II, and generating reactive oxygen species (Chabner et al., 2001; Longley and Johnston, 2005). Other chemotherapeutic agents in use for solid tumors include camptothecin analogs, which inhibit topoisomerase I, and nitrogen mustards, which alkylate DNA (Chabner et al., 2001). Because many alkylating agents require activation in the liver, mechlorethamine is frequently used with cultured cells, because it does not require conversion to an active form. The proteins that regulate susceptibility to chemotherapy are potential therapeutic targets for cancer, and altering their expression or activity could improve clinical chemotherapy responsiveness.

Hpr6 is a member of the heme-1 domain family of proteins, which includes the human Hpr6 and Dg6 proteins (Gerdes et al., 1998), the rodent 25-Dx (Selmin et al., 1996), and IZA proteins (Raza et al., 2001) and the budding yeast Dap1p (damage resistance protein) (Hand et al., 2003). The Hpr6/25-Dx/IZA/Dap1p proteins contain a central heme-1 domain that shares homology with cytochrome b5 (Mifsud and Bate-man, 2002). Similar to cytochrome b5, IZA and Dap1p bind to heme (Min et al., 2004; Mallory et al., 2005a). 25-Dx is a variant of IZA (Min et al., 2004), suggesting that it binds to heme, and we show in the present study that Hpr6 binds to heme. Heme is composed of protoporphyrin IX and ferrous iron and is a cofactor in numerous reactions in energy production and metabolism.

Hpr6 is the homolog of Dap1p, and mutants lacking Dap1p are sensitive to a DNA-alkylating agent and inhibitors of sterol biosynthesis (Hand et al., 2003). These defects can be suppressed by high levels of heme or the cytochrome P450 protein Cyp51p/Erg11p (Mallory et al., 2005a), a heme-binding protein, suggesting a role for Dap1p in heme transport or metabolism. In rodents, IZA binds to heme (Min et al., 2004) and localizes to the adrenal inner zones (Raza et al., 2001), where it likely up-regulates the steroid biosynthetic activities of cytochrome P450 proteins (Min et al., 2004). Because Dap1p directs resistance to xenobiotic damaging agents, we have tested the extent to which Hpr6 performs a similar function.

Hpr6 is overexpressed in clinical tumor samples, including
approximately 50% of breast tumors, and is readily detectable in cancer cell lines (Crudden et al., 2005), suggesting a role for Hpr6 in tumor cell survival. Multiple microarray studies have also identified Hpr6 as an up-regulated gene in tumor samples (Difilippantonio et al., 2003; Kim et al., 2004; Irby et al., 2005). The rodent Hpr6 homolog was originally identified as 25-Dx because it was up-regulated in liver tumors induced by dioxin (Selmin et al., 1996), but the function of Hpr6 in tumors has not been analyzed previously. Human Hpr6 was originally named as a human membrane progesterone receptor (Gerdes et al., 1998) based on its homology to putative progesterone-binding proteins (Falkenstein et al., 1996) and is listed in databases as PGRMC1 for progesterone receptor membrane component 1 or HMPR for human membrane progesterone receptor. Hpr6 is often identified as PGRMC1 in microarray studies, but progesterone binding for this protein has not been demonstrated.

To test the role of Hpr6 in resistance to chemotherapeutic agents, we inhibited Hpr6 expression and measured the susceptibility of cells with inhibited Hpr6 function to chemotherapeutic drugs. In cells where Hpr6 expression was inhibited by RNAi, we detected a significant increase in cell death after chemotherapy. We then found that purified Hpr6 bound to heme, whereas a mutation in the heme-1 domain inactivates heme binding. Overexpression of the heme binding defective mutant in breast cancer cells increased doxorubicin susceptibility, indicating that heme binding is critical for Hpr6 activity. These findings indicate that Hpr6 mediates resistance to chemotherapeutic agents in breast cancer and is a promising target for therapeutic intervention.

