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Yeast Cysteine Proteinase Gene *ycp1* Induces Resistance to Bleomycin in Mammalian Cells

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SUMMARY

Tumor resistance to the glycopeptide anticancer drug bleomycin (BLM) has been suggested to involve metabolic inactivation by BLM hydrolase. Direct evidence for this hypothesis is lacking due to difficulties in obtaining full-length BLM hydrolase cDNA from mammalian cells. In the present investigation, we used the yeast cysteine proteinase gene *ycp1*, a homologue of the mammalian BLM hydrolase gene, to provide direct evidence of the importance of BLM metabolism in BLM resistance. Transfection of *ycp1* into NIH 3T3 cells induced resistance of these cells to BLM. The *ycp1*-transfected cells also metabolized BLM A_2 to its inactive metabolite deamido-BLM A_2 to a much greater extent. The *ycp1*-induced BLM resistance was completely reversed by the cysteine proteinase inhibitor E-64, a known inhibitor of BLM hydrolase. Transfection of NIH 3T3 cells with the plasmid pUT533-Sh *ble*, a bacterial BLM resistance gene that encodes a 14-kDa protein that does not metabolize BLM, also induced BLM resistance, but this resistance was not overcome by E-64. The results demonstrate that increased BLM hydrolase activity in NIH 3T3 cells causes BLM resistance and that inhibition of BLM metabolism sensitizes these cells to BLM. Thus, the molecular approach described in the present study directly implicates BLM hydrolase in BLM resistance.

BLM, an antitumor glycopeptide isolated from *Streptomyces verticillus*, is used in the treatment of several human cancers, including testicular carcinomas, lymphomas, and certain types of squamous cell carcinomas (1–4). BLM is a common component of several combination regimens in cancer treatment because it lacks typical side effects, such as bone marrow, hepatic, and renal toxicities, that are usually associated with traditional cytotoxic agents (2). However, the use of BLM as an anticancer drug is limited by two major obstacles: BLM-induced pulmonary fibrosis and tumor resistance (3, 5, 6).

Several mechanisms have been proposed for human tumor resistance to BLM; these include decreased drug uptake, increased drug efflux, increased repair of BLM-induced DNA damage, and metabolic inactivation (1–3). The discovery of a BLM-inactivating activity in normal and tumor tissues led to the suggestion that metabolic inactivation may have an important role in BLM resistance (7–12). The enzyme responsible for inactivating BLM was later named BLM hydrolase and shown to convert BLM to its inactive deamido metabolite by hydrolyzing the amide bond of the β-aminoalanine amide moiety at the carboxyl terminus of BLM (9–12). During the past three decades, there have been several conflicting reports concerning the role of BLM hydrolase in tumor resistance (for a review, see Ref. 1). Most of the reports focused on correlating the levels of BLM hydrolase activity to the levels of BLM resistance in human tumor cell lines. Recently, inhibitors of BLM hydrolase were used to investigate the role of BLM metabolism in tumor resistance, but the results remain correlative (13–17).

In an attempt to more directly address this question, we (18–21) and others (22) have purified BLM hydrolase to homogeneity and biochemically characterized it. We found BLM hydrolase to be a cytosolic, 250-kDa protein composed of five identical 50-kDa subunits (19). BLM hydrolase recognized and inactivated several BLM analogues, including BLM A_2, the major component of the clinically used mixture (20). Of great interest was the fact that the ability to hydrolyze BLM was inhibited by cysteine proteinase inhibitors (21). Furthermore, we isolated and sequenced a partial-length cDNA from a rabbit liver cDNA library and found that BLM hydrolase contains the active site of cysteine proteinases (21). We confirmed that BLM hydrolase is a novel cysteine proteinase by showing that BLM hydrolase hydrolyzes cysteine proteinase substrates such as arginine-4-methyl-7-

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ABBREVIATIONS:: BLM, bleomycin; Ycp1, yeast cysteine proteinase; E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane; DMEM, Dulbecco's modified Eagle medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPLC, high performance liquid chromatography; kb, kilobase(s).

coumarylamide and that the activity was inhibited by the cysteine proteinase-specific inhibitor E-64 (21).

