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Regulation of the Stability of P-Glycoprotein by Ubiquitination

Zhigang Zhang, Jin-Ying Wu, William N. Hait, and Jin-Ming Yang

Departments of Pharmacology and Medicine, the Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, New Brunswick, New Jersey

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ABSTRACT

Ubiquitination plays a crucial role in regulating protein turnover. Here we show that ubiquitination regulates the stability of the *MDR*1 gene product, P-glycoprotein, thereby affecting the functions of this membrane transporter that mediates multidrug resistance. We found that P-glycoprotein was constitutively ubiquitinated in drug-resistant cancer cells. Transfection of multidrug-resistant cells with wild-type ubiquitin or treatment with an *N*-glycosylation inhibitor increased the ubiquitination of P-glycoprotein and increased P-glycoprotein degradation. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), a proteasome inhibitor, induced accumulation of ubiquitinated P-glycoprotein, suggesting the involvement of the proteasome in the turnover of the transporter. Treatment of multidrug-resistant cells with 12-*O*-tetradecanoylphorbol-13-acetate, a phorbol ester that increases the phosphorylation of P-glycoprotein through activation of protein kinase C, or substituting phosphorylation sites of P-glycoprotein by nonphosphorylatable residues did not affect the ubiquitination of the transporter. Enhanced ubiquitination of P-glycoprotein resulted in a decrease of the function of the transporter, as demonstrated by increased intracellular drug accumulation and increased cellular sensitivity to drugs transported by P-glycoprotein. Our results indicate that the stability and function of P-glycoprotein can be regulated by the ubiquitin-proteasome pathway and suggest that modulating the ubiquitination of P-glycoprotein might be a novel approach to the reversal of drug resistance.

Drug resistance is a major impediment to successful cancer chemotherapy. Multidrug resistance (MDR) mediated by P-glycoprotein (P-gp), the product of the *MDR*1 (*ABCB1*) gene, is believed to be one of the major causes of failure of cancer therapy. P-gp is a 150- to 180-kDa heavily glycosylated and phosphorylated plasma membrane protein that functions as a drug transporter. Overexpression of P-gp confers resistance to a variety of structurally and functionally diverse anticancer drugs such as paclitaxel, doxorubicin, and vinblastine. Despite promising early studies showing that blocking of P-gp by pharmacological means could sensitize drug-resistant cells, the ultimate goal of restoring drug sensitivity has met with limited success in clinical trials. Therefore, we and others began to elucidate the factors that control P-gp synthesis. For example, we have demonstrated that several substances control the expression of *MDR*1 through activation of phospholipase C and that the transcriptional modulation of *MDR*1 expression by phospholipase C is mediated by the Raf–mitogen-activated protein kinase pathway (Yang et al., 2001). We recently have become interested in the factors that regulate P-gp stability.

P-gp, at steady state, is located in the plasma membrane and undergoes endocytosis and recycling (Kim et al., 1997). Experimentally induced alterations in trafficking of P-gp can change the steady-state distribution of the transporter, thereby affecting the MDR phenotype (Kim et al., 1997). Proteasome inhibitors can decrease the activity of P-gp by preventing its maturation and localization in the plasma membrane (Loo and Clarke, 1999). These studies indicate that reducing the content of P-gp within the plasma membrane by perturbing its subcellular localization, inhibiting its synthesis, or facilitating its degradation might be effective approaches to modulating MDR in cancer cells.

P-gp is a relatively stable protein with a half-life of 14 to 17 h (Muller et al., 1995); its immature, core-glycosylated, or glycosylation-deficient forms have much shorter half-lives (approximately 3 h) (Loo and Clarke, 1994, 1999). *N*-glycosylation was shown to contribute to the stability of P-gp (Schinkel et al., 1993), and inhibiting glycosylation reduced membrane-associated P-gp and altered the MDR phenotype (Kramer et al., 1995). In addition, a protease-sensitive site was found in the first extracellular loop near the consensus glycosylation sites of P-gp, and proteolytic enzymes were

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ABBREVIATIONS: MDR, multidrug resistance; P-gp, P-glycoprotein; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

shown to play an important role in proper P-gp folding (Loo and Clarke, 1998).

It is now appreciated that ubiquitination, a reaction in which ubiquitin molecules are covalently ligated to substrate proteins via isopeptide bonds formed through its C-terminal glycine to the ϵ-amino group of lysine residues, plays a crucial role in degrading certain membrane proteins. For example, ubiquitination was found to be required for degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) by the proteasome (Ward et al., 1995). A number of other plasma membrane proteins, such as the epithelial Na^+ channel (Staub et al., 1997), epidermal growth factor receptor (Galcheva-Gargova et al., 1995), and platelet-derived growth factor receptor (Mori et al., 1992), are ubiquitinated before proteasomal or lysosomal degradation.

Despite a previous study showing that inhibition of calpain-mediated proteolysis caused the accumulation of ubiquitinated P-gp (Ohkawa et al., 1999), little is known about the factors that regulate P-gp ubiquitination or the impact of the modification on P-gp function. Therefore, the aim of this study was to explore the role of ubiquitination in the stability of P-gp, the factors that control the process, and the possible effect on the function of the transporter. We demonstrate here for the first time that the stability of P-gp is regulated by the ubiquitin-proteasome pathway in MDR cancer cells. Furthermore, we show that inhibition of *N*-glycosylation enhances the ubiquitination and degradation of P-gp, thereby reducing the function of the multidrug transporter.

Materials and Methods

Cell Lines and Culture. The MDR human breast cancer cell lines MCF-7/AdrR and MCF-7/BC-19 and their parental, sensitive line MCF-7 were kindly supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE). They were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. MCF-7/AdrR was developed by step-wise selection (Cohen et al., 1986), and MCF-7/BC-19 is an *MDR*1-transfected MCF-7 cell line (Yu et al., 1991). The MDR human oral carcinoma line KBV-1 (Ueda et al., 1986) and the sensitive parental line KB3-1, and murine NIH3T3 fibroblasts and their *MDR*1 transfectants N3V2400 (wild-type P-gp), N4V600, and N5V2400 (phosphorylation-defective mutants of P-gp) were provided by Dr. Michael Gottesman of the National Cancer Institute (Bethesda, MD), and were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under conditions identical with those described above. For KBV-1, 1 μg/ml vinblastine was added in the medium for the maintenance of the MDR phenotype. For N3V2400, N5V2400, and N4V600, 2400 ng/ml and 600 ng/ml vincristine were added in the medium, respectively. Human ovarian carcinoma cell lines, A2780 and A2780Dx5, were provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY) and were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under condition identical with those described above except that for A2780Dx5, 2 μM doxorubicin was added to the medium for the maintenance of the MDR phenotype (Alaoui Jamali et al., 1989). All cultures were checked routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 3 months, and new lines were obtained from frozen stocks.

