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Overproduction of a 37.5-kDa Cytosolic Protein Structurally Related to Prostaglandin F Synthase in Ethacrynic Acid-Resistant Human Colon Cells

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SUMMARY

We report the initial identification of a 37.5-kDa putative aldo-keto reductase in human colon carcinoma cells. An amino-terminal trypsin fragment was sequenced and found to be identical to bovine prostaglandin F synthase in 19 of 21 amino acids. Levels of this cytosolic human aldo-keto reductase, assessed by immunoblots using polyclonal antibodies raised against this protein, increased 30-fold in cells resistant to the Michael reaction acceptor ethacrynic acid and increased with time and ethacrynic acid concentration after treatment of wild-type cells. Induction of the reductase appeared to be cell type and drug specific. It was induced by the Michael reaction acceptors dimethyl maleate, *t*-butylhydroquinone, and hydroquinone but not by the nitrogen mustard chlorambucil. Ethacrynic acid and dimethyl maleate induced the reductase in a second human colon cell line but not in human prostate cells. NADPH-dependent metabolism of aldoketo reductase substrates by cytosol from colon but not prostate cells was enhanced 2–3-fold when cells were grown in the presence of either ethacrynic acid or dimethyl maleate. The discrepancy between induced reductase activity and protein levels may be due to the multiplicity of constitutively expressed NADPH-dependent reductases that compete for substrate. Ethacrynic acid-resistant cells exhibited low levels of cross-resistance to Adriamycin, mitomycin C, and the bovine prostaglandin F synthase substrates phenylglyoxal and prostaglandin D_2. Thus, significant overexpression of a human aldo-keto reductase structurally related to bovine prostaglandin F synthase may result from exposure of cells to Michael reaction acceptors and may give rise to an enhanced capacity to metabolize exogenous and endogenous substrates, thereby contributing to the drug-resistant phenotype.

It has recently become popular to approach the problem of clinical drug resistance by modulating the cellular targets presumed responsible for resistance. The increased expression of drug-metabolizing enzyme systems such as GST (EC 2.5.1.18) confers resistance by increasing the potential to detoxify drug substrates. To circumvent the clinical problem of nitrogen mustard resistance, the transition-state GST inhibitor EA may be useful where GST class α overexpression occurs in mustard-resistant tumors.

EA inhibits GST activity in human lymphocytes *in vivo* (1), sensitizes alkylator-resistant cell lines from both rats and humans to the nitrogen mustards chlorambucil and Melphalan (2, 3), and sensitizes human tumor xenografts to Melphalan (4). EA inhibits the GST class α-catalyzed conjugation of chlorambucil to glutathione in a concentration-dependent manner (5) at concentrations that approximate clinically achievable serum values (1).

Cells can be made resistant to EA. EA-resistant human HT29 colon carcinoma cells exhibit approximately 2-fold resistance to EA and Melphalan and increased levels of reduced glutathione and GST class π but not GST class α (6). GST-π activity and protein are induced 2-fold, resulting from a 2–3-fold increase in GST-π transcript. EA is a substrate for GST-π isolated from EA-resistant HT29 cells.

EA can be classified structurally as a Michael reaction acceptor. The α,β-unsaturated carbonyl moiety is characteristically contained in certain GST inducers and substrates and NAD(P)H:quinone oxidoreductase (EC 1.6.99.2) inducers and is represented in compounds such as quinones, maleates, cinnamates, fumarates, and α,β-unsaturated lactones (7–10).

EA was originally used as a diuretic. It produces venodilation in the kidney that is due in part to increased prostaglandin release (11). Prostaglandins are important regulators of cell growth and function, the profiles of which are frequently altered

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ABBREVIATIONS: GST, glutathione *S*-transferase; EA, ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl)phenoxy)acetic acid]; B-PGFS, bovine prostaglandin F synthase; PGD_2, prostaglandin D_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGH_2, prostaglandin H_2; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PGFS, prostaglandin F synthase; ARE, antioxidant-responsive element.

in neoplastic tissue (12). Here we report on the overproduction in EA-resistant HT29 colon cells of a putative human aldo-keto reductase (H-37) that is structurally related to NADPH-dependent lung B-PGFS (EC 1.1.1.188) (13–16). We sought to determine whether H-37 might be induced by other Michael reaction acceptors and whether cytosol from resistant cells and drug-treated wild-type cells could metabolize B-PGFS substrates. Metabolism of generic aldoketo reductase substrates by cytosol from resistant cells and colon cells treated with Michael reaction acceptors increased in parallel (but nonstoichiometrically) with induction of H-37 by these agents. Overexpression of cytosolic H-37 enzyme was consistent with an active role in conferring cellular resistance (via detoxification) to EA and aldoketo reductase substrates.

Experimental Procedures

Materials. Freund's adjuvant and all drugs and substrates were purchased from Sigma. Polyvinylidene difluoride membranes were obtained from Millipore. ^{125}I-Protein A was purchased from Amersham. Cell culture media and reagents were obtained from GIBCO BRL.