Materials and Methods

Cell Lines, Culturing, and Infection. MDA-MB-231 human breast cancer cells were a kind gift from Dr. Carolyn Sartor (University of North Carolina, Chapel Hill, NC) and were maintained in Dulbecco’s modified Eagle’s medium with 10% Serum Supreme (Fisher Scientific Co., Pittsburgh, PA) and penicillin and streptomycin. Cells were grown at 37°C in a humidified chamber containing 5% CO₂ in air. Cells were visualized using a VistaVision inverted microscope attached to a Sony DCS-F717 digital camera (VWR, West Chester, PA). For infection, cells were incubated with varying doses of Ad-LacZ or Ad-Hpr6 abolished or 4 h prior to drug treatment. Infections were performed in normal culture medium (see above).

Fig. 1. Hpr6 was induced by doxorubicin. MDA-MB-231 cells were untreated (lane 1), treated with 0.5 or 1 µM doxorubicin (lanes 2 and 3), or 0.25 µM camptothecin (lane 4) for 3 days, and Hpr6 expression was analyzed by Western blot. The -fold increase in Hpr6 expression (bottom panel) is derived from the Hpr6/tubulin ratio from each sample. Tubulin is included as a control for equal protein loading (bottom panel). dox, doxorubicin; cmp, camptothecin.

Expression Plasmids and Viral Preparation. The 3’ end of the HPR6 open reading frame containing the D120G mutation was amplified from the plasmid IMAGE 3254089 (Research Genetics, Huntsville, AL) using the primers HPR-D120G-F (5’-TACGGGCCC-GAGGGCGCCATATGCGGTCTTCTGGAAGGAGGTCAGGGG-CCCTGGC-3’) and HPR-690R-APA (5’-GAGCACTTGCTATAAAGTTTCTCGAGGGGCCCACA-3’) and cloned into Apal site of the plasmid pRC38 (Hamb and Craven, 2003). The resulting plasmid, pRC42, contained the full-length HPR6 open reading frame with the D120G mutation. The HPR6-D120G open reading frame was cloned into the BglII and XhoI sites of pShuttle-CMV (provided by the University of North Carolina at Chapel Hill adenoviral core facility), resulting in the plasmid pRC45. The Ad-Hpr6 abolished adenovirus was isolated by the University of North Carolina at Chapel Hill adenoviral core facility, and the adenovirus was amplified by the Medical College of Wisconsin adenoviral core facility.

RNAi. MDA-MB-231 cells were plated at a density of 500,000 cells/100-mm tissue culture dish. RNA oligonucleotide duplexes were diluted to 220 nM in 1 ml of Opti-MEM medium (Invitrogen, Carlsbad, CA). A 1:6 suspension of Oligofectamine (Invitrogen) in Opti-MEM was added to the RNAi duplex solution according to the manufacturer’s instructions, and the mixture was incubated for 15 to 20 min. During the incubation, plated MDA-MB-231 cells were washed once and overlaid with 4.4 ml of Opti-MEM. The RNAi suspension was added to the cells to a final concentration of 40 nM RNAi, a dose that was determined to be optimal for MDA-MB-231 cells. After 4 h at 37°C, 2.8 µl of culture medium containing 30% Serum Supreme was added to the cells and the cells were incubated overnight. The
cells were then trypsinized and plated at a density of 5000 cells/well in a 96-well dish or 500,000 cells/plate in a 100-mm dish.

**Fusion Protein Plasmids, Preparation, and Analysis.** Hpr6-GST fusion proteins contained amino acids 43 to 195 of the Hpr6 open reading frame. Hpr6 was amplified using the primers HPR + 126F-Bam and HPR + 566R-Xho with the plasmid pRC40 as a template. PCR products were cloned into the pCR2.1 plasmid (Invitrogen) forming the plasmid pRC44. The Hpr6 fragment was then cloned into the BamHI and XhoI sites of pGEX-4T-1 (GE Healthcare, Little Chalfont, Buckinghamshire, UK), forming the plasmid pRC46. To introduce the D120G mutant, the plasmid pRC46 was digested with ApaI and XhoI and ligated to the 260-bp ApaI-XhoI fragment of the plasmid pRC45, forming the plasmid pGC4.