Antibodies and Reagents. Polyclonal antibody mdr (Ab-1), recognizing human P-gp, and C219 anti-P-gp monoclonal antibody were obtained from Calbiochem (San Diego, CA). Polyclonal antiubiquitin antiserum was purchased from Sigma-Aldrich (St. Louis, MO), and monoclonal antiubiquitin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Additional reagents were purchased from the following sources: Ubiquitinated Protein Enrichment Kit and MG-132, from Calbiochem; Tunicamycin, from Sigma-Aldrich; LipofectAMINE 2000, from Invitrogen (Carlsbad, CA); Protein-A Sepharose CL-4B, ECL Western blotting analysis kit, and [^{35}S]methionine (in curies per millimole), from Amersham Biosciences Inc. (Piscataway, NJ).

Immunoprecipitation and Immunoblotting. Confluent cells in 150-mm dishes were washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), scraped off the dishes, and pelleted at 800*g* for 10 min. Cell pellets were then lysed in cold TNT buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 10 mM iodoacetamide, 1 mM PMSF, and 1% aprotinin) for 45 min with occasional rocking. The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000*g* for 40 min at 4°C. Identical amounts (2 mg of protein) of precleared cell lysates were immunoprecipitated with 1 μg mdr (Ab-1) or antiubiquitin polyclonal antibodies by overnight incubations at 4°C after adjusting the volumes to 0.5 ml with cold NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mM PMSF, and 1% aprotinin). The immune complexes were precipitated with Protein-A Sepharose CL-4B and washed three times with TNT buffer, once with NET buffer, and once with PBS.

Immunoprecipitated proteins were eluted with Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, and 0.001% bromphenol blue) and resolved by SDS-PAGE. Transfer of proteins to nitrocellulose was carried out by the method of Towbin et al. (40). The blots were incubated in blocking solution consisting of 5% milk and 0.1% bovine serum albumin in PBS plus 0.1% Tween 20 at room temperature for 1 h, then immunoblotted with anti–P-gp, antiubiquitin, or anti–β-actin monoclonal antibodies. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham).

Preparation of Cytosolic and Membrane Fractions. Membrane and cytosolic fractions were prepared in homogenization buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM iodoacetamide, 0.2 mM PMSF, and 20 μM *N*-acetyl-leucinyl-leucinyl-norleucinal-H), as described previously (VanSlyke and Musil, 2002). In brief, cells plated in 150-mm culture dishes were grown to 95% confluence and then homogenized in homogenization buffer. Membrane and cytosolic fractions were separated by centrifugation at 100,000*g* for 60 min at 4°C.

Plasmids and Transfection. Plasmids PCW7 (wild-type ubiquitin) and PCW8 (ubiquitin K48R mutant) were kindly provided by Dr. Ron Kopito (Stanford University, Stanford, CA). Cells were transfected with the plasmids using LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol. In brief, 90% confluent cells grown in 100-mm dishes were washed twice with serum-free medium before the addition of 25 μg of plasmid DNA and 60 μl of LipofectAMINE 2000 in 3 ml of serum-free media. After 4-h incubation at 37°C, 7 ml of medium containing 10% fetal bovine serum was added. Incubations were continued at 37°C for another 48 h.

Metabolic Labeling and Pulse-Chase Analysis. Metabolic labeling was performed as described previously (Muller et al., 1995). In brief, MCF-7/AdrR cells were plated in 100-mm dishes and grown to 60 to 70% confluence in complete growth medium, then [^{35}S]methionine (10 μCi/ml) was added. After 48 h of incubation, the cells were washed and incubated in complete growth medium containing 450 μg/ml methionine. The labeled proteins were chased at 0, 6, 12, 24, and 48 h. P-gp levels were determined by immunoprecipitation with an anti-P-gp antibody, mdr (Ab-1), and resolved by 7% SDS-PAGE. The gels were fixed with 10% methanol/5% glycerol, dried, and exposed for 5 days to X-ray film with an intensifying screen at −70°C.

Measurement of Doxorubicin Accumulation. Control or ubiquitin- or tunicamycin-treated cells were incubated with 25 μM of doxorubicin for 2 h. At the end of incubation, cells were washed three times with PBS and observed under a fluorescence microscope with 100× magnification (Nikon ECLIPSE TE200; Nikon Inc., Melville, NY).

Assay of Drug Sensitivity. Cells grown in 96-well plates were transfected with PCW7 or control vector. Twenty-four hours later, various concentrations of drug were added, and the cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for another 72 h. At the end of incubation, the viability of cells was determined using Promega's CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Results

Ubiquitination of P-gp and Its Effect on P-gp Turnover. To investigate whether P-gp was ubiquitinated in MDR cells, cell lysates were immunoprecipitated with an anti-P-gp antibody followed by immunoblotting with an antiubiquitin antibody, or by a reciprocal approach. As shown in Fig. 1, A and B, ubiquitinated P-gp was found in all MDR cell lines tested, including the *MDR*1 transfectant, MCF-7/BC19, and drug-resistant lines whose expression of P-gp was induced by step-wise selection with chemotherapeutic drugs (MCF-7/AdrR, KBV-1, and A2780Dx5). Ubiquitinated P-gp was also detected using polyubiquitin-affinity beads composed of a glutathione *S*-transferase fusion protein containing a ubiquitin-associated sequence (Chen et al., 2001; Chen and Madura, 2002) conjugated to glutathione-agarose, then immunoblotted with an anti-P-gp antibody (Fig. 1C). Ubiquitination of P-gp was only detected in the plasma membrane fraction (Fig. 2).