We attempted previously to implicate the role of BLM hydrolase in BLM resistance with molecular approaches but were not successful because of difficulties encountered in isolating full-length mammalian BLM hydrolase cDNA (21). In the present study, we used the recently isolated yeast cysteine proteinase gene *ycp1* (23), a homologue of the mammalian BLM hydrolase gene, to demonstrate for the first time that BLM hydrolase induces resistance to the anticancer agent BLM.

Experimental Procedures

Plasmids. The plasmid pUT533-Sh *ble* (a gift from Prof. G. Tiraby, Universite Paul Sabatier, Toulouse, France) was derived from the pUT plasmid family (24) and contains a human cytomegalovirus enhancer and promoter, SV40 mRNA splice site and polyadenylation signal sequence (Fig. 1). The plasmid also contains a bacterial gene, Sh *ble*, which encodes a protein that can bind to BLM and inactivates it via a mechanism unrelated to BLM metabolism (25, 26). By modifying pUT533-Sh *ble*, we constructed a new mammalian expression vector named pUT533a (Fig. 1). In pUT533a, the Sh *ble* gene was replaced with the 36-base pair multicloning sequence from pBluescript KS− (Stratagene). To subclone the *ycp1* gene into pUT533a, the *ycp1* gene from the yeast genomic library clone 47C (23) was digested with *Acc*I. The resulting 4.1-kb fragment, which contains the entire uninterrupted open reading frame (1449 base pairs) of *ycp1* as well as its 5′ and 3′ flanking regions, was inserted into the *Acc*I site of pUT533a (Fig. 1). The pSV2-neo plasmid, which provides a selective marker in G418 medium (27), was purchased from American Type Culture Collection.

Transfection. The recipient NIH 3T3 cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin at 37° and 10% CO_2 atmosphere. Plasmid DNAs were introduced into the recipient cells by a liposome-mediated electroporation method. The pSV2-neo plasmid was cotransfected as a selective marker. The liposomes were kindly provided by Dr. L. Huang (University of Pittsburgh, Pittsburgh, PA) (28). Briefly, 10 μg of plasmid DNAs (in DMEM), with 0.5 μg of pSV2-neo plasmid, was first mixed with 100 μl liposomes (2 μmol/ml, premixed with Hanks' solution; BRL) and incubated at room temperature for ~30 min. The plasmid/liposome mixture was then added to the recipient NIH 3T3 cell suspension (3×10^6 cells) and electroporated at 220 V, 960 μF (Gene Pulser Transfection Apparatus, Bio-Rad). After electroporation, the cells were split into 10 Petri dishes (60 mm) with regular DMEM. On the second day, the cells were changed to DMEM with 0.5 mg/ml G418 (Sigma Chemical Co.). The G418-resistant colonies were isolated 2–3 weeks after the onset of selection, and single colonies were established into independent cell lines.

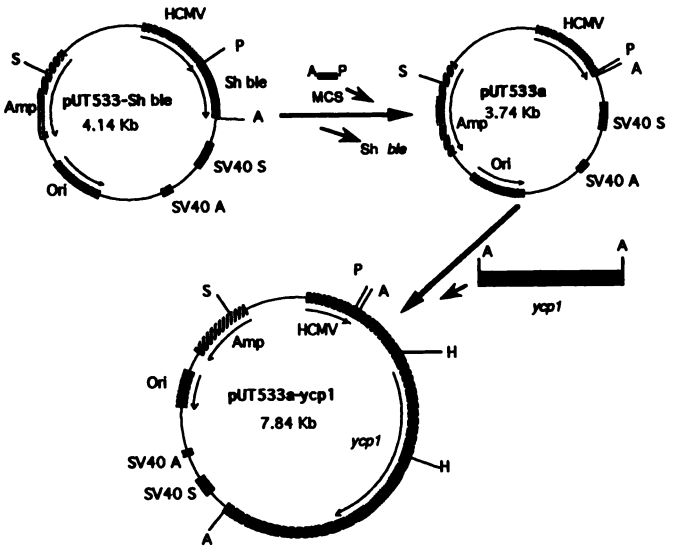

Fig. 1. Cloning of the *ycp1* gene into a mammalian expression vector. A 4.1-kb *ycp1* gene fragment was inserted into the mammalian expression vector pUT533a, as described in Experimental Procedures. *SV40 S*, SV40 mRNA splice site; *SV40 A*, SV40 polyadenylation signal sequence; *MCS*, multicloning sequence; *A*, *Acc*I; *H*, *Hind*III; *P*, *Pst*I; *S*, Sca I.