Fusion protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Fisher Scientific Co.), and cells were lysed in the B-PER (bacterial protein extraction) reagent (Pierce, Rockford, IL) and bound to glutathione-agarose beads (Pierce). At this stage, the GST-Hpr6-bound columns were visibly brown due to bound heme, whereas the GST-Hpr6-D120G-bound columns were white due to the inability of this mutant to bind heme. After estimating protein quantity by SDS-polyacrylamide gel electrophoresis, 100 μg of Hpr6 or Hpr6-D120G proteins were liberated by digestion with 1 U of thrombin for 2 h and a scan of absorbance from 350 to 550 nm was determined using a Beckman Coulter DU640 spectrophotometer (Fullerton, CA). Proteins were reduced with the addition of 1 mg of sodium hydrosulfite.

**Drug Treatments and Viability Assays.** Doxorubicin (Sigma-Aldrich, St. Louis, MO) was added to culture media and incubated for 5 days. Cells were incubated with mechlorethamine (kindly provided by Dr. Robert Orlowski, University of North Carolina, Chapel Hill, NC) or camptothecin (Sigma-Aldrich) for 72 h. For measurements of cell growth, transfected cells were counted and plated in 96-well dishes and then treated with doxorubicin, camptothecin, or mechlorethamine for 72 to 96 h. Cells were then grown in media containing 0.5 mg/ml MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) for 1 to 2 h. MTT-containing media were then removed and replaced with 100 μl of dimethyl sulfoxide. The plates were incubated for 20 min on a rotating platform, and the $A_{595}/A_{650}$ was determined using an MR600 Microplate Reader (Dynex Technologies, Chantilly, VA). The percentage of viable cells was calculated as absorbance of cells treated with drug divided by the average absorbance of untreated cells. In each case, the results shown indicate representative results of at least three independent experiments. Trypan blue assays were performed by incubating cells in 0.4% trypan blue (Sigma-Aldrich) for 5 min and counting the percentage of blue cells, which are inviable cells that are unable to

![Fig. 3.](https://i.imgur.com/3H5Q5QG.png)  

**Fig. 3.** Hpr6 binds to heme, and Hpr6-D120G lacks heme binding. A, purification of GST-Hpr6 (lane 1) and GST-Hpr6-D120G (lane 2) fusion proteins. Proteins were purified and analyzed by SDS-polyacrylamide gel electrophoresis. B, absorbance scans of Hpr6 (top line) and Hpr6-D120G (bottom line) from 360 to 580 nm. The scan shows a peak at 400 nm for Hpr6 that is characteristic of heme-binding proteins. The 400-nm peak was absent in the Hpr6-D120G scan. C, diagram of the domain structure of Hpr6 (top), the region of Hpr6 purified as a GST fusion protein (second from top), the site of the D120G mutation within the GST-Hpr6 fusion protein (second from bottom), and the site of the D120G mutation within the Hpr6$^{HBD}$ construct (bottom).
exclude the dye. Trypan blue measurements were also performed in triplicate.

**Western Blots.** Western blots of cultured cells were performed as described previously (Yang et al., 2003). The antibodies used were as follows: anti-HA (HA11; BabCO, Richmond, CA), tubulin (Fisher Scientific Co.), poly(ADP-ribose) polymerase (PARP; Santa Cruz Biotechnologies, Santa Cruz, CA), Hpr6 (Meyer et al., 1998), and Cyp21 (Corgen LLC, Guilford, CT). The antibody to Cyp1A1 was a kind gift from Dr. Harry Gelboin (The Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD). The intensities of the Hpr6 and tubulin bands were quantitated by densitometry using ImageQuant software (GE Healthcare), and the ratio of Hpr6 to tubulin was calculated from these values.