To determine the effect of ubiquitination on P-gp stability, we transiently transfected the MDR MCF-7 cells with a wild-type ubiquitin plasmid, PCW7, or a dominant-negative ubiquitin, PCW8 (K48R mutant), in which the invariant lysine at position 48 was replaced by arginine (Finley et al., 1994). The lysine 48 is the site of isopeptide linkage to other ubiquitin molecules and is required for the generation of multiubiquitin chains that mark proteins for degradation (Ward et al., 1995; Ciechanover and Schwartz, 1998). The K48R ubiquitin mutant (PCW8) produces ubiquitin chain termination and accumulation of incompletely ubiquitinated proteins that are not targeted for proteasomal degradation (Ward et al., 1995; Yu and Kopito, 1999). Transfection of wild-type ubiquitin increased accumulation of ubiquitinated P-gp (Fig. 3B) as well as other ubiquitinated cellular proteins (Fig. 3A). This increase in ubiquitination was accompanied by a reciprocal decrease in P-gp (Fig. 3, A and B). In contrast, transfection of the dominant-negative mutant of ubiquitin caused an increase in P-gp content (Fig. 3C), indicating that disruption of P-gp ubiquitination decreased its degradation. Neither wild-type nor dominant-negative ubiquitin affected the content of β-actin, a protein whose monoubiquitination does not trigger proteolysis (Ball et al., 1987; Hicke, 2001).

Inhibition of Glycosylation Increases Ubiquitination and Turnover of P-gp. Because *N*-glycosylation is linked to P-gp stability (Schinkel et al., 1993), we studied whether ubiquitination of P-gp was influenced by its glycosylation state. Tunicamycin, a compound that inhibits the formation of N-linked oligosaccharide chains (Elbein, 1987), increased the ubiquitination of P-gp (Fig. 4). In pulse-chase experiments, the degradation of P-gp in tunicamycin-treated cells (half-life, ~4 h) was approximately 3-fold faster than that in vehicle-treated cells (half-life, ~12 h) (Fig. 5). These data provide a clear link between glycosylation, ubiquitination, and degradation of P-gp.

Phosphorylation Does Not Affect P-gp Ubiquitination. The ubiquitination of certain plasma membrane proteins is regulated by phosphorylation (Hicke et al., 1998; Marchal et al., 1998). Because P-gp is also phosphorylated (Aftab et al., 1994), we sought to determine whether the ubiquitination state of P-gp was controlled by phosphoryla-

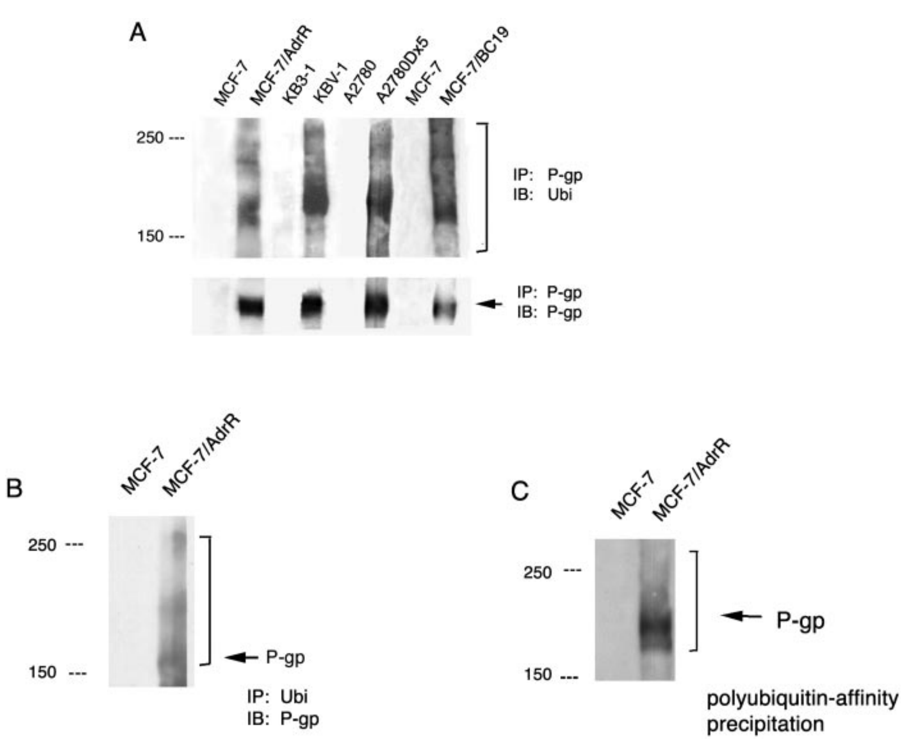

Fig. 1. P-gp is ubiquitinated in MDR cancer cells. A, cell lysates (2 mg of protein) were subjected to immunoprecipitation with 1 μg anti–P-gp polyclonal antibody (mdr Ab-1) followed by immunoblotting with a monoclonal antiubiquitin (top) or anti-P-gp (C219) (bottom) antibody, as described under *Materials and Methods*. B, the proteins immunoprecipitated with a polyclonal antiubiquitin antibody were immunoblotted with an anti–P-gp antibody. C, cell lysates were incubated with polyubiquitin affinity beads, then examined by immunoblotting with a monoclonal anti-P-gp antibody, C219. Results are representative of at least three similar experiments.

tion. Cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is known to increase the phosphorylation of P-gp through activation of protein kinase C (Fine et al., 1988; Aftab et al., 1994). Figure 6A shows that treatment of MCF-7/AdrR cells with TPA did not affect the level of P-gp ubiquitination. We also compared the ubiquitination of P-gp in NIH3T3 cells transfected with wild-type or phosphorylation-defective P-gp mutants. N3V2400 cells express wild-type P-gp, whereas N4V600 cells express a mutant P-gp in which serines at positions 661, 667, 671, 675, and 683 were replaced by nonphosphorylatable alanine residues. N5V2400 cells express the mutant-carrying aspartic acid residues at the respective positions to mimic permanently phosphorylated serine residues (Germann et al., 1996). N3V2400, N4V600, and N5V2400 lines express similar levels of P-gp, but N4V600 and N5V2400 exhibit no detectable levels of P-gp phosphorylation (Germann et al., 1996). As shown in Fig. 6B, the ubiquitination of P-gp in these three cell lines was identical. These experiments demonstrate that the phosphorylation state of P-gp had no effect on its ubiquitination.