Southern blot analysis. The high-molecular-weight genomic DNAs were isolated according to the method described by Ausubel *et al.* (29). For Southern blot analysis, 40 μg genomic DNAs were first digested with the restriction endonuclease *Hind*III for 7 hr and run in 0.8% agarose gel overnight. The DNA fragments were then transferred from the gel to nitrocellulose membranes. The DNA probe used for detection was from a *Hind*III digestion of pUT533a-*ycp1* that released a 1.1-kb *ycp1* core fragment. This 1.1-kb fragment was labeled with [^{32}P]dCTP (NEN) with the use of a nick-translation kit (BRL). The hybridization was performed at 42° in 25 mM KPO_4, pH 7.4, 5× SSC, 5× Denhardt's solution (0.1% each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin), 50 μg/ml salmon sperm DNA, 50% formamide, and 10% dextran sulfate (29), with 500,000 cpm/ml of the *ycp1* probe. The final wash was at 42° in 0.25× SSC and 0.1% sodium dodecyl sulfate solution. Autoradiography was then performed by exposing the membrane to X-ray film at −70°.

MTT assay. The cell resistance to BLM was determined with the previously described MTT microculture assay (16). Cells were seeded at a density of 2000 cells/well in a 100 μl volume into 96-well microliter plates with the use of a multichannel pipet. After 3 hr, BLM (in 100 μl medium) was added in serial dilutions to the wells. After 4 days of growth in the presence or absence of BLM, the medium was replaced with 100 μl of MTT (1 mg/ml) in DMEM. The tetrazolium/formazan reaction was allowed to proceeded for 3 hr at 37°, after which the solution containing the unreacted MTT was removed and replaced with 100 μl dimethylsulfoxide. After 5 min of shaking to solubilize all dye, the absorbance at a wavelength of 540 nm was determined spectrophotometrically (Titertek Multiskan, Flow Laboratories, McLean, VA).

BLM hydrolase activity assay. The metabolism of BLM was determined with the HPLC method that we described previously, which separates BLM A_2 from its inactive metabolite deamido-BLM A_2 (dA_2) (18). Cells were homogenized in lysis buffer (20 mM Tris, pH 7.5, 200 μM $CaCl_2$, and 2 mM dithiothreitol) and centrifuged at 20,000 × *g* for 30 min (4°), followed by a 100,000 × *g* ultracentrifugation of the supernatants at 4° for 1 hr. The 100,000 × *g* supernatants were incubated with 60 μM BLM A_2 (Bristol-Myers Squibb Laboratories) in a 50-μl reaction buffer (20 mM Tris, pH 7.5) at 37° for 15 hr. The reactions were stopped by the addition of 40 μl methanol and 10 μl 7.5 mM $CuSO_4$ and injected into a C8 reverse-phase HPLC column (25 cm × 4.6 mm; 5 μm particle size; Rainin Dynamax). The BLM A_2 metabolites were eluted at 1 ml/min with 17% methanol, 7.2% acetonitrile, 0.8% acetic acid, 2 mM heptanesulfonic acid, and 25 mM triethylamine, pH 5.5. BLM A_2 and BLM dA_2 were detected by absorbance at 292 nm (18).

Results and Discussion

Soon after the discovery of the antitumor antibiotic BLM by Umezawa *et al.* in the mid-1960s, a BLM-inactivating activity was identified by the same group (7–12). It was then suggested that this enzyme, termed BLM hydrolase, might be responsible for the resistance of some human tumors to BLM. Indirect evidence (mainly correlative) for and against this suggestion has been reported throughout the past three decades, but direct evidence demonstrating that BLM hydrolase can induce resistance to BLM is lacking due to difficul-

ties in obtaining full-length BLM hydrolase gene or cDNA (21). Recently, a yeast cysteine proteinase gene *ycp1* was cloned (23) and shown to encode a protein with 43% identical homology and 78% similarity to our previously reported partial-length BLM hydrolase from rabbit liver (21). Furthermore, the identical homology increases to >90% around the BLM hydrolase active site, and the enzyme recognized and hydrolyzed cysteine proteinase substrates. More recently, three other groups independently cloned the same yeast gene (30–32). The third group (32) found the yeast BLM hydrolase to bind DNA and, more recently, published its crystal structure (33). In the present study, we used the *ycp1* gene to demonstrate that BLM hydrolase can induce BLM resistance.

