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A protein disulfide-thiol interchange activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl)urea (LY181984)

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Abstract

Plasma membrane vesicles isolated from HeLa cells grown in suspension culture contain a protein disulfide-thiol interchange (protein disulfide-like) activity. The activity was estimated from the restoration of activity to inactive (scrambled) pancreatic RNAase. RNAase activity was measured either by hydrolysis of cCMP or by a decrease in acid precipitable yeast RNA. The ability of plasma membrane vesicles to restore activity to inactive (scrambled) pancreatic ribonuclease was inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl)urea (LY181984). The activity correlated with that of a cyanide-resistant NADH oxidase also associated with the plasma membrane vesicles that exhibited a similar pattern of drug response. The activity was stimulated by reduced glutathione and inhibited by oxidized glutathione but did not depend on either for activity. The antitumor sulfonylurea-inhibited activity was greatest in the presence of reduced glutathione and least in the presence of oxidized glutathione. The antitumor sulfonylurea-inhibited activity was unaffected by a monoclonal antibody to protein disulfide isomerase. Also the antitumor sulfonylurea-inhibited activity was unaffected by peptide antisera to the consensus active site sequence of protein disulfide isomerase. Thus the antitumor sulfonylurea-inhibited activity appeared to reside with a novel cell surface protein capable of oxidation of both NADH and protein thiols and of carrying out a protein disulfide isomerase-like protein disulfide-thiol interchange activity in the absence of NADH or other external reductants.

Keywords: Diarylsulfonylurea; Sulfonylurea; Protein disulfide-thiol interchange; Protein disulfide isomerase; NADH oxidase; Plasma membrane; HeLa

Abbreviations: LY181984, *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl)urea; LY181985, *N*-(4-methylphenylsulfonyl)-*N′*-(phenyl)urea; Sulofenur, LY186641, *N*-(5-indanylsulfonyl)-*N′*-(4-chlorophenyl)urea; cCMP, cyclic 3′,5′-cytidine monophosphate; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; DMSO, dimethylsulfoxide; RNAase, ribonuclease

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1. Introduction

Homogenates, total particulate fractions and plasma membranes of cultured HeLa S cells bound the tritiated antitumor sulfonylurea [^{3}H]LY181984 [*N*-(4-methylphenyl)-*N′*-(4-chlorophenyl)urea] with high affinity (K_d of 20 to 50 nM) [1]. Highest affinity binding was to purified plasma membranes (K_d of 25 nM). In a subsequent report [2], a binding protein, apparently with one or more thiols in the active site, was identified and labeled with radioactive thiol reagents in antitumor sulfonylurea protection experiments. Binding proteins of M_r ca. 34 kDa were labeled [2].

By analogy with earlier findings in plants [3], the binding activity was suggested to correlate with a 34 kDa drug-inhibited NADH oxidase [4]. An NADH oxidase activity of the plant plasma membrane had been reported to respond to auxin regulators of plant growth [3] and to be drug-inhibited by herbicidal sulfonylureas [5] active, as well, as plant growth retardants [6]. However, the plasma membrane NADH oxidase of plants was resistant not only to inhibition by cyanide but also proceeded in an argon atmosphere [7]. These findings prompted us to seek electron acceptors other than oxygen for the NADH oxidation. During the course of these studies, an ability of the plasma membranes to catalyze a protein disulfide isomerase-like protein disulfide-thiol exchange activity was revealed [7]. This activity of the plant plasma membranes was stimulated by auxin regulators of plant growth in parallel to the oxidation of NADH [8]. In this report, we demonstrate an ability of isolated plasma membrane vesicles from HeLa cells to catalyze a protein disulfide-thiol exchange activity. The activity, like the NADH oxidase of the HeLa plasma membrane [4], is inhibited by the antitumor sulfonylurea LY181984, but not by a structurally-related but antitumor-inactive sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N′*-(phenyl)urea (LY181985). The drug responsiveness of the HeLa activity provides an important parallel between the protein disulfide-thiol interchange activity and that of the NADH oxidase of the plasma membrane. The latter was growth factor- and hormone-stimulated with rat liver plasma membranes as well as resistant to cyanide [9,10]. Upon transformation, the growth factor- and hormone-responsiveness of the plasma membrane NADH oxidase appeared to be lost [11,12]. The response to sulfonylurea provided an important additional line of evidence of the existence in mammalian plasma membranes of a protein with both NADH oxidase and protein disulfide-thiol interchange activities altered in transformation.