**Reverse Transcription-PCR.** RNA was purified using the RNAeasy kit from QIAGEN (Valencia, CA) and reverse-transcribed using SuperScript II reverse transcriptase and random hexamers (Invitrogen). PCR reactions were performed using Taq polymerase (Genscript, Piscataway, NJ) in an Eppendorf Master Cycler (Eppendorf North America, Westbury, NY) using 25 to 35 cycles of a program consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR reactions contained primers to HPR6 and to actin, which was an internal control for equal loading of the cDNA. DNA was then visualized by electrophoresis in 2.5% agarose 1000 (Invitrogen). The primer sequences were HPR6/M1 (5′-CTCGAGACTGCACTTCTGGACTGCACT-3′) and HPR6/M2 (5′-GGGACACCTGTTGTCCTTG-3′) and HPR6/Actin1 (5′-GCAAACACTGTGCTTATATTCTG-3′).

**Doxorubicin Staining and Flow-Cytometry Analysis.** For doxorubicin visualization, MDA-MB-231 cells were plated in six-well culture slides and infected with 1000 pfu/cell Ad-LacZ or Ad-Hpr6hbd for 24 h. The cells were then incubated with 1 μM doxorubicin for 24 h, washed three times with phosphate-buffered saline, fixed with formaldehyde, and visualized using a Zeiss fluorescence microscope. For fluorescence-activated cell sorting analysis, MDA-MB-231 cells were plated at a density of 500,000 cells/100 mm2 dish and then infected with 1000 pfu/cell Ad-LacZ or Ad-Hpr6hbd for 24 h. The cells were treated with 1 μM doxorubicin for 6 h, and the drug was removed and replaced with normal medium. The cells were then harvested, washed once with phosphate-buffered saline, and analyzed by fluorescent-activated cell sorting at the University of Kentucky Flow Cytometry Facility.

### Results

**Hpr6 Expression Is Induced by Doxorubicin.** Hpr6 is expressed in a number of breast cancer cell lines (Cruden et al., 2005), and we measured the extent to which Hpr6 expression is altered by chemotherapeutic drugs. Hpr6 expression was induced by 0.5 and 1 μM topoisomerase II inhibitor doxorubicin (Fig. 1, lanes 1–3) and 0.25 μM topoisomerase I inhibitor camptothecin (Fig. 1, lane 4) in MDA-MB-231 breast cancer cells. These findings are consistent with our observations with a cDNA microarray that Hpr6 is transcriptionally induced by doxorubicin in MDA-MB-231 cells (Cruden et al., 2005).

**Inhibiting Hpr6 Expression Increases Chemotherapy Susceptibility.** In some cases, genes that are induced by a damaging agent regulate susceptibility to that agent. To determine the role of Hpr6 in doxorubicin susceptibility, we inhibited Hpr6 expression using RNAi. MDA-MB-231 cells were transiently transfected with oligonucleotide duplexes consisting of a random sequence (Con) or a sequence targeting Hpr6 (Hpr6i), and Hpr6 inhibited Hpr6 transcript levels by 62% relative to actin (Fig. 2A). The viability of Con- and Hpr6i-transfected cells was analyzed by MTT assay four days after transfection and was not significantly different in the two cell populations (P = 0.17).

Hpr6 inhibition caused a significant increase in susceptibility to doxorubicin (Fig. 2B, closed triangles) and camptothecin (Fig. 2B, open triangles) compared with control cells (Fig. 2B, open and closed squares). Measurements were performed in triplicate, and error bars indicate mean ± S.D. (Fig. 2B). The difference between Hpr6i- and control-transfected cells was highly significant at doses of 0.5 and 0.125 μM doxorubicin (P = 0.006 and 5 × 10−5, respectively, by Student’s t test) and 0.5 μM camptothecin (P = 0.002). We conclude that Hpr6 regulates susceptibility to two different chemotherapeutic drugs. Hpr6 inhibition also caused a moderate increase in susceptibility to mechlorethamine (83% ± 0.3 for the control cells versus 65% ± 7.5 for Hpr6i-transfected cells), but the result was marginally significant (P = 0.05).