Cell culture and cytosol isolation. HT29 and LS174T human colon carcinoma cells were maintained in 25 ml of minimum essential medium with 10% fetal calf serum and 4 mM L-glutamine, in 150- × 20-mm dishes, as described (6). These cells were plated at a density of 1.5×10^6 cells/dish (5–10 dishes/condition) on day 0. Cells were exposed to drug at various time periods before cytosol isolation on day 4. EA-resistant cells were maintained continuously in 72 μM EA. Revertant HT29 cells were obtained by removing drug from the medium for one or two cycles of passaging. After washing with phosphate-buffered saline, cells were homogenized in buffer containing 10 mM potassium phosphate, pH 7.4, 150 mM KCl, 0.5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Cytosol was obtained from cell homogenates by centrifugation at 10,000 × *g* for 20 min, followed by centrifugation of the latter supernatant for 1 hr at 100,000 × *g*. For determination of protein half-life, cells were treated with 200 μg/ml cycloheximide at time zero before isolation at different intervals.

Enzyme assay. Enzyme activity toward various aldoketo reductase substrates in HT29 cell cytosol was measured by following the disappearance of NADPH (90 μM) at 340 nm (extinction coefficient, 6500 M^{-1} cm^{-1}). The reaction buffer contained 100 μM potassium phosphate, pH 6.5 (activity was higher at pH 6.5 than at pH 7.0 or pH 7.4). Dicumarol (10 μM; 0.1% dimethylsulfoxide vehicle) was used in some samples to inhibit NAD(P)H:quinone oxidoreductase. Except for PGD_2, substrate concentrations were saturating and NADPH disappearance was linear with time and protein concentration. To monitor PGD_2 metabolism, 0.65 mM substrate and 0.5 mg of cytosol were used. Samples containing boiled cytosol were used as blanks.

Cytotoxicity assay (colony formation). Logarithmic phase cells were plated at a density of 300 cells/well in plastic culture dishes and were treated with drug 24 hr later. Cells were exposed continuously to drug until fixed with ethanol (95%) and stained with crystal violet (0.1%) for colony counting. Results are expressed as percentage of control survival. Each data point is reported as the mean ± standard error (17).

Protein separation (SDS-PAGE), antibody production, and immunoblotting. SDS-PAGE, electrophoretic transfer, and immunoblotting were accomplished by standard procedures (18, 19). SDS (0.025%) was included in the transfer buffer. H-37 protein was purified from 2.0 mg of cytosol after SDS-PAGE separation and transfer to polyvinylidene difluoride membranes. H-37 was excised directly from membranes after identification by Ponceau stain (0.1%) and comparison with wild-type samples. Peptide sequence analysis was performed by Edman degradation after elution of the protein from membrane strips, trypsin digestion of the protein, and high performance liquid chromatographic separation of the quantitatively most important protein fragment. For antibody production, membrane strips containing protein from four gels were dissolved in a small volume of dimethylsulfoxide and mixed with Freund's complete or incomplete adjuvant, for the first and subsequent challenges, respectively. Adult female New Zealand white rabbits were challenged four times, on weeks 1, 6, 9, and 13. Sera were obtained from anesthetized rabbits by cardiac puncture on week 14. IgG was purified from sera on a Protein A-Sepharose column, as described by Duignan *et al.* (20), and was used in immunoblot incubations as primary antibody (at 10 μg/ml). Immunodetection was achieved by specific binding of ^{125}I-Protein A to IgG, followed by autoradiography for 24–72 hr. Measurement of H-37 induction was determined by absorbance densitometry using a Pharmacia LKB Ultrascan XL densitometer.

Results

Overproduction of a 37.5-kDa cytosolic protein, determination of peptide sequence, and antibody specificity. The increased production by EA-resistant HT29 cells of a 37.5-kDa cytosolic protein is illustrated by a silver-stained 10% polyacrylamide gel in Fig. 1 (*lane 2*, resistant; *lane 3*, wild-type). This increase was mimicked in a concentration-dependent manner by exposing wild-type HT29 cells to sublethal concentrations of EA for 48 hr (Fig. 1, *lanes 4-7*). Treatment of wild-type cells with chlorambucil for 48 hr did not cause an increase in this protein (Fig. 1, *lanes 8* and *9*). Resolution of this protein from EA-resistant cell cytosol by 10% SDS-PAGE permitted microsequencing. The protein was found to be amino-terminally blocked, thus necessitating microsequencing of high performance liquid chromatography-separated trypsin fragments. As shown in Fig. 2, the amino terminus starting at amino acid 11 was identical in 19 of 21 amino acids to lung B-PGFS, a monomeric protein of 36,666 Da (13, 14). We have called this putative human aldo-keto reductase H-37.

Additional protein from cytosol was resolved by SDS-PAGE, to raise antibodies in adult female rabbits. Polyclonal antibod-

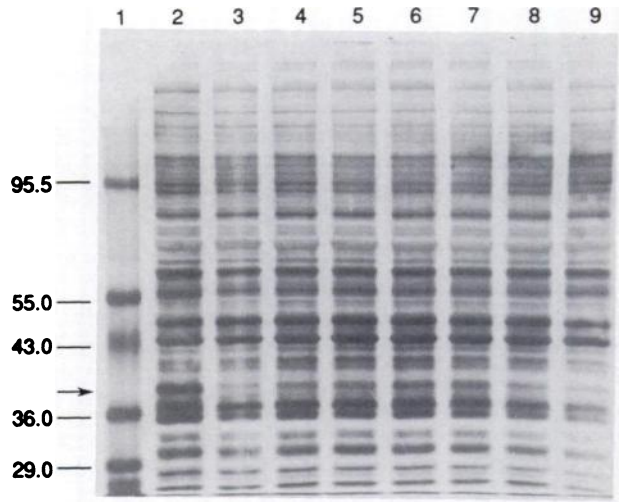

Fig. 1. Silver stain detection of cytosolic proteins from human colon carcinoma HT29 cells treated with EA or chlorambucil for 48 hr (separation by 10% SDS-PAGE; 100 μg of protein/lane). *Lane 1*, molecular mass markers; *lane 2*, resistant cells; *lane 3*, wild-type control cells; *lane 4*, wild-type cells treated with 10 μM EA; *lane 5*, wild-type cells treated with 20 μM EA; *lane 6*, wild-type cells treated with 40 μM EA; *lane 7*, wild-type cells treated with 50 μM EA; *lane 8*, wild-type cells treated with 10 μM chlorambucil; *lane 9*, wild-type cells treated with 30 μM chlorambucil. *Arrow*, 37.5-kDa protein.