The sulfonylureas represent a novel series of synthetic organic compounds identified as having activity against human solid tumors in vivo [13–15]. Known collectively as antitumor diarylsulfonylureas (sulfonylureas), the compounds have a high degree of efficacy and a relatively low toxicity [13,15]. Additionally, their mechanism of action, while unknown, is apparently unrelated to previously described classes of oncolytic agents [16]. One member of the series, Sulofenur, progressed in evaluation to Phase I [15,16] and Phase II [17] clinical trials. The sulfonylureas were identified as the result of a program of screening against in vivo murine solid tumors implanted subcutaneously [14].

Despite considerable clinical and laboratory data, the site of antitumor sulfonylurea action has remained elusive [13,18]. The drugs were membrane active and weak uncouplers of mitochondrial oxidative phosphorylation [18–21], a response given by sulfonylureas both active and inactive as antitumor agents [21]. There was no evidence for cell cycle specificity of the drugs and no inhibition of DNA, RNA or protein synthesis [16,18]. The sulfonylureas exhibited few, if any, mechanistic parallels to other known antitumor agents [22]. Their mode of action was expected to be unique and, as shown by the present study, appears to involve inhibition of a cell-surface protein disulfide-thiol interchange protein with NADH oxidase activity.

2. Materials and methods

2.1. Growth of cells

HeLa S cells were grown on Minimal Essential Medium (S-MEM) (Joklik modified) with glutamine (244 mg/l) and phosphate (1.3 g/l Na_2HPO_4) and without $CaCl_2$ plus 5% donor horse serum. Gentamycin sulfate (50 mg/l) and sodium bicarbonate (2 g/l) were added.

2.2. Purification of plasma membranes from HeLa cells

HeLa cells grown as suspension cultures were collected by centrifugation for 6 min at $1\,000 \times g$. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 40 sec at 10 000 rpm using a 10 ST-probe and 7 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at $175 \times g$ to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at $15\,000 \times g$ to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approximately 1 ml per pellet from $5 \cdot 10^8$ cells. The resuspended membranes then were loaded onto the two-phase system constituted on a weight basis as follows. The two-phase system contained 6.6% (w/w) Dextran T-500 (Pharmacia), 6.6% (w/w) poly(ethylene glycol) 3350 (Fisher), and 0.2 M potassium phosphate buffer (pH 7.2) [23]. The homogenate (2 g) was added to the two-phase system and the weight of the system was brought to 16 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm ($150 \times g$) in a Sorvall HB 4 rotor for 5 min. The upper phases were carefully withdrawn with a Pasteur pipette, divided in half and transferred into 40 ml plastic centrifuge tubes and diluted with cold l mM $NaHCO_3$ and centrifuged at $10\,000 \times g$ in a HB rotor for 30 min. The purity of the plasma membrane was determined to be $>90\%$ by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

2.3. Estimation of protein disulfide-thiol interchange activity

Two methods were employed to determine protein disulfide-thiol interchange activity. Both involved restoration of ribonuclease activity by refolding of reduced and randomly oxidized (scrambled) and initially inactive ribonuclease A.