First, we determined whether Ycp1 is capable of metabolizing BLM *in vitro*. Homogeneously purified Ycp1 (10 μg) (23) was incubated with BLM A_2 overnight, and the reaction mixture was analyzed with HPLC as described in Experimental Procedures. Fig. 2 shows the HPLC profiles of reaction mixtures that contained BLM A_2 alone (Fig. 2A), BLM A_2 and Ycp1 (Fig. 2B), or BLM A_2, Ycp1, and the cysteine proteinase inhibitor E-64 (Fig. 2C). Fig. 2 shows that Ycp1 was able to convert BLM A_2 to its inactive metabolite dA_2 and that this metabolic inactivation was completely inhibited by E-64, a known BLM hydrolase inhibitor (13, 21). Thus, the yeast cysteine proteinase Ycp1 is capable of metabolizing BLM.

We then investigated whether *ycp1* gene can induce BLM resistance in NIH 3T3 cells. The *ycp1* gene (23) was subcloned into a mammalian expression vector under the control of a human cytomegalovirus enhancer and promotor (Fig. 1) as described in Experimental Procedures. The *ycp1*-containing plasmid (pUT533a-*ycp1*) and a neomycin resistance gene-containing plasmid (pSV2-neo) were then cotransfected into NIH 3T3 cells by a liposome-mediated electroporation method, as described in Experimental Procedures. We also transfected NIH 3T3 cells with an empty vector plasmid pUT533a (Fig. 1) as a negative control. As a positive control, we used pUT533-Sh *ble*, which contains the bacterial BLM resistance gene Sh *ble* that encodes a protein that does not metabolize BLM (25, 26). After selection in G418 medium, colonies were obtained at a frequency of 300–350/3 × 10^6 transfected cells. A number of stable G418-resistant cell clones were isolated and expanded into individual cell lines.

To determine whether the exogenous *ycp1* DNA has been integrated into the transfected cells, we digested the high-molecular-weight chromosomal DNA from the transfected cell lines with *Hind*III, separated the digests by electrophoresis, and performed Southern blot analysis with a ^{32}P-labeled *ycp1* fragment as a probe, as described in Experimental Procedures. As expected, complete *Hind*III digestion of pUT533a-*ycp1* plasmid (Fig. 1) contained the 1.1-kb *ycp1* fragment (Fig. 3, *lane 1*). Fig. 3 also shows that DNAs from NIH 3T3 cells (*lane 3*), cells transfected with pUT533a (*lane 4*), and cells transfected with pUT533-Sh *ble* (*lane 2*) did not contain the *ycp1* gene fragment. In contrast, DNAs from two cell lines (K and G) isolated from clones that were transfected with pUT533a-*ycp1* did contain the *ycp1* fragment (*lanes 5 and 6*, respectively). Fig. 3 also shows that clone K incorporated higher copy numbers of the *ycp1* gene and therefore was used for further investigation.