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          11        20        30
B-PFS NDGHFIPVLGFGTYAPEEVPK
H-37  NDGHFMPVLGFGTYAPAEVPK
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Fig. 2. Peptide fragment sequence of H-37 and comparison with B-PGFS (13, 14).

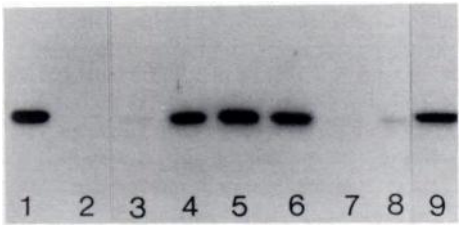

Fig. 3. Western blot of cytosol from HT29 human colon carcinoma cells using anti-H-37 IgG (100 μg of protein/lane). *Lane 1*, resistant cells; *lane 2*, wild-type control cells; *lane 3*, revertant cells; *lanes 4-9*, wild-type cells treated with drug 48 hr before cytosol isolation; *lane 4*, 20 μM EA; *lane 5*, 50 μM EA; *lane 6*, 80 μM dimethyl maleate; *lane 7*, 10 μM chlorambucil; *lane 8*, 80 μM hydroquinone; *lane 9*, 80 μM *t*-butylhydroquinone.

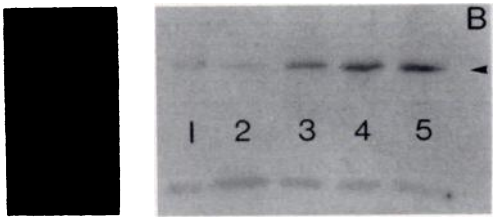

Fig. 4. Western blot of cytosol from HT29 cells, illustrating time dependence of the increase in glyoxalase I and H-37 levels with EA treatment. *Arrowhead*, H-37. A, *Lane 1*, 100 μg of EA-resistant cytosol; *lane 2*, 250 μg of wild-type cytosol. B, Wild-type HT29 cells treated with 40 μM EA for various times before cytosol isolation (100 μg of protein/lane). *Lane 1*, 0 hr; *lane 2*, 6 hr; *lane 3*, 24 hr; *lane 4*, 48 hr; *lane 5*, 72 hr. The blot was probed sequentially with anti-H-37 IgG and anti-human glyoxalase I IgG.

ies raised against H-37 were then tested for their specificity. As shown in Fig. 3, antibodies recognized a single band at 37.5 kDa, corresponding to H-37. As determined by densitometry of autoradiographs, compared with wild-type levels (Fig. 3, *lane 2*) production of H-37 was induced 30-fold in EA-resistant cells (Fig. 3, *lane 1*). Induction was reversible upon removal of the selection pressure (EA) for a single cycle of cell passaging (Fig. 3, *lane 3*). No other immuno-cross-reactivity was detected in cytosolic preparations by this method. In addition, H-37 was not recognized in microsomes or enriched membrane preparations from either wild-type or EA-resistant cells (data not shown). Induction of H-37 in resistant cells could be mimicked in wild-type cells by treating them with EA. Compared with wild-type levels at 0 hr (Fig. 4B, *lane 1*; H-37 is upper band), 40 μM EA caused a 2.4-fold increase in H-37 within 6 hr of exposure (Fig. 4B, *lane 2*) and further induced it to high levels at 72 hr of exposure (20-fold) (Fig. 4B, *lane 5*). Cotreatment of wild-type cells with 200 μg/ml cycloheximide and 40 μM EA for 24 hr prevented the increase in H-37 (data not shown). As also shown in Fig. 4A, levels of the enzyme glyoxalase I were approximately 2–3-fold higher in wild-type cells, compared with EA-resistant cells (glyoxalase I is the lower band). EA treatment of wild-type HT29 cells produced no significant changes in glyoxalase I levels with time (Fig. 4B).[1]

[1] S. Ranganathan and K. D. Tew. Analysis of glyoxalase I from normal and tumor tissue from human colon. Submitted for publication.

Induction specificity by Michael reaction acceptors in human colon cells. EA induced H-37 in wild-type HT29 cells in a concentration-dependent manner (Fig. 3, *lanes 4* and *5*). In contrast, concentrations of chlorambucil that produce toxicity approximately equivalent to that produced by the EA concentrations used did not cause an increase in H-37 (Fig. 3, *lane 7*). As with GST-π overexpression in EA-resistant cells (6), it was thought that the observed patterns of H-37 induction might be due to the Michael addition chemistry of EA that is characteristic of compounds containing α,β-unsaturated carbonyl moieties. Treatment with 80 μM concentrations of the Michael reaction acceptors dimethyl maleate, *t*-butylhydroquinone, and hydroquinone resulted in marked induction of H-37. Dimethyl maleate induced H-37 to levels comparable to those of the EA-resistant phenotype (27-fold) (Fig. 3, *lane 6*). Hydroquinone caused an increase in H-37 to intermediate levels (7-fold) (Fig. 3, *lane 8*). *t*-Butylhydroquinone (80 μM) induced H-37 approximately 20-fold (Fig. 3, *lane 9*).