In the first method (Method I), restoration of activity to ribonuclease A activity was measured using a spectrophotometric assay based on hydrolysis of cCMP as the RNAase substrate [24]. Scrambled RNAase (0.36 mg) was incubated together with 0.45 mM cCMP in 50 mM Tris-Mes buffer (pH 6.5), at 30°C in a final volume of 3 ml. In the absence of membranes, the scrambled RNAase was inactive (2% of the native RNAase). In the presence of membranes, the scrambled RNAase became active as evidenced from an increase in A_{296} from the RNAase catalyzed hydrolysis of cCMP. A variation of this assay was to preincubate scrambled RNAase with HeLa plasma membranes for 20 min in the presence or absence of 1 μM LY181984 added as a DMSO solution (using DMSO as control) and in the presence or absence of 1 μM reduced or oxidized glutathione or a mixture of both. After this 20 min of reactivation of RNAase, cCMP was added to a final concentration of 0.45 mM to initiate the reaction. The increase of the absorbance at 296 nm was recorded over 25 min and the concentrations of cCMP were determined ($\epsilon = 0.19$ mM^{-1} cm^{-1}). Measurements were with a Hitachi U3210 spectrophotometer with a thermostated cell compartment maintained at 30°C with continuous stirring.

The second method (Method II) followed the procedure of Kalnitsky et al. [25] where the rate of hydrolysis of yeast RNA was determined by measuring the amount of acid-soluble oligonucleotides liberated. Here, 250 μl of 0.15 mM sodium acetate buffer (pH 7.0), 550 μl of reagent grade water, 10 μl of HeLa plasma membranes (140 to 180 μg protein) and 50 μl of scrambled or reduced RNAase (0.36 mg) were preincubated in a 1.5 ml Eppendorf centrifuge tube for 20 min at 37°C. For the assay, 100 μl was transferred to each of 4 tubes. Yeast RNA (100 μl, 0.33 mg/ml, pH 5.0) was added. After 0 (two tubes) and 10 min (two tubes), the reaction was stopped by the addition of 100 μl of 25% perchloric acid containing 0.75% uranyl acetate. The tubes were cooled in an ice bath for 5 min and clarified by centrifugation (2 min, microfuge). Then 75 μl of the clear supernatant from each tube was diluted to 3.0 ml with water. The absorbance was determined at 260 nm against an unincubated blank. The ab-

sorbance at 0 min averaged from the two determinations was then subtracted from the absorbance at 10 min averaged from the two determinations. One unit was defined as an increase in absorbance of 1.0 at A_{260} at 37°C and pH 5.0.

Protein was estimated by the method of Smith et al. [26] with bovine serum albumin as the standard.

2.4. Preparation of scrambled RNAase substrate

To prepare the oxidized, denatured RNAase substrate (scrambled RNAase), native RNAase A (Sigma, Type 1-AS from bovine pancreas) (30 mg/ml) was incubated 1 h at 35°C in 50 mM Tris-acetate (pH 8.6), containing 9 M urea and 130 mM DTT [27].

The fully reduced protein was isolated by adjusting the pH to 4.0 with glacial acetic acid followed by elution from a column of Sephadex G-25 with degassed 0.1 M acetic acid and used directly or further processed to produce scrambled RNAase. Protein concentration was estimated from spectrophotometric measurement at 280 nm using native RNAase A as standard. For preparation of scrambled RNAase, the reduced RNAase was diluted to ~ 0.5 mg/ml with 0.1 M acetic acid. Solid urea then was added to a final concentration of 10 M after which 0.1 M sarcosine hydrochloride was added and the pH adjusted to 8.5 with 1 M Tris. The mixture then was incubated in the dark for 2–3 days during which time the protein was randomly oxidized. The scrambled product was recovered by acidification to pH 4 with glacial acetic acid and elution from Sephadex G-25 in 0.1 M acetic acid. Fractions containing protein were pooled, adjusted to pH 8 and stored at 4°C. Free thiol groups, as determined using 5,5′-dithio(2-nitrobenzoic acid) [28], were 80 to 90% oxidized.

2.5. Source of antisera

Antiprotein disulfide isomerase antibody was a mouse monoclonal antibody (SPA-891) from Stress-Gen Biotechnologies (Victoria, Canada). The immunogen was protein disulfide isomerase purified from bovine liver and the antisera cross-reacted with protein disulfide isomerase from human, monkey, rat, mouse and hamster cell lines. The peptide antibody was to Cys-Tyr-Ala-Pro-Trp-Cys-Gly-His-Cys-Lys-Gln-Leu-Ala-Pro-Ile conjugated to keyhole limpet hemocyanin and supplied as whole rabbit sera. The peptide was synthesized and the antisera prepared by Immuno-Dynamics, La Jolla, CA (Antisera #1472). The preimmune sera were from the same source as the peptide antisera.