The Proteasome Is Involved in the Degradation of P-gp. To assess whether P-gp was subject to proteasomal or lysosomal degradation, we treated the drug-resistant MCF-7 cells with MG-132, monensin, or NH_4Cl and then assayed the ubiquitination of P-gp. Treatment with the proteasome inhibitor MG-132 increased both P-gp and its ubiquitinated form in MCF-7/AdrR cells (Fig. 7A), whereas the lysosome inhibitors monensin and NH_4Cl had no effect on the content of P-gp or its ubiquitinated form (Fig. 7B). These experiments indicate that the proteasome, but not the lysosome, is involved in the P-gp turnover.

Effect of Ubiquitination on P-gp Function. We also studied the effect of ubiquitination on the function of P-gp and drug sensitivity. Figure 8A shows that, compared with controls, transfection of MCF-7/AdrR cells with the wild-type ubiquitin construct PCW7 enhanced the intracellular accumulation of doxorubicin, a P-gp–transportable chemotherapeutic drug. Tunicamycin, an inhibitor of *N*-glycosylation that increased ubiquitination and degradation of P-gp (Fig. 4), also increased intracellular doxorubicin accumulation (Fig. 8B). In addition, the sensitivity of MDR MCF-7 cells to

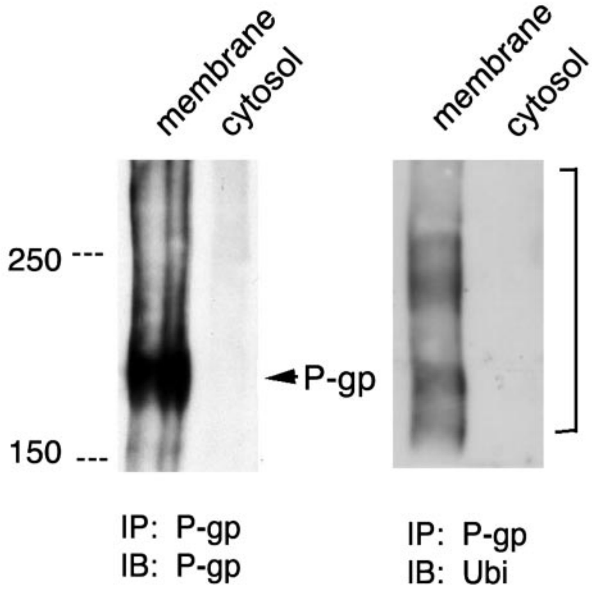

Fig. 2. Subcellular distribution of the ubiquitinated P-gp. Crude membrane and cytosol preparations from the human MDR breast cancer cell line, MCF-7/AdrR, were subjected to immunoprecipitation with 1 μg of anti–P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-P-gp (C219) or antiubiquitin antibody, as described in Fig. 1A. Results are representative of three similar experiments.

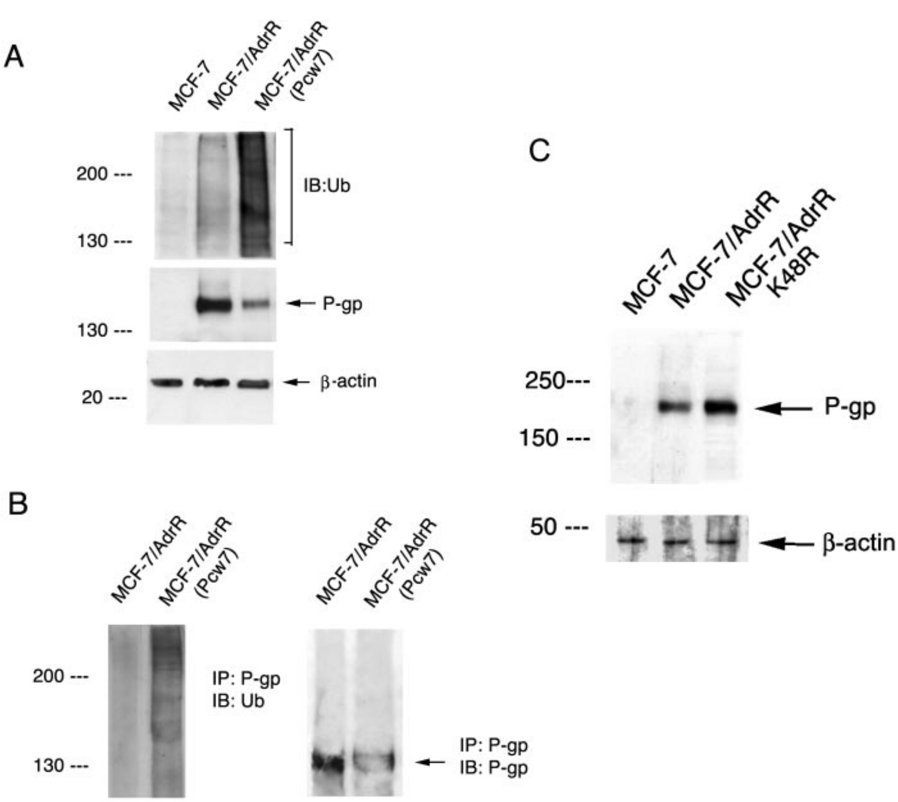

Fig. 3. Effects of exogenous ubiquitin on ubiquitination of P-gp. MDR MCF-7 cells were transfected with wild-type ubiquitin (A and B) or mutant ubiquitin K48R (C). Forty-eight hours later, the cells were lysed, and the cell lysates were analyzed by immunoblotting (A and C) or immunoprecipitation followed by immunoblotting (B), as indicated. Results are representative of three similar experiments.