Because basal levels of H-37 in wild-type cells were low and inducer specificity in causing H-37 overexpression was observed in colon HT29 cells, we compared these results with data from other human cell lines to determine potential tissue response specificity (Fig. 5). H-37 was not detected in untreated LS174T human colon carcinoma cells but was induced by both EA and dimethyl maleate (Fig. 5, *lanes 2-4*) to levels comparable to those of the EA-resistant HT29 phenotype (Fig. 5, *lane 1*). A lower molecular mass protein (approximately 36,000 Da) that was not induced by EA treatment was recognized in LS174T cells. Anti-H-37 IgG did not recognize H-37 in untreated or EA-treated human prostate DU145 cells (Fig. 5, *lanes 5* and *6*). Anti-H-37 antibody did not cross-react with any proteins from either untreated wild-type, EA-treated wild-type, or chlorambucil-resistant rat Walker breast carcinoma cells, at 3 times the titer used in immunoblots for human cytosol (data not shown).

Fig. 6 shows the effect of cycloheximide treatment on H-37

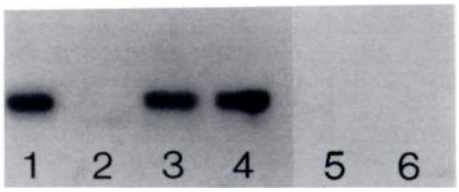

Fig. 5. Western blot of cytosol from DU145 and LS174T cells (100 μg/lane). *Lane 1*, EA-resistant HT29 cells; *lane 2*, untreated wild-type LS174T cells; *lane 3*, wild-type LS174T cells treated with 50 μM EA for 48 hr; *lane 4*, wild-type LS174T cells treated with 80 μM dimethyl maleate for 48 hr; *lane 5*, untreated wild-type DU145 cells; *lane 6*, wild-type DU145 cells treated with 40 μM EA for 48 hr.

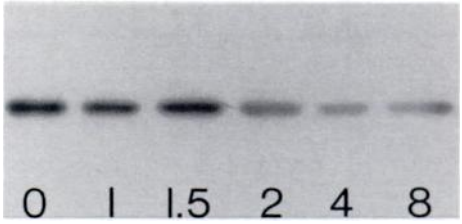

Fig. 6. Determination of H-37 protein half-life in EA-resistant HT29 cells by cycloheximide treatment. *Numbers*, hours after treatment of cells with 200 μg/ml cycloheximide. Cytosol from cycloheximide-treated cells was isolated and analyzed for H-37 levels by Western blotting, as described in Experimental Procedures.

protein levels in cytosol from resistant cells. The protein half-life determined by this method was approximately 2–4 hr. Cycloheximide treatment did not induce H-37 in either wild-type (data not shown) or EA-resistant HT29 cells.

Assessment of NADPH- and NADH-dependent cytosolic reductase activities. B-PGFS substrates include PGD_2, PGH_2, 9,10-phenanthrenequinone, phenylglyoxal, menadione, and other quinones and aldehydes (15). Therefore, we sought to determine whether cytosol from wild-type and drug-treated cells could metabolize some of these substrates. NADPH is the preferred cofactor of B-PGFS and so was used for this purpose. Total phenylglyoxal reductase activity is reported in Table 1. In every case except chlorambucil treatment, total NADPH-dependent activity in HT29 cytosol increased with drug treatment. The induction of this activity was prevented by cotreatment with 200 μg/ml cycloheximide for 24 hr (data not shown). Activity from EA-treated wild-type cells increased with both time and concentration. Activities in the cytosol from the EA-resistant cells were approximately 2 times those of wild-type cells and reverted to wild-type levels after removal of EA from the cell culture medium. Dimethyl maleate was the most potent inducer of this activity (40 μM, 2.2-fold; 80 μM, 3.3-fold). *t*-Butylhydroquinone (80 μM) also induced this activity 2-fold (data not shown). Similar results for phenylglyoxal reductase were obtained with LS174T colon carcinoma cells. Activity in DU145 cell cytosol was not changed by EA treatment.

Total HT29 cytosolic 9,10-phenanthrenequinone and PGD_2 reductase activities are reported in Tables 2 and 3, respectively. As in the case of phenylglyoxal reductase, 9,10-phenanthrenequinone and PGD_2 turnover was increased 2–3-fold in the resistant phenotype. 9,10-Phenanthrenequinone reductase activity also returned to wild-type levels in the revertant cells.