**Hpr6 Binds to Heme, and the D120G Mutation in the Heme-1 Domain Blocks Heme-Binding Activity.** Homologues of Hpr6 bind to heme (Min et al., 2004; Mallory et al., 2005a). We purified a GST fusion protein containing amino acids 43 to 195 of Hpr6 (Fig. 3A) and found that purified Hpr6 had an absorbance peak at 400 nm that shifted to 420 nm after reduction with sodium hydrosulfite (Fig. 3B), as is characteristic of heme-binding proteins. Hpr6 and its homologues share the sequence FYGP-x-GPY-x-x-FAG-x-DASR-x-LA within their heme-1 domain (Mifsud and Bateman, 2002), and an Asp-to-Gly mutation in this sequence inactivates the yeast Dap1p protein (Mallory et al., 2005a). We made the analogous D120G mutation in Hpr6 (Fig. 3D) and found that the 400-nm absorbance peak was absent in the Hpr6-D120G protein (Fig. 3C). As a result, we did not detect a peak at 420 nm in the Hpr6-D120G mutant after hydrosulfite treatment. We then measured the A400 in triplicate preparations of Hpr6 and Hpr6-D120G proteins, and the difference in absorbance between the two proteins was statistically significant (P = 0.03). By adding heme to purified Hpr6, we estimated the K_D for heme as 0.4 mM, whereas the K_D for...
Hpr6-D120G was 1.9 mM, a 5-fold decrease. We conclude that Hpr6 is a heme-binding protein and that Hpr6-D120G is an inactive form of the protein.

The Ad-Hpr6hbd Adenovirus Increases Chemotherapeutic Drug Susceptibility. To test the role of heme binding in Hpr6-mediated drug resistance, we prepared an adenovirus called Ad-Hpr6hbd encoding the Hpr6-D120G protein (diagrammed in Fig. 3D). We infected MDA-MB-231 human breast cancer cells with Ad-Hpr6hbd, resulting in efficient, dose-dependent expression of Hpr6hbd (Fig. 4A, top, lanes 1–3) that was absent in cells infected with a control Ad-LacZ adenovirus (Fig. 4A, top, lane 4). Hpr6hbd was overexpressed relative to the endogenous Hpr6 protein when detected with antibodies to the HA epitope tag (Fig. 4B, top) and total Hpr6 (Fig. 4B, middle).

Ad-Hpr6hbd-infected cells suffered a significant loss of viability after doxorubicin treatment compared with cells infected with a control virus (Fig. 5, A–C). Loss of viability in Ad-Hpr6hbd-expressing cells was significant at doses of 0.5 (P = 0.0003), 1 (P = 0.0002), and 1.5 μM doxorubicin (P = 0.008). In the absence of doxorubicin, Ad-Hpr6hbd had no detectable effect on tumor cell survival or proliferation (Fig. 5, A–C, 0 dose of doxorubicin). Because the MTT assay measures mitochondrial activity, it is formally possible that Hpr6hbd-expressing cells are viable but have limited mitochondrial activity. To test this possibility, we measured viability of control and Hpr6hbd-expressing cells before and after doxorubicin treatment by trypan blue assays (Fig. 5B). As expected, Ad-Hpr6hbd-expressing cells exhibited decreased viability after doxorubicin treatment (Fig. 5B, 1/9262 M dose of doxorubicin; P = 0.0006). Doxorubicin susceptibility was dependent on the dose of Ad-Hpr6hbd (Fig. 5C; P = 0.0002 at 1000 pfu/cell and P = 0.0001 at 2000 pfu/cell). We conclude that the heme binding activity of Hpr6 regulates doxorubicin susceptibility in MDA-MB-231 breast cancer cells.

Ad-Hpr6hbd also increased susceptibility to the topoisomerase I and II inhibitors camptothecin and etoposide. MDA-MB-231 cells were infected with Ad-LacZ or Ad-Hpr6hbd and treated with 125 to 1000 nM camptothecin. Ad-Hpr6hbd significantly increased sensitivity to camptothecin at a dose of

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125 nM (Fig. 5D, P = 0.005). In contrast, Ad-Hpr6<sup>hbd</sup> had no effect on the susceptibility of MDA-MB-231 cells to the alkylating agent mechlorethamine (data not shown). We conclude that the effects of Ad-Hpr6<sup>hbd</sup> are relatively specific and do not include all classes of damaging agents and drugs.