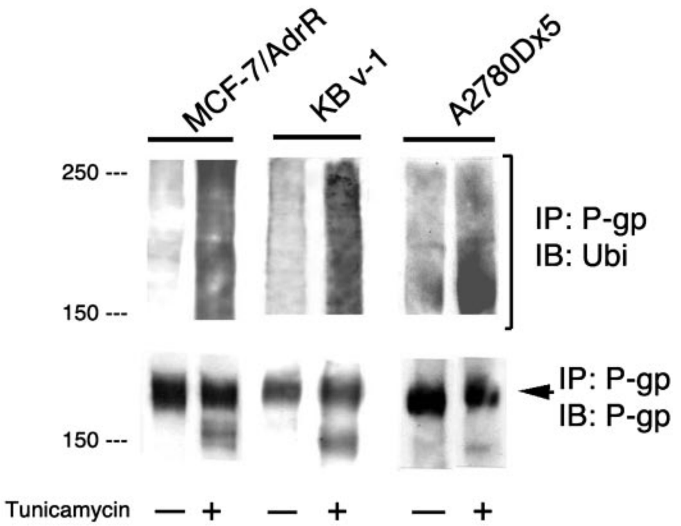

Fig. 4. Effect of tunicamycin on ubiquitination of P-gp. MDR cancer cells were treated with tunicamycin (5 μg/ml) for 10 h, and then cell lysates were prepared as described under *Materials and Methods*. Equal amounts (2 mg) of cell lysates were immunoprecipitated with 1 μg of anti–P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal antiubiquitin (top) or anti–P-gp (C219) (bottom) antibody. Results are representative of three similar experiments.

another P-gp–transportable drug, vinblastine, was increased by transfection with wild-type ubiquitin (Fig. 9). In contrast, ubiquitin transfection did not alter the sensitivity to a non–P-gp substrate drug, hydroxyurea, in MDR cancer cells (Fig. 9).

Discussion

In the current study, we investigated the role of ubiquitination in the stability and function of P-gp, an ATP-dependent drug transporter that mediates resistance to a variety of structurally and functionally diverse chemotherapeutic agents. We found that P-gp is ubiquitinated (Fig. 1) and that increasing ubiquitination of P-gp by transfection with wild-type ubiquitin or by treatment with the *N*-glycosylation inhibitor, tunicamycin, increases P-gp degradation (Figs. 3, A and B, 4, and 5), reduces the function of the protein (Fig. 8), and selectively increases the sensitivity of MDR cells to P-gp transportable cytotoxic drugs (Figs. 8 and 9). These results indicate that modification of P-gp via ubiquitination can affect the stability and activity of this drug transporter and suggest the potential for circumvention of P-gp–mediated drug resistance by modulating the ubiquitin pathway.

Covalent modification of cellular proteins with ubiquitin is associated with the regulation of diverse cellular processes,

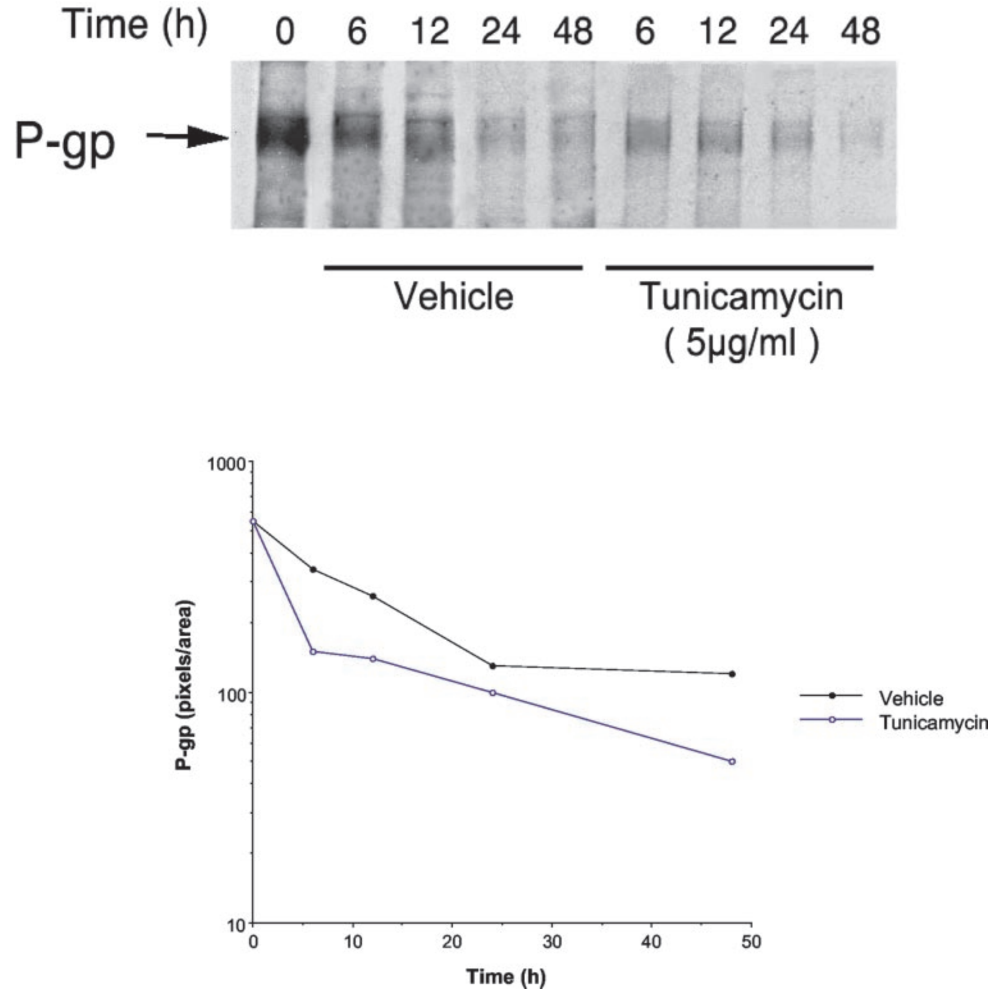

Fig. 5. Effect of tunicamycin on degradation rate of P-gp in MDR MCF-7 cells. MCF-7/AdrR cells were labeled with [^{35}S]methionine (10 μCi/ml) for 48 h and then chased with an excess of unlabeled methionine in the presence or absence of tunicamycin (5 μg/ml) for the indicated times. P-gp was immunoprecipitated with an anti–P-gp antibody, mdr (Ab-1), and analyzed by 7% SDS-PAGE and autoradiography, as described under *Materials and Methods*. Results are representative of three similar experiments.