3. Results

When estimated using cCMP as substrate [26] (Method I), HeLa plasma membranes restored activity to scrambled RNAase (Table 1). Restoration of

Table 1
Protein disulfide-thiol interchange activity of HeLa plasma membranes (Method I)

Treatment	nmol/min per mg protein		Change due to LY181984
	LY181984	total	
None	None	36 ± 4 [a]	−25
	1 μM	11 ± 8 [b]	
1 μM reduced glutathione (GSH)	None	75 ± 4 [c]	−37
	1 μM	38 ± 3 [a]	
1 μM oxidized glutathione (GSSG)	None	26 ± 8 [a]	− 8
	1 μM	18 ± 6 [b]	
GSH + GSSG	None	72 ± 24 [c]	−53
	1 μM	19 ± 8 [b]	
Scrambled RNAase	–	8 ± 3 [d]	

All treatments contained 360 μg scrambled RNAase and 200 μg HeLa plasma membrane protein except for scrambled RNAase alone where the plasma membranes were omitted.

Numbers not followed by the same letters were significantly different from each other ($P < 0.02$) as determined by two-tailed *t*-test. The inhibitions by LY181984 with no additions and in the presence of GSH were highly significant ($P < 0.001$).

Results are averages of three determinations, each with a different preparation of plasma membranes ± S.D. Specific activities are based on plasma membrane protein.

RNAase activity was accelerated by addition of 1 μM reduced glutathione (GSH) and was slowed by 1 μM oxidized glutathione (GSSG) (Table 1). Restoration of activity to scrambled RNAase by HeLa plasma membranes was inhibited by 1 μM LY181984. When corrected for activity in the absence of plasma membranes, the inhibition by LY181984 was between 80 and 90% in the absence of GSH or GSSG or with GSH and GSSG in equal proportions. The increment of sulfonylurea-inhibited activity was greatest with GSH alone or an equal mixture of GSH and GSSG and was least with GSSG alone (Table 1).

The concentration dependency of the sulfonylurea inhibition exhibited an EC_{50} of about 30 nM (Fig. 1). The sulfonylurea was added as a DMSO solution (final DMSO concentration 0.1%). DMSO controls were evaluated and the solvent, on average, was without effect. Also without effect was LY181985, a homolog of LY181984 inactive as an antitumor agent, at concentrations equivalent to those for the antitumor-active LY181984 (Fig. 1).

When RNAase was assayed by the Kalnitsky et al. method [25] (Method II), plasma membranes of HeLa cells also exhibited a protein disulfide-thiol interchange activity as evidenced by the restoration of activity to scrambled RNAase (Table 2). This activity was stimulated by 1 μM reduced glutathione (GSH) and was inhibited by 1 μM LY181984. This method provided a more direct assay of ribonuclease activity based on the hydrolysis of acid-precipitable RNA.

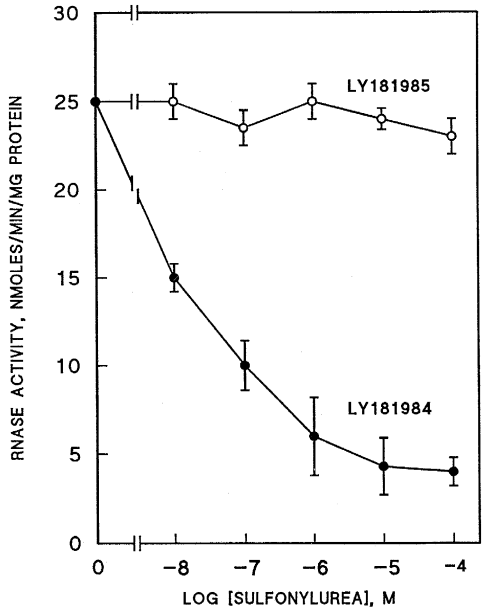

Fig. 1. Response to sulfonylurea concentration of the protein disulfide-thiol interchange activity of HeLa plasma membranes. The activity was inhibited when treated with the active antitumor sulfonylurea LY181984 but not by the inactive sulfonylurea LY181985. The specific activity is calculated after 20 min of reactivation on the basis of the amount of plasma membrane protein in the assay.