Because many cytosolic reductases are expressed in cells, it was thought that both constitutive and induced cytosolic activities reported in Tables 1 and 3 (NADPH-dependent activity) for HT29 cells might be due in part to NAD(P)H:quinone oxidoreductase or NADH reductases that can use NADPH. NAD(P)H:quinone oxidoreductase was therefore probed, and enzyme activities using NADH (90 μM) as cofactor were tested. NAD(P)H:quinone oxidoreductase was constitutively expressed but found to be decreased reproducibly by 30% in cytosol from EA-resistant HT29 cells.[2] To probe the role of NAD(P)H:quinone oxidoreductase in phenylglyoxal and 9,10-phenanthrenequinone reductase activities, 10 μM dicumarol was used as an inhibitor. In every case, dicumarol inhibited approximately 35–45% of total phenylglyoxal reductase activity (Table 4). 9,10-Phenanthrenequinone reductase was reduced by up to 60% in both uninduced and induced samples, suggesting that much of this activity was indeed due to this enzyme (Table 2). Table 2 also shows levels of phenylglyoxal and 9,10-

[2] A. Jaiswal, Fox Chase Cancer Center, personal communication,

TABLE 1

NADPH-dependent phenylglyoxal reductase activity (total) in cytosol isolated from HT29, DU145, and LS174T Cells

Expt.	Cell line	Treatment (before isolation)	Drug	Reductase activity[a]	*n*[b]	Fold increase
		hr	*μM*	*nmol of NADPH/min/mg of protein*		
I	HT29(WT)[c]		None	7.09 ± 0.66	9	
	HT29(R)	Continuous exposure	E Acid, 72	13.02 ± 0.92[d]	9	1.8
	HT29(RV)		None	7.17 ± 0.26	4	1.0
			EA			
	HT29(WT)	6	40	10.06	2	1.4
		24	40	11.89	2	1.7
		48	40	14.42	2	2.0
		72	40	17.09 ± 2.12[d]	4	2.4
		48	10	8.47 ± 1.35	3	1.2
		48	20	9.95 ± 1.75[d]	4	1.4
		48	40	14.20 ± 1.08[d]	4	2.0
		48	50	14.92 ± 2.22[d]	4	2.1
			Chlorambucil			
		48	10	6.87	2	1.0
		48	30	8.37	2	1.2
			Dimethyl maleate			
		48	40	15.95 ± 1.82[d]	4	2.2
			80	23.17 ± 2.57[d]	7	3.3
			Hydroquinone			
		48	40	14.47 ± 2.12[d]	6	2.0
		48	80	11.94 ± 0.73[d]	6	1.7
II	DU145(WT)	48	None	4.02	2	
			EA			
		48	20	4.00	2	1.0
		48	40	3.12	2	0.8
III	LS174T(WT)	48	None	9.29 ± 0.25	5	
			EA			
		48	20	9.74 ± 0.96	4	1.0
		48	40	13.73 ± 0.47[d]	4	1.5
		48	50	21.82	2	2.3
		48	Dimethyl maleate, 80	31.68 ± 0.84[d]	3	3.4

[a] Values are mean ± standard error.
[b] Each sample isolation (*n*) was analyzed in duplicate.
[c] WT, wild-type phenotype; R, resistant phenotype; RV, revertant phenotype.
[d] Statistically significant difference from wild-type control ($p < 0.05$). Analysis by *t* test for independent groups, unknown variance.

TABLE 2

9,10-Phenanthrenequinone and phenylglyoxal reductase activity in cytosol isolated from HT29 cells

	9,10-Phenanthrenequinone reductase		Phenylglyoxal reductase, NADH[c]
	NADPH[a]	NADH[b]	
	nmol/min/mg		nmol/min/mg
A. Total			
WT[d]	61.79 ± 15.52	32.07 ± 9.05	0.72
R	141.66 ± 10.98[e]	61.37 ± 9.90[e]	1.67
RV	67.35 ± 10.55	ND[f]	ND
B. Dicumoral insensitive			
WT	20.57 ± 4.06	2.40 ± 0.47	0.71
R	54.42 ± 5.97[e]	7.22 ± 1.31[e]	1.21
RV	25.85 ± 6.05	ND	ND

[a] n = 5, wild-type; 8, resistant; 4, revertant, representing duplicate analyses of each isolation (n).
[b] n = 3, representing duplicate analyses of each isolation (n).
[c] Results represent duplicate analyses of two isolations per condition (n = 2).
[d] WT, wild-type phenotype; R, resistant phenotype; RV, revertant phenotype.
[e] Statistically significant difference from wild-type control ($p < 0.05$). Analysis by t test for independent groups, unknown variance.
[f] ND, not determined.

TABLE 3

Drug-metabolizing enzymes in HT29 cells

	Activity[a]	
	WT	R
	nmol/min/mg	
Glyoxalase I[b]	246	198
GST[c]	90	160
NADPH-cytochrome P-450 reductase[d]	3.12	3.76
PGD_2 reductase[e]		
A. Total	0.25	0.55
B. Dicumoral insensitive	0.26	0.62

[a] Except for NADPH-cytochrome P450 reductase, which was measured in isolated microsomes, samples were from duplicate (glyoxalase I, NADPH-cytochrome P-450 reductase, and PGD_2 reductase) or quadruplicate (GST) HT29 cell cytosol isolates and were measured in duplicate. WT, wild-type phenotype; R, resistant phenotype.
[b] Glyoxalase I assay was performed as described by Oray and Norton (47).
[c] GST activity was assessed by using chlorodinitrobenzene as substrate (49).
[d] Reductase was assayed by using 40 nmol of cytochrome c, as described by Vermilion and Coon (48).
[e] NADPH was used as cofactor. Activity was assessed as described in Experimental Procedures.

phenanthrenequinone reductase activity obtained when NADH was used as cofactor. Compared with activities with NADPH as cofactor, activities were 6–7-fold lower for phenylglyoxal reductase and 2–3-fold lower for 9,10-phenanthrenequinone reductase. Aldehyde reductase, which can metabolize both phenylglyoxals and 9,10-phenanthrenequinone, is an inefficient catalyst of 9,10-phenanthrenequinone reduction with NADPH as cofactor and can metabolize phenylglyoxal 30 times faster than it can metabolize 9,10-phenanthrenequinone when NADPH is used (22). This is the opposite of what was observed with colon cell cytosolic reductase activity, where 9,10-phenanthrenequinone metabolism was markedly higher than that of phenylglyoxal, suggesting that aldehyde reductase is not expressed in these cells.