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including stress response, oncogenesis, transcription, protein turnover, organelle biogenesis, DNA repair, and cell-cycle control (Peters et al., 1998). Although most of the proteins subjected to ubiquitin conjugation identified to date are cytoplasmic or nuclear, the ubiquitination of membrane proteins has been reported recently. For example, receptors for epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor were found to be ubiquitinated, and ubiquitination was shown to participate in cell-surface signaling (Mori et al., 1992; Stang et al., 2000). The role of ubiquitination in endocytosis and degradation of the yeast ATP-binding cassette transporters has also been reported (Kolling and Hollenberg, 1994; Egner and Kuchler, 1996). Here, we demonstrate for the first time that ubiquitination of the human multidrug transporter, P-gp, a member of the ATP-binding cassette family, is involved in regulating its turnover and function. It would be of interest to find whether other human multidrug transporters, such as

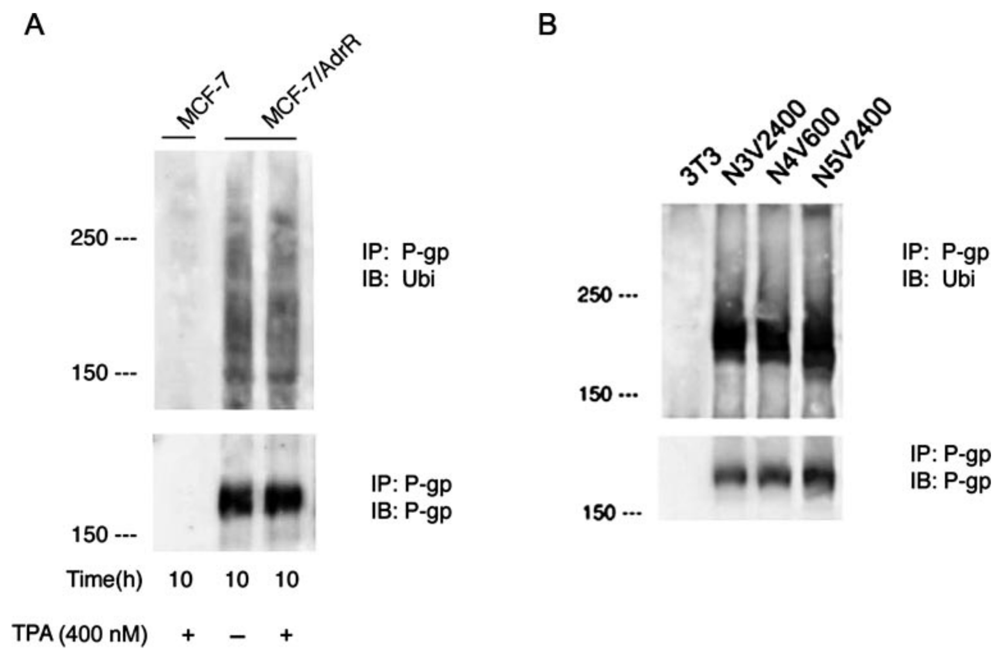

Fig. 6. Phosphorylation does not affect ubiquitination of P-gp. A, cells were treated with 400 nM TPA for 10 h, and then cell lysates were prepared as described under *Materials and Methods*. Equal amounts (2 mg) of cell lysates were subjected to immunoprecipitation with 1 μg of anti–P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal antiubiquitin (top) or anti–P-gp (C219) (bottom) antibody. B, cell lysates (2 mg of protein) prepared from parental NIH3T3 cells and from *MDR*1 transfectants expressing wild-type (N3V2400) or phosphorylation-defective mutants of P-gp were subjected to immunoprecipitation with 1 μg of anti–P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal antiubiquitin (top) or anti–P-gp (C219) (bottom) antibody, as described under *Materials and Methods*. Results are representative of two similar experiments.

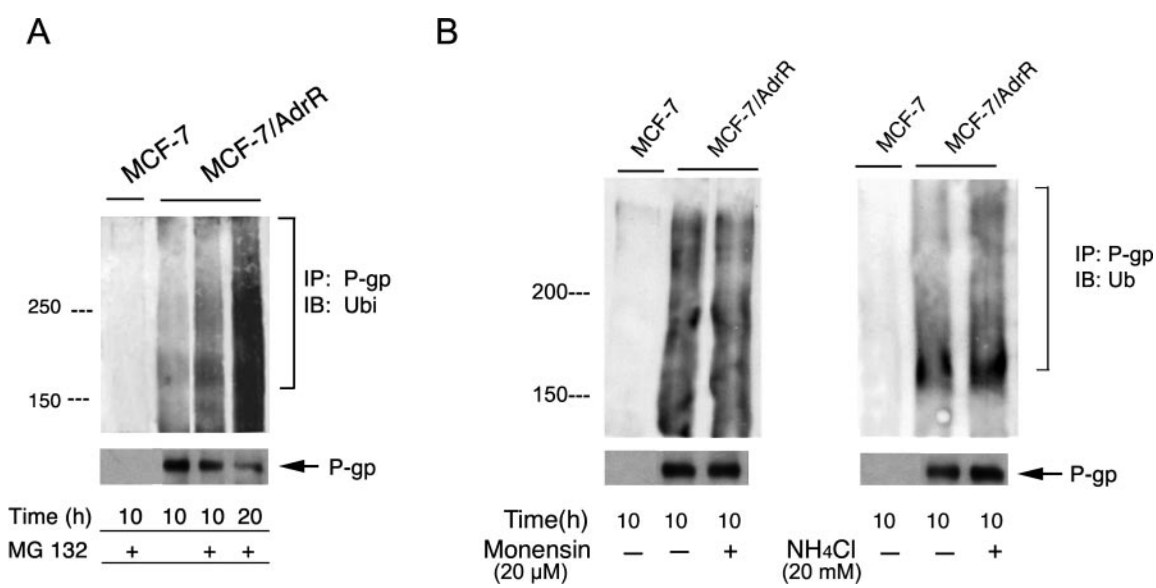

Fig. 7. Effect of the inhibitors of proteasome or lysosome on ubiquitination of P-gp. Cells were treated for 10 and 20 h with the proteasome inhibitor MG-132 (10 μM) or with the lysosome inhibitors monensin (20 μM) and NH_4Cl (20 mM). Cell lysates were immunoprecipitated with 1 μg of anti–P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal antiubiquitin antibody, as described under *Materials and Methods*. Results are representative of three similar experiments.

multidrug resistance-associated protein, breast cancer resistance protein, and lung cancer resistance protein are ubiquitinated, because these ABC transporters share many similarities (Gottesman et al., 2002).