To investigate whether *ycp1* induces BLM resistance, we

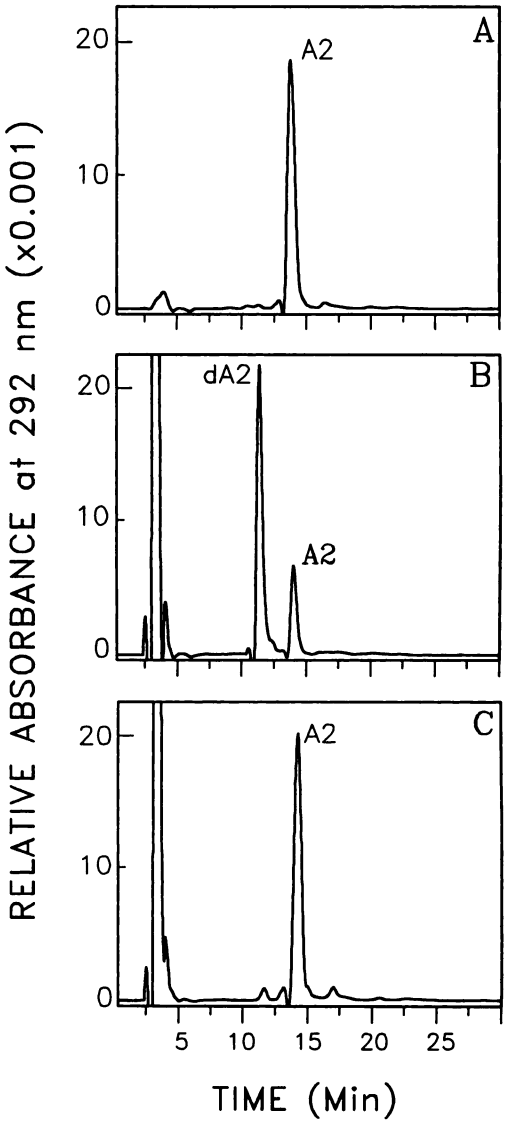

Fig. 2. Yeast cysteine proteinase has BLM hydrolase activity. Homogeneously purified yeast cysteine proteinase (Ycp1) (10 μg) was incubated with BLM A_2 for 15 hr, and the reaction mixture was then analyzed with HPLC, as described in Experimental Procedures. A, BLM A_2 only. B, BLM A_2 and Ycp1. C, BLM A_2, Ycp1, and E-64.

exposed the various transfectants to a range of BLM concentrations and determined their survival after 4 days in an MTT proliferation assay, as described in Experimental Procedures. Fig. 4 shows that the parent cell line, NIH 3T3 cells, were sensitive to BLM and that at 70 nM, BLM inhibited their growth by 50% (IC_{50} = 70 ± 9 nM) (Table 1). Similarly, NIH 3T3 cells transfected with the empty vector pUT533a were also sensitive to BLM (IC_{50} = 100 ± 27 nM). In contrast, NIH 3T3 cells transfected with pUT533a-*ycp1*(sense) were highly resistant to BLM (IC_{50} = 549 ± 49 nM). We also isolated three other pUT533a-*ycp1*(sense)-transfected clones (G, W, and M) that also showed an increased resistance to BLM (IC_{50} = 460, 504, and 511 nM, respectively). Data represent the average of two independent experiments. Transfection of NIH 3T3 cells with pUT533-Sh *ble* also induced high resistance levels to BLM (IC_{50} = 828 ± 153 nM) (Table 1). This is consistent with previous reports that showed that the bacterial BLM resistance gene Sh *ble* induces BLM re-

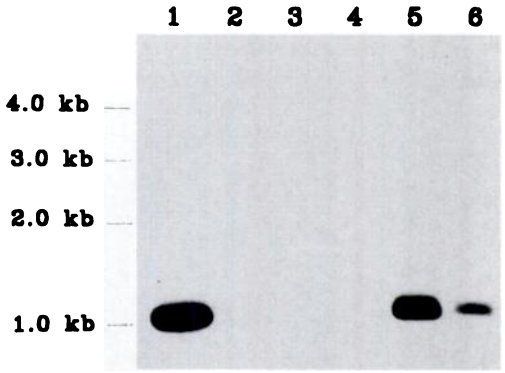

Fig. 3. Southern blot analysis. The genomic DNAs from various transfectants were completely digested by *Hind*III, separated on an agarose gel, and hybridized with a *ycp1* probe, as described in Experimental Procedures. *Lane 1*, pUT533a-*ycp1* plasmid. *Lane 2*, DNA from pUT533-Sh *ble*-transfected cells. *Lane 3*, DNA from the parent NIH 3T3 cells. *Lane 4*, DNA from pUT533a-transfected cells. *Lanes 5* and *6*, DNA from pUT533a-*ycp1*-transfected cells (*lane 5*: clone K; *lane 6*: clone G). The data are representative of three independent experiments.