We also measured additional relevant enzyme activities. GST, glyoxalase I, and NADPH-cytochrome P-450 reductase (microsomes) activities are reported in Table 3. Except for GST and PGD_2 reductase activities, no differences were observed for samples from wild-type versus EA-resistant cells.

Response of HT29 cells to cytotoxic agents and B-PGFS substrates. The apparent enhanced capacity for metabolism of B-PGFS substrates provided the rationale for measuring cellular responses to a number of potential H-37 drug substrates. The EA-resistant HT29 cells, which do not exhibit the multidrug-resistant phenotype (6), were approximately 2-fold resistant to PGD_2 and 1.6-fold resistant to phenylglyoxal. These cells did not exhibit resistance to 9,10-phenanthrenequinone (data not shown) but were 1.8- and 2.2-fold resistant to Adriamycin and mitomycin C, respectively (Fig. 7). Upon removal of EA for a single cycle of passaging, cells reverted and exhibited resistance to Adriamycin at levels intermediate between those of wild-type and resistant cells. PGD_2, which is much more toxic than the B-PGFS products $PGF_{2\alpha}$ and its stereoisomer $9\alpha,11\beta$-PGF_2, is converted spontaneously to Δ^{12}-prostaglandin J_2, a potent inhibitor of cell growth (23). We therefore tested the effects of low level pretreatment with PGD_2 or $PGF_{2\alpha}$ on colony formation response to Adriamycin and mitomycin C. $PGF_{2\alpha}$ pretreatment (24 hr) at nontoxic concentrations (0.5 μM) rendered the EA-resistant cells more resistant to these drugs. PGD_2 pretreatment (nontoxic, 0.5 μM) (24 hr) rendered both the wild-type and EA-resistant cells more susceptible to these drugs.

Discussion

We report the marked induction in EA-resistant cell cytosol of a protein (H-37) that appears to be related to both drug and prostaglandin metabolism. H-37 is similar in molecular mass and structure to B-PGFS, suggesting that they are homologous forms. Conclusive proof of their structural similarity awaits cloning of H-37. The effect of EA on levels of H-37 appears to be cell type specific, in that it was marked in two types of human colon carcinoma cells, did not occur in human prostate carcinoma cells, and was not apparent in rat breast carcinoma Walker cells. The latter finding might be explained by the possible inability of anti-H-37 antibody to cross-react with related proteins from other species even at higher titers. Anti-PGFS antibody recognized a second minor protein in LS174T cells that was not affected by drug treatment. Two-dimensional nonequilibrium isoelectric focusing immunoblots of cytosol from EA-resistant cells revealed a single H-37 protein (data not shown). Thus, there was no additional evidence to suggest that there are other H-37 colon isozymes.

The Michael reaction acceptors tested in this study induce H-37, whereas the structurally unrelated nitrogen mustard chlorambucil does not, suggesting that alkylation mediated by bischloroethylating drugs does not induce this protein. A number of drugs structurally unrelated to these agents, including arachidonic acid, buthionine sulfoximine, diamide, and phorbol 12-myristate 13-acetate, also did not induce H-37 (data not shown). Talalay and co-workers (7, 9, 10) have made the compelling case that most inducers of phase II protective enzymes contain or acquire by metabolism distinctive electrophilic centers. Many such compounds are classical Michael reaction acceptors that are susceptible to attack by nucleophiles and are electrophilic by conjugation with electron-withdrawing groups. Their structures include $CH_2{=}CH\text{-}Z$, $Z'\text{-}CH{=}CH\text{-}Z$ (also quinones), and $R\text{-}C{\equiv}C\text{-}Z$, where Z is an electron-withdrawing group (9, 10). EA, dimethyl maleate, t-butylhydroquinone, and hydroquinone contain or are converted to compounds that contain α,β-unsaturated carbonyl groups. The inducing signal may be due to either the parent drug or metabolite, depending on whether it requires reversible oxidation to the

TABLE 4

NADPH-dependent phenylglyoxal reductase activity (dicumoral-insensitive) in cytosol isolated from HT29, DU145, and LS174T cells

Expt.	Cell line	Treatment (before isolation)	Drug	Reductase activity[a]	n[b]	Fold increase
		hr	*μM*	*nmol of NADPH/min/mg of protein*		
I	HT29(WT)[c]		None	5.33 ± 0.44	9	
	HT29(R)	Continuous exposure	EA, 72	10.07 ± 0.84[d]	9	1.9
	HT29(RV)		None	4.59 ± 0.52	4	0.9
			EA			
	HT29(WT)	6	40	6.24	2	1.2
		24	40	7.48	2	1.4
		48	40	9.33	2	1.8
		72	40	12.67 ± 1.72[d]	4	2.4
		48	10	6.75 ± 1.29	3	1.3
		48	20	8.40 ± 1.30[d]	4	1.6
		48	40	9.98 ± 1.66[d]	4	1.9
		48	50	13.55 ± 1.06[d]	4	2.5
			Chlorambucil			
		48	10	4.87	2	0.9
		48	30	6.15	2	1.2
			Dimethyl maleate			
		48	40	11.08 ± 1.23[d]	4	2.1
		48	80	16.73 ± 1.85[d]	7	3.1
			Hydroquinone			
		48	40	8.53 ± 1.20[d]	6	1.6
		48	80	7.90 ± 0.67[d]	6	1.5
II	DU145	48	None	3.42	2	
			EA			
		48	20	3.32	2	1.0
		48	40	2.96	2	0.9
III	LS174T	48	None	8.94 ± 0.49	5	
			EA			
		48	20	8.10 ± 0.70	4	0.9
		48	40	11.75 ± 0.22[d]	4	1.3
		48	50	18.30	2	2.0
		48	Dimethyl maleate, 80	22.53 ± 1.37[d]	3	2.5