**Hpr6<sup>hbd</sup> Does Not Induce Cell Death via a Classical Apoptotic Pathway.** Doxorubicin frequently induces cell death via the apoptotic pathway. We detected rounded cells after infection with Ad-Hpr6<sup>hbd</sup> and treatment with doxorubicin, although the majority of the cells had a wasted necrotic morphology (Fig. 6A, right). These cells were less evident in cells infected with a control adenovirus (Fig. 6A, left). In spite of the fact that the cells were rounded, we were unable to detect classical markers of apoptosis, including cleavage of PARP (Fig. 6B), the focal adhesion kinase, endonucleolytic cleavage of chromosomal DNA, or nuclear condensation (data not shown).

**Hpr6 Does Not Regulate Doxorubicin Accumulation.** We tested the possibility that Hpr6 regulates drug susceptibility by altering intracellular accumulations of chemotherapeutic drugs. To test this, cells were infected with Ad-LacZ or Ad-Hpr6<sup>hbd</sup>, treated with doxorubicin, and examined by fluorescence microscopy. We were unable to detect any difference in doxorubicin accumulation in Ad-LacZ versus Ad-Hpr6<sup>hbd</sup>-infected cells (Fig. 7A). We then measured doxorubicin concentrations after 24 h of treatment by fluorescence-activated cell sorting and were unable to detect any differences in doxorubicin levels (Fig. 7B). We conclude that Hpr6 does not have a significant effect on the intracellular accumulation of doxorubicin. Hpr6<sup>hbd</sup> expression also had no effect on the stability of the cytochrome P450 proteins Cyp1A1 and Cyp21 (Fig. 6B, top middle and bottom middle).

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**Fig. 6.** Hpr6<sup>hbd</sup> induces cell death but does not induce apoptosis. A, morphology of MDA-MB-231 cells infected with Ad-LacZ (left) or Ad-Hpr6<sup>hbd</sup> (right) and treated with 1 μM doxorubicin for 72 h. Ad-Hpr6<sup>hbd</sup>-infected, doxorubicin-treated cells generally had a rounded or necrotic morphology. B, Hpr6<sup>hbd</sup> does not alter the levels of apoptosis or two cytochrome P450 proteins in doxorubicin-treated cells. MDA-MB-231 cells were infected with Ad-LacZ (lanes 1 and 3) or Ad-Hpr6<sup>hbd</sup> (lanes 2 and 4) and left untreated (lanes 1 and 2) or were treated with 1 μM doxorubicin (lanes 3 and 4). The cells were then lysed and analyzed for the migration of PARP (top), Cyp1A1 (top middle), or Cyp21 (bottom middle). Ad-Hpr6<sup>hbd</sup> did not increase levels of p85<sub>PARP</sub> or p112<sub>PARP</sub>, a marker for apoptosis, either before or after treatment with doxorubicin, and Hpr6<sup>hbd</sup> did not alter the levels of Cyp1A1 or Cyp21.

**Fig. 7.** Hpr6<sup>hbd</sup> does not alter the accumulation of doxorubicin. A, MDA-MB-231 cells were infected with either Ad-LacZ (left) or Ad-Hpr6<sup>hbd</sup> (right) and treated with 1 μM doxorubicin for 24 h. Cells were then fixed and visualized using bright-field microscopy (top) or fluorescence (bottom). In both groups of infected cells, doxorubicin accumulated in the nucleus to similar extents. B, MDA-MB-231 cells were infected with either Ad-LacZ (left) or Ad-Hpr6<sup>hbd</sup> (right) and left untreated (top) or were treated with 1 μM doxorubicin for 24 h (bottom). Cells were then analyzed by fluorescence-activated cell sorting.
Discussion

Breast cancer is usually treated with a combination of chemotherapeutic agents that includes doxorubicin or related anthracyclines. We have shown that Hpr6 regulates the susceptibility of breast cancer cells to doxorubicin and other chemotherapeutic drugs. We have also shown that Hpr6 is a heme-binding protein and that expression of an Hpr6 mutant that cannot bind heme increases chemotherapy susceptibility in breast cancer cells. These findings suggest that Hpr6 increases cancer cell survival during chemotherapy.