Increases in ubiquitination by transfection with wild-type ubiquitin leads to a decrease in the content of P-gp (Fig. 3, A and B), whereas introduction of dominant-negative ubiquitin results in an increase in P-gp content (Fig. 3C). These data suggest that modification by ubiquitin is important for degradation of P-gp. We also found that degradation of P-gp seems to be mediated through the proteasomal rather than lysosomal pathway. This conclusion is derived from the results using the proteasome inhibitor MG-132, which increases the accumulation of P-gp and the ubiquitinated form of the transporter (Fig. 7A), and the lysosome inhibitors monensin and NH_4Cl, which do not do so even at high concentrations (Fig. 7B). This result is consistent with previous observations that the proteasome is involved in degradation of the misfolded, core-glycosylated, or glycosylation-deficient mutant of P-gp (Loo and Clarke, 1998; Gribar et al., 2000). For example, Loo and Clarke found that proteasome inhibitors prevented degradation of the proteolytic digestion products of P-gp (Loo and Clarke, 1998). Gribar et al. (2000) showed that treatment with proteasome inhibitors increased glycosylation-deficient P-gp. Therefore, the ubiquitinated forms of P-gp may represent immature or misfolded forms of the transporter. Our result differs from that of Ohkawa et al. (1999), who used a low concentration of lactacystin as proteasome inhibitor. Degradation of membrane proteins by either proteasomes or lysosomes, or by both, has been observed. For example, CFTR, an ABC transporter, is degraded by the proteasome (Ward et al., 1995), and Ste2p, a G protein-coupled plasma membrane receptor, is degraded in the lysosome (Hicke and Riezman, 1996). Both the proteasome and the lysosome are involved in the degradation of the epithelial Na^+ channel (Staub et al., 1997). In addition, degradation of membrane proteins by calpain has also been reported (Zaidi and Narahara, 1989; Salamino et al., 1994; Ohkawa et al., 1999).

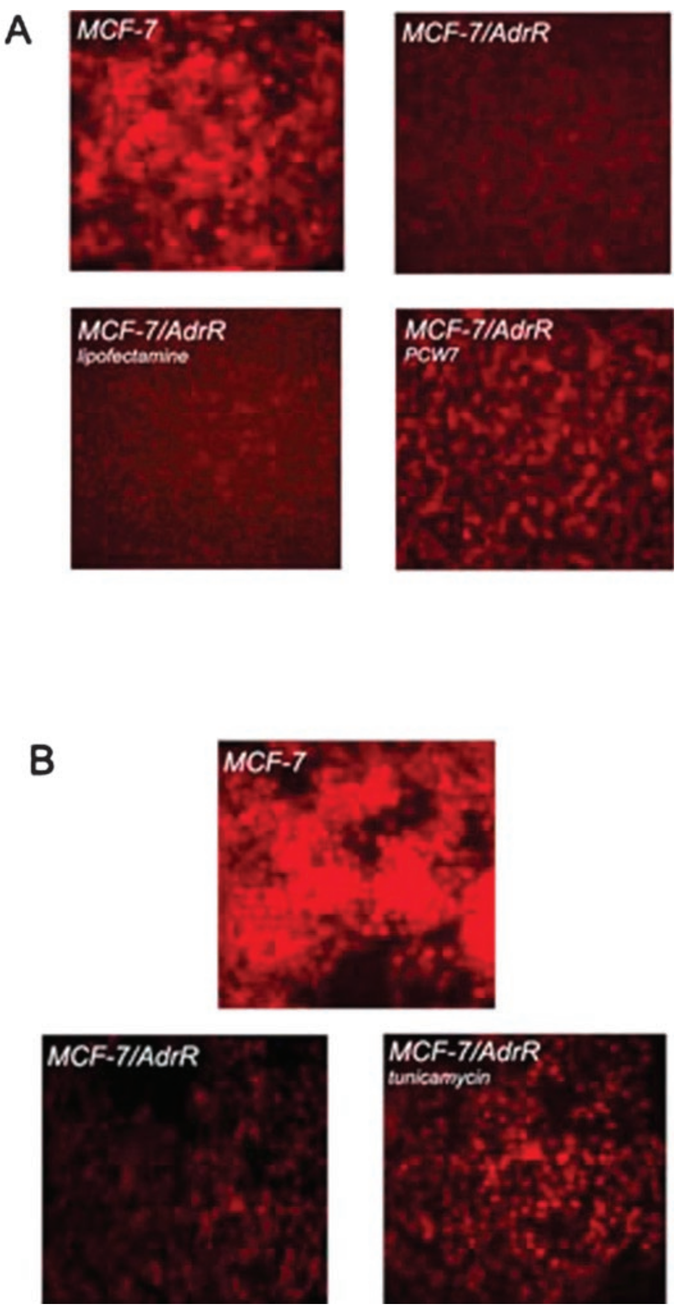

Fig. 8. Effect of exogenous ubiquitin (A) and tunicamycin (B) on doxorubicin accumulation in MCF-7/AdrR cells. MCF-7/AdrR cells grown in 100-mm culture dishes were transfected with the wild-type ubiquitin vector, PCW7 (A), or treated for 10 h with tunicamycin (5 μg/ml) or with vehicles. Cells were then incubated with 25 μM doxorubicin for 2 h, followed by washing three times with PBS. Doxorubicin accumulation was observed under a fluorescence microscope with 100× lens. Results are representative of two similar experiments.