[a] Values are mean ± standard error.
[b] Each sample isolation (*n*) was analyzed in duplicate.
[c] WT, wild-type phenotype; R, resistant phenotype; RV, revertant phenotype.
[d] Statistically significant difference from wild-type control ($p < 0.05$). Analysis by *t* test for independent groups, unknown variance.

cognate quinone. An important qualification should be made about EA pharmacological activity; although the reactivity of EA with nucleophiles such as sulfhydryls likely plays a role in both its ability to induce phase II enzymes and its ability to inhibit GST, its sulfhydryl reactivity is not an absolute requirement for other actions such as the diuretic-saluretic response (11).

Both H-37 and GST-π (6) are induced by EA and thus may be coordinately regulated in HT29 cells. A number of early-response genes are induced by drugs and growth factors that initiate a host of protein-DNA interactions that regulate transcription. Several important DNA motif sequences to which activating proteins might bind have been identified in the 5′ flanking regions of the rat and human GST-π genes (putative tetradecanoylphorbol acetate-responsive elements) (24–26), the rat GST-α gene (xenobiotic-responsive elements and ARE) (27–31), and the NAD(P)H:quinone oxidoreductase NQO1 gene (xenobiotic-responsive elements and ARE) (32). The ARE are required for transcriptional activation by antioxidants, redox-labile quinones, and hydrogen peroxide (30, 31). The determination of the molecular mechanisms of H-37 induction by Michael addition acceptors would require similar characterizations of regulatory elements in the H-37 gene.

B-PGFS can metabolize a variety of carbonyl-containing compounds, including PGD_2 (to $9\alpha,11\beta$-PGF_2) and PGH_2 (to $PGF_{2\alpha}$) (15). The consistent parallel increases in NADPH-dependent B-PGFS substrate reductase activities in cytosol samples containing increased H-37 protein suggest conservation of PGFS function. A few explanations could account for the lack of stoichiometric correlation between the 30-fold increases in production of H-37 protein and the 2–3-fold increases in B-PGFS substrate reductase activities, i.e., (a) a number of NADPH-dependent reductase enzymes that display varying degrees of affinity for these substrates are constitutively expressed, (b) post-translationally modified H-37 protein in resistant cells exhibits lower affinity for substrates than does nonmodified H-37 protein from wild-type cell cytosol, or (c) not all increased H-37 is functional.

It is clear that cells express a number of cytosolic reductases. By measurement of substrate activity or protein levels (immunoblotting) and by testing for cofactor requirements for 9,10-phenanthrenequinone and phenylglyoxal reductase, we have attempted to show that neither NAD(P)H:quinone oxidoreductase, glyoxalase I, aldehyde reductase, nor other NADH-dependent reductases are likely major components of induced cellular reductase activity that could contribute to the observed resistant phenotype. However, some of them do represent significant components of constitutive activity [e.g., NAD(P)H:quinone oxidoreductases and glyoxalase I] and could mask the role of marked H-37 protein induction in reductase activity in treated and resistant cells.