One potential model is that Hpr6 alters doxorubicin susceptibility by regulating the activity of cytochrome P450 proteins. In rats, the Hpr6 homolog IZa localizes to the adrenal inner zones, the zona fasciculata, and zona reticularis (Laird et al., 1988), and a monoclonal antibody to IZa inhibited two reactions catalyzed by cytochrome P450 in the adrenal gland (Laird et al., 1988). Furthermore, IZa elevated cycP21/steroid 21-hydroxylase activity in transfected cells (Min et al., 2004). In yeast, the Hpr6 homolog Dap1p regulates sterol synthesis and damage resistance (Hand et al., 2003) by stabilizing the cytochrome P450 protein Cycp11a/Erg11p (Mallory et al., 2005a). However, Hpr6 did not alter the expression of the cytochrome P450 proteins Cycp1a1 or Cycp21 in MDA-MB-231 cells (Fig. 6). Thus, Hpr6-mediated chemotherapy susceptibility does not correlate with a role for Hpr6 in regulating cytochrome P450 proteins. One possible explanation is that the Hpr6-related protein Dg6 (Gerdes et al., 1998) supplements the loss of Hpr6 in maintaining cytochrome P450 protein levels and that Hpr6 performs a damage function that is distinct from Dg6.

The structural basis through which Hpr6 <i>hbd</i> disrupts Hpr6 function is unknown. One likely mechanism for Hpr6 <i>hbd</i> is competition with endogenous Hpr6 for binding to substrates and associated proteins. However, there is evidence that Hpr6 homologues function as a covalently bound dimer (Min et al., 2004). Thus, Hpr6 <i>hbd</i> could bind directly to wild-type Hpr6, forming an inactive complex. Inactive heterodimer formation is a common mechanism among dominant-negative mutants (Rishi et al., 2004; Zhu et al., 2004), including drug resistance proteins (Kage et al., 2002) and cytochrome proteins (Curry et al., 2004). However, covalently bound Hpr6-Hpr6 <i>hbd</i> dimers would have been detected on Western blots of infected cells (such as those in Fig. 4), but these bands were not observed.

Because the Hpr6 homolog Dap1p regulates sterol synthesis (Hand et al., 2003; Mallory et al., 2005a), it is possible that Hpr6 also regulates the synthesis of sterols. Indeed, Hpr6 binds to the sterol regulatory protein Insigni-1 (Yang et al., 2002; Suchanek et al., 2005), which in turn regulates the sterol regulatory element-binding protein transcription factor via binding to sterol regulatory element-binding protein cleavage-activating protein (Yang et al., 2002; Anderson, 2003). If Hpr6 inhibition decreased sterol synthesis, this could alter the turnover of transmembrane receptors, a process that is dependent on sterol-rich lipid rafts (Le Roy and Wran, 2005). Several receptors have been implicated in doxorubicin susceptibility in breast cancer, including the hepatocyte growth factor receptor (Fan et al., 1998; Gao et al., 2001), although scatter factor failed to protect MDA-MB-231 cells from doxorubicin-mediated cell death (Fan et al., 1998). However, the relationship among Hpr6, sterol synthesis, and damage signaling is only speculative at the present.

In conclusion, our results are consistent with a model in which Hpr6 functions in protecting tumor cells from chemotherapeutic agents. In normal tissues, Hpr6 is highly expressed in liver and kidney (Gerdes et al., 1998; Hand and Craven, 2003). When overexpressed in breast tumors, Hpr6 could be corrupted to protect tumor cells from xenobiotic compounds, providing resistance to chemotherapeutic drugs. Our results indicate that inhibiting Hpr6 increases the ability of chemotherapeutic drugs to kill cancer cells, suggesting that Hpr6 is a target for intervention in cancer therapy.

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