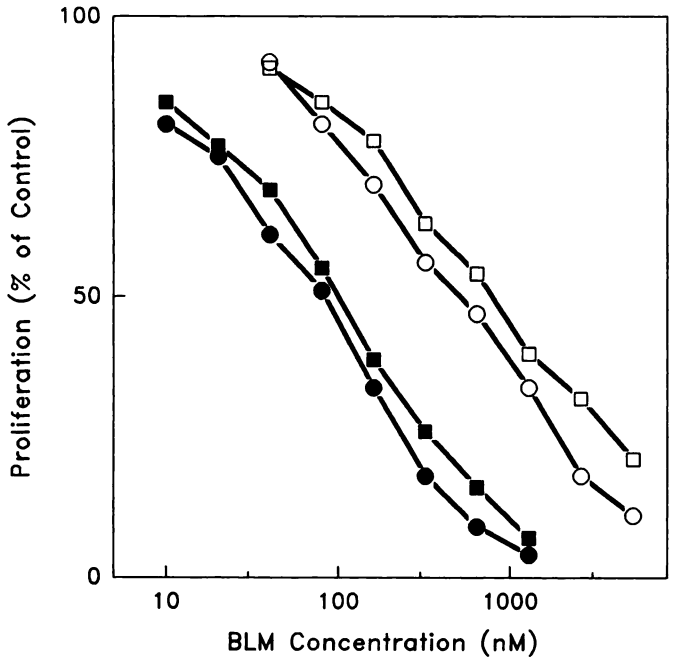

Fig. 4. *ycp1*-transfected cells are more resistant to BLM. Cell resistance to BLM was determined by MTT assay, as described in Experimental Procedures. ●, Parent NIH 3T3 cells. ■, Empty vector (pUT533a)-transfected cells. ○, pUT533a-*ycp1*-transfected cells. □, pUT533-Sh *ble*-transfected cells. The data for each cell line are representative of at least three independent experiments.

sistance in mammalian cells (26, 34, 35). The pUT533-Sh *ble* cell line serves as a positive control of BLM resistance that is independent of BLM metabolism (see below). Transfection of NIH 3T3 cells with a plasmid where the *ycp1* gene was inserted in the 3′-5′ antisense orientation did not induce BLM resistance (IC_{50} = 100 ± 12 nM) (Table 1). Furthermore, we isolated two other antisense clones, and neither one showed increased resistance to BLM (data not shown). Thus, the results clearly demonstrate that *ycp1* is able to induce BLM resistance in NIH 3T3 cells and that 7-fold higher concentrations of BLM were needed to inhibit the growth of the transfectants compared with the parent cell line.

If the *ycp1*-induced BLM resistance is due to an increased

TABLE 1

BLM resistance levels and BLM hydrolase activity

Cell lines	IC_{50}[a]	BLM hydrolase activity
	nM	% BLM dA_2 formed
NIH 3T3	70 ± 9	16 ± 10
Vector	100 ± 27	19 ± 9
ycp1 (sense)	549 ± 49	54 ± 6
ycp1 (antisense)	100 ± 12	17 ± 10
Sh *ble*	828 ± 153	20 ± 12

Data are the average of three independent experiments with standard deviations.

[a] BLM concentrations that inhibit cell growth by 50%.

ability of the transfectants to inactivate BLM, pUT533a-*ycp1*-transfected cells should have higher levels of BLM hydrolase activity. The ability of the various cell lines to metabolize BLM was determined by HPLC, as described in Experimental Procedures. Fig. 5A shows that NIH 3T3 cell lysate was able to metabolize BLM A_2 to its inactive metabolite BLM dA_2 but only to a small extent. BLM dA_2 represented only 16 ± 10% of total BLM (Table 1). Similarly, NIH 3T3 cells transfected with pUT533a (Fig. 5B) also metabolized only 19 ± 9% BLM. In contrast, *ycp1*(sense)-transfected cells (Fig. 5C) metabolized 54 ± 6% BLM (Table 1). However, Sh *ble*-transfected cells metabolized only 20 ± 12% BLM (Fig. 5D), a value similar to the parent NIH 3T3 cells (Table 1). Also, cells transfected with *ycp1* antisense plasmid did not show any increase in BLM metabolism (Fig. 5E). Thus, the *ycp1* gene enhanced the ability of NIH 3T3 cells to inactivate