PGD_2 and PGF_2 metabolites are formed in most tissues in

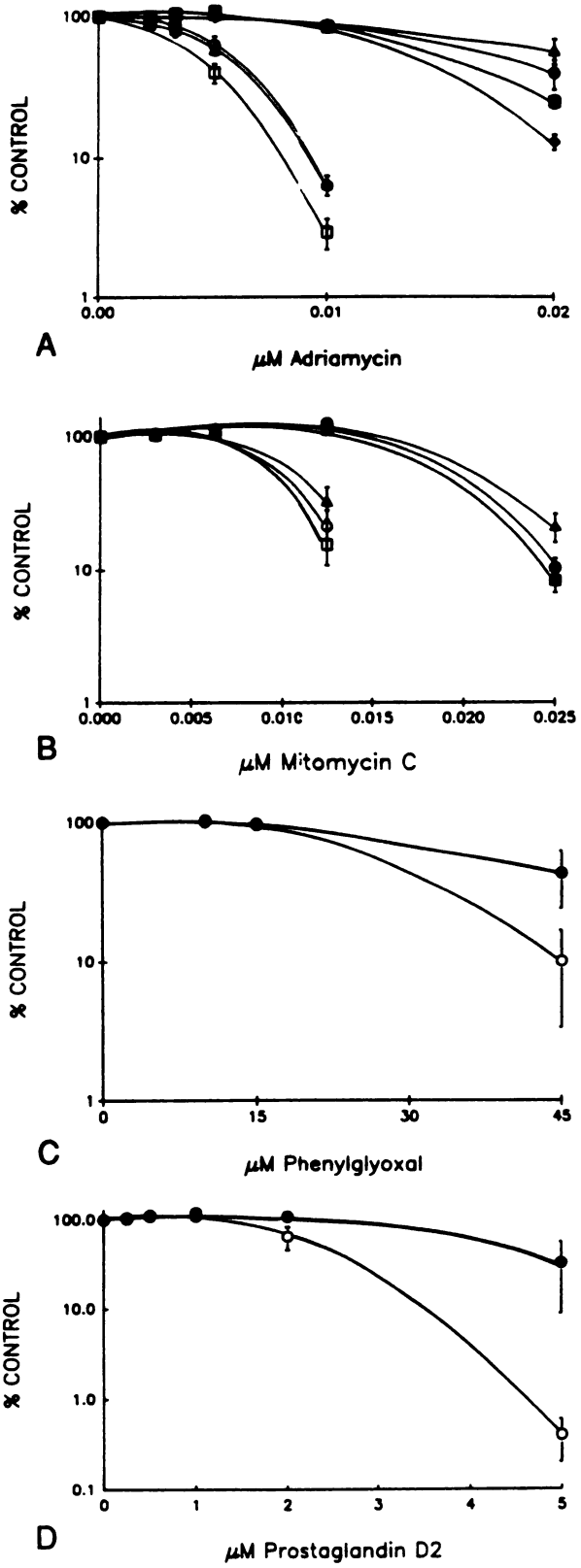

Fig. 7. Colony formation assay. Response to drug by wild-type compared with EA-resistant HT29 cells. ●, EA-resistant HT29 cells; ▲, EA-resistant HT29 cells pretreated with 0.5 μM $PGF_{2\alpha}$; ■, EA-resistant HT29 cells pretreated with 0.5 μM PGD_2; ○, wild-type HT29 cells; △, wild-type HT29 cells pretreated with 0.5 μM $PGF_{2\alpha}$; □, wild-type HT29 cells pretreated with 0.5 μM PGD_2; ◆, revertant HT29 cells. Cells were exposed to drug continuously.

varying amounts but are produced at altered levels in several histological types of lung cancer (12). They evoke a broad range of physiological effects. $PGF_{2\alpha}$ mediates contraction of uterine muscle and enhances type IV collagenase production in tumor cells *in vivo* (33). Because tissue-specific biosynthesis of prostaglandins is determined in large measure by the distribution of individual enzymes responsible for their formation, in theory agents that enhance PGFS activity could have a secondary impact on growth, metastases, and smooth muscle contraction. Given the relatively fast induction capacity and short half-life of H-37, it is conceivable that colon epithelial cells regulate surrounding longitudinal or circular smooth muscle by H-37-mediated short term alterations in local prostaglandin release.

An important pharmacological question remains to be addressed, i.e., does H-37 overexpression possibly confer resistance to the drugs tested by (a) alteration of physiologically active PGD_2 and PGF_2 metabolites or (b) increased drug detoxification? As a means of testing PGFS activity in HT29 colon cells, we measured cell conversion of [^{14}C]arachidonic acid to PGF_2 metabolites by thin layer chromatography and we measured intracellular endogenous levels of PGD_2 by radioimmunoassay. However, no apparent differences in arachidonic acid-induced release of $PGF_{2\alpha}$ and PGF_2 into extracellular medium (total PGF_2 series, pmol/10^6 cells/hr: wild-type, 2.77; resistant, 2.26) or steady state intracellular endogenous PGD_2 levels (pg/10^6 cells: wild-type, 2.80 ± 1.08; resistant, 3.18 ± 1.78) between wild-type and resistant cells were observed. It is conceivable that the primary function of H-37 in these cells is to metabolize exogenous compounds rather than endogenous prostaglandins. However, it is important to note that there are several cellular enzymes competing for synthesis of both PGF_2 metabolites and PGD_2 that might, in theory, quickly compensate for changes in prostaglandin levels caused by H-37 overexpression (34–41).

The fact that increased production of H-37 occurs in parallel with cross-resistance to the clinically important drugs mitomycin C and Adriamycin could be of consequence in understanding the role of drug metabolism in resistance to these compounds. Adriamycin is thought to act by release of superoxide anions after initial metabolism to a hydroquinone (42). Increased levels of GST-π and GSH in EA-resistant cells may play some role in protection against Adriamycin-induced lipid peroxidation, although Adriamycin has not been shown to be a substrate for GST-π. Mitomycin C appears to require reductive activation to genotoxic metabolites for its activity. It may be reduced by xanthine oxidase, NAD(P)H:quinone oxidoreductase, and NADPH-cytochrome P-450 reductase (43–46). The relative levels of these enzymes are important determinants of the number of alkylations achieved. Aldoketo reductase activity may be an additional determinant, in that mitomycin C and Adriamycin could be substrates for H-37. Both of these compounds contain carbonyl moieties (as does EA) that could serve as potential sites for H-37-mediated reduction. The finding that nontoxic concentrations of a PGFS product ($PGF_{2\alpha}$) and substrate (PGD_2) could alter the cytotoxicity of these compounds by competitive inhibition or by stimulation of a signal transduction pathway is also of potential interest for modulation of drug resistance.

In conclusion, a putative human aldo-keto reductase that is expressed in human colon cells and is structurally related to B-PGFS has been identified. Significant overexpression of this enzyme may result from cellular exposure to Michael addition

acceptors. Protein production is fast (6 hr), reversible, and extensive (30-fold) and may give rise to an enhanced capacity to metabolize exogenous and endogenous substrates, thereby contributing to the drug-resistant phenotype. Finally, the availability of a selective polyclonal antibody against H-37 should allow for the screening for expression of this protein in a number of different drug-resistant cell lines and human tumors.

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