The ubiquitination-proteasome system is known to be involved in the degradation of short-lived, mutant, or misfolded proteins. P-gp is a relatively stable protein with a half-life of 14 to 17 h (Muller et al., 1995). Inhibition of *N*-glycosylation increases the ubiquitination (Fig. 4), decreases the stability (Fig. 5), and reduces the function (Fig. 8B) of P-gp. Immature, core-glycosylated, or glycosylation-deficient P-gp has a shorter half-life of approximately 3 h (Loo and Clarke, 1994, 1999; Gribar et al., 2000). In addition, Loo and Clarke (1994) demonstrated that mutant forms of P-gp which are unable to fold into the mature forms are rapidly degraded. From the analysis of deletion mutants, Schinkel et al. (1993) proposed that *N*-glycosylation contributes to proper routing or stability of P-gp. Furthermore, inhibiting *N*-glycosylation of P-gp has been shown to decrease drug resistance in cancer cells overexpressing the transporter (Kramer et al., 1995). These data suggest that core-glycosylated, immature, or glycosylation-deficient P-gp are the targets of ubiquitination-proteasome system, and inhibition of *N*-glycosylation can decrease the stability of P-gp by increasing ubiquitination/proteasome-mediated degradation. Ubiquitinated P-gp is only detected in the plasma membrane fraction (Fig. 2), probably because the majority of cellular P-gp is located on the cell surface, and the intracellular P-gp pool may represent newly synthesized, properly folded protein (Kim et al., 1997; Ohkawa et al., 1999).

The ubiquitin-mediated turnover of P-gp has important functional consequences. For example, transfection of wild-type ubiquitin increased the ubiquitination and degradation of P-gp (Fig. 3B) and also restored intracellular accumulation of and sensitivity to drugs transported by P-gp (Figs. 8 and 9). Although expression of a dominant-negative ubiquitin increased P-gp abundance (Fig. 3C), this did not detectably

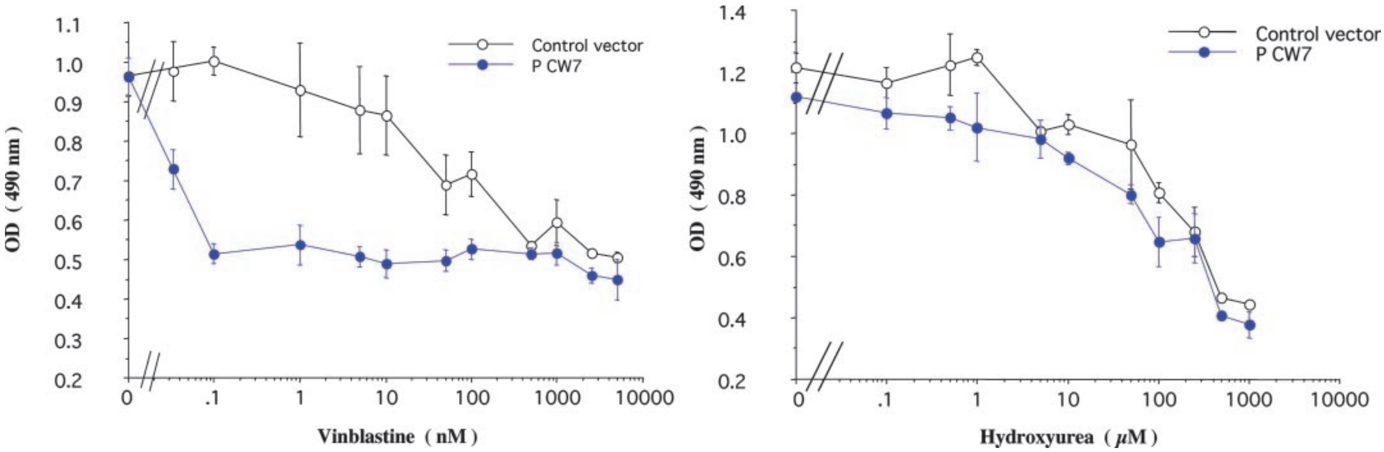

Fig. 9. Effect of exogenous ubiquitin on the sensitivity of MCF-7/AdrR cells to drugs. Transfected cells in 96-well plates in growth medium were incubated at 37°C for 72 h in the presence of varying concentrations of vinblastine or hydroxyurea. The viability of cells was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Each point represents the mean ± standard deviation of quadruplicate determinations. Results are representative of three similar experiments.

increase activity of the already active transporter (data not shown).

Using a web-based algorithm, PESTFind (http://www.at.embnet.org/embnet/tools/bio/PESTfind/), we did not find in P-gp any possible PEST sequence, a region usually considered to control the ubiquitination and degradation of short-lived proteins (Kornitzer et al., 1994; Marchal et al., 1998). This might account for the relative stable nature of P-gp. However, a PEST sequence is not always required for protein ubiquitination and degradation (Van Antwerp and Verma, 1996; Chen and Clarke, 2002). For example, the PEST sequence was found to play no significant role in ubiquitination and degradation of CFTR (Chen and Clarke, 2002). What trigger(s) the ubiquitination of membrane proteins is currently unclear. Although P-gp contains no PEST sequences, there are 85 lysine residues for potential ubiquitination. It is unfortunate that mapping of critical ubiquitination site(s) on P-gp through systematic mutation seems unrealistic, because mutation of a ubiquitinated lysine typically results in modification of a second nonphsyiological site (Hou et al., 1994).

Ubiquitination of P-gp is not affected by phosphorylation. We found that neither treatment with a protein kinase activator (TPA) that is known to increase P-gp phosphorylation nor abolishment of phosphorylation by mutating phosphorylation sites has any effect on the ubiquitination level of P-gp (Fig. 6). These experiments suggest that ubiquitination of P-gp is independent of phosphorylation. Phosphorylation was reported to play a role in controlling ubiquitination of other plasma membrane proteins (Hicke et al., 1998; Marchal et al., 1998), but it did not seem to affect ubiquitination of the yeast ABC transporter Ste6 (Kolling, 2002).

In summary, our results demonstrate that the ubiquitination-proteasome system plays a role in the turnover of P-gp, thereby affecting the function of the drug transporter. Therefore, modification of P-gp via ubiquitin-proteasome pathway might represent a novel strategy for modulating MDR in cancer cells.

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Address correspondence to: Dr. Jin-Ming Yang, The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, 195 Little Albany Street, New Brunswick, NJ 08901. E-mail: jyang@umdnj.edu