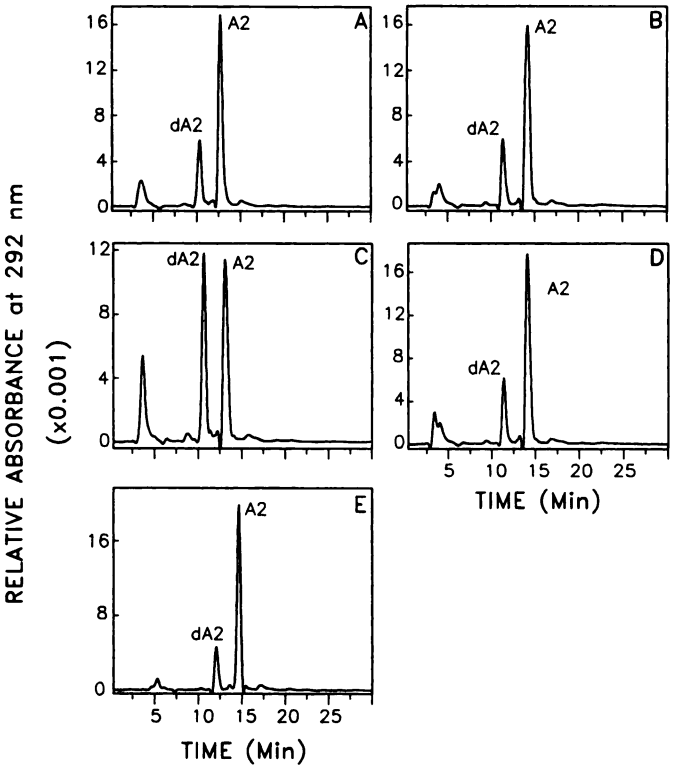

Fig. 5. *ycp1*-transfected cells have increased BLM hydrolase activity. Lysates from various transfectants were isolated, and BLM hydrolase activity was determined with HPLC analysis, as described in Experimental Procedures. A, Parent NIH 3T3 cells. B, Empty vector pUT533a-transfected cells. C, pUT533a-*ycp1*(sense)-transfected cells. D, pUT533-Sh *ble*-transfected cells. E, pUT533-*ycp1*(antisense)-transfected cells. The data for each cell line are representative of three independent experiments.

BLM. The results of Fig. 4 coupled with those of Fig. 5 and Table 1 show a very strong correlation between the ability of the *ycp1*-transfected cells to inactivate BLM and their resistance to BLM. Sh *ble*-transfected cells showed high levels of BLM resistance even though their ability to metabolize BLM was low. This is not surprising because Sh *ble* gene encodes a 14-kDa protein that is known not to metabolize BLM but rather to cause BLM resistance by physically binding BLM and preventing it from degrading DNA (25, 26).

To confirm that the *ycp1*-induced BLM resistance was due to increased metabolism in these cells, we carried out inhibition studies with the cysteine proteinase inhibitor E-64, a known inhibitor of BLM hydrolase (21). We first demonstrated that the increased BLM hydrolase activity in the *ycp1*-transfected cells can be inhibited by E-64. Lysates (0–80 μg) from these cells were incubated with BLM A_2 in the presence or the absence of E-64. As shown in Fig. 6, the amount of BLM A_2 converted to BLM dA_2 was proportional to the amount of lysates used and 80 μg of cell lysates was able to convert 70% of BLM A_2 to BLM dA_2. In contrast, when E-64 was present in the reaction mixture with BLM A_2 and cell lysates, no BLM A_2 was metabolized to BLM dA_2 (Fig. 6). These results clearly demonstrate that the *ycp1*-induced increase in BLM metabolism can be completely blocked by E-64. If metabolism is responsible for the increased BLM resistance, inhibition of this metabolism by E-64 should reverse BLM resistance. Fig. 7 shows that *ycp1*-transfected cells, which had an IC_{50} value of 549 ± 49 nM in the absence of E-64, had an IC_{50} value of 76 ± 22 nM when treated with E-64. This IC_{50} value is very similar to the IC_{50} value of the parent NIH 3T3 cells (70 nM) and the empty vector pUT533a-transfected cells (100 nM). Thus, E-64 is able to sensitize cells that have high resistance to BLM. In contrast, cells transfected with Sh *ble* were not sensitized to BLM by E-64 (Fig. 7) confirming that Sh *ble*-induced BLM resistance is not due to an increased ability of these cells to metabolize BLM by BLM hydrolase. Furthermore, the fact that E-64 did not sensitize Sh *ble*-transfected cells argues strongly that the effect of E-64 on *ycp1*-transfected cells is a specific effect due to inhibition of BLM hydrolase activity.

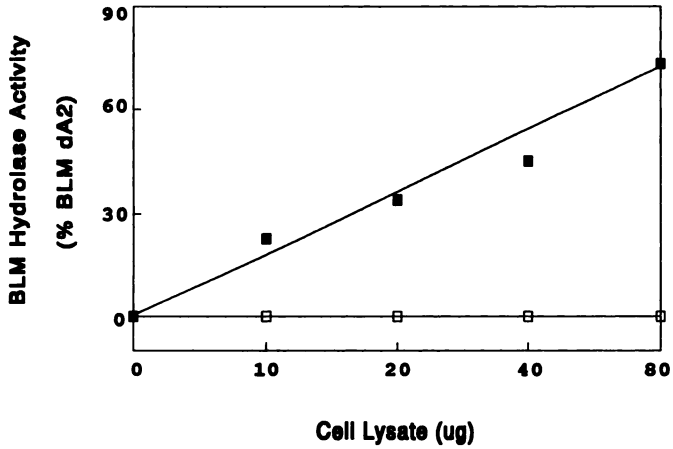

Fig. 6. E-64 blocks BLM hydrolase activity from pUT533a-*ycp1*-transfected cells. Lysates from the pUT533a-*ycp1*-transfected cells were incubated with BLM A_2 in the presence (□) or the absence (■) of E-64. The amount of BLM dA_2 formed was then determined with HPLC, as described in Experimental Procedures. The data are representative of 2 independent experiments.

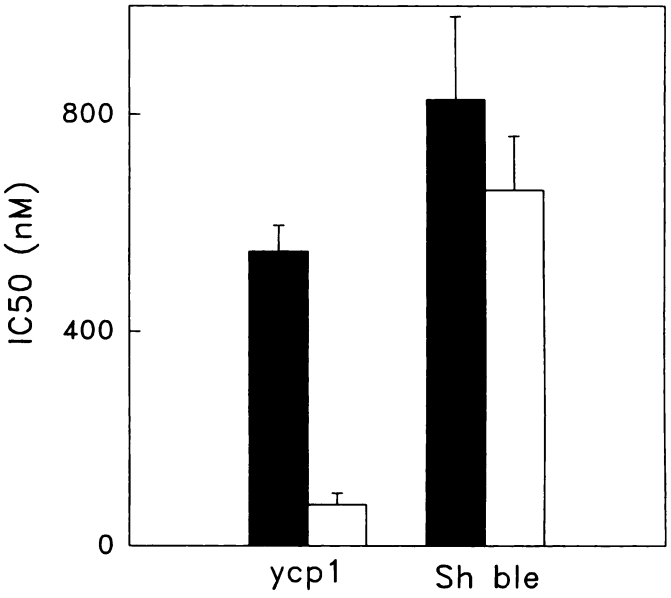

Fig. 7. Reversal of *ycp1*-transfected cell resistance to BLM by E-64. The ability of BLM to inhibit the cell growth of *ycp1*- or Sh *ble*- transfected cells was determined by MTT assay in the presence (□) or absence (■) of E-64, as described in Experimental Procedures. The data for each cell line are the average of three independent experiments with standard deviations.

The results of the present study provide the first direct evidence demonstrating that increasing cellular BLM hydrolase activity results in increased BLM resistance. The data give support for the involvement of metabolic inactivation in BLM resistance and argue strongly for the use of BLM hydrolase inhibitors as modulators that would increase the spectrum of tumors that can be treated with BLM.

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