As with Method I (Table 1), the increment of

Table 2
Effect of sulfonylurea and thiol compounds on protein disulfide-thiol interchange of HeLa plasma membranes (Method II)

Addition (1 μM)	LY181984	A/20 min	
		total	change due to LY181984
None	None	0.041 ± 0.006 [a]	−0.012
	1 μM	0.029 ± 0.003 [b]	
DMSO	None	0.034 ± 0.001 [a]	−0.008
	1 μM	0.026 ± 0.005 [b]	
1 μM reduced glutathione (GSH)	None	0.049 ± 0.002 [a,c]	−0.014
	1 μM	0.035 ± 0.004 [a,b]	
1 μM oxidized glutathione (GSSG)	None	0.036 ± 0.005 [a,b]	−0.003
	1 μM	0.033 ± 0.002 [b]	
GSH + GSSG	None	0.051 ± 0.003 [a]	−0.016
	1 μM	0.035 ± 0.003 [a,b]	
Scrambled RNAase	–	0.024 ± 0.004 [b]	

All treatments contained 360 μg scrambled RNAase and 200 μg HeLa plasma membrane except for scrambled RNAase alone where the plasma membranes were omitted.
Numbers not followed by the same letters were significantly different as determined by two-tailed t-test ($P < 0.05$). Results are averages of 3–6 determinations, each with a different preparation of plasma membranes ± S.D. Specific activities are based on plasma membrane protein.

Table 3
Effect of sulfonylureas and thiol compounds on activity of native RNAase at pH 6.0 and pH 6.8

Sulfonylurea	nmol/min per mg	
	pH 6.0	pH 6.8
None	150	210
1 μM LY181984	140	210
1 μM LY181985	150	210

Assay was by Method I.

sulfonylurea inhibition determined by Method II was greatest with GSH present or with a balanced redox system containing both GSH and GSSG compared to DMSO alone and was least in the presence of GSSG alone (Table 2). The blank rate was determined to be the same both in the presence and absence of GSH or GSSG [28] and was unaffected by DMSO or LY181984. There was no effect of LY181984 alone on active RNAase (Table 3).

The activity inhibited by the antitumor sulfonylurea LY181984 appeared not to be that of a classic protein disulfide isomerase. Under conditions where the activity of authentic protein disulfide isomerase was inhibited by the antisera, monoclonal antisera to authentic protein disulfide isomerase or polyclonal antisera to a consensus peptide sequence of protein disulfide isomerase containing the active site cysteines both failed to inhibit the protein disulfide-thiol interchange activity of HeLa plasma membranes inhibited by LY181984 as determined either by Method

Table 4
Response to antisera directed to protein disulfide isomerase of the protein disulfide-thiol interchange activity stimulated by reduced glutathione (GSH) and inhibited by LY181984 of HeLa plasma membranes by Method I

Antisera	nmol/min per mg protein			
	no GSH	no GSH + 1 μM LY181984	1 μM GSH	1 μM GSH + 1 μM LY181984
None	35 ± 2 [a]	9 ± 3 [a]	72 ± 3 [a]	38 ± 3 [a]
Preimmune	25 ± 3 [b]	4 ± 2 [a]	63 ± 8 [a,b]	29 ± 4 [b]
Peptide antisera 1472	30 ± 5 [a,b]	6 ± 2 [a]	60 ± 5 [b]	40 ± 4 [a]
Anti-PDI MAB (StressGen SPA-891)	36 ± 3 [a]	18 ± 3 [b]	55 ± 7 [b]	39 ± 8 [a,b]

Antisera were added to 3 ml of the assay mixture containing ca. 200 μg of HeLa plasma membrane protein at a final dilution 1:2 400. Following a 20 min preincubation with antisera plus plasma membranes and scrambled RNAase, the cCMP substrate was added to initiate the reaction.
Numbers not followed by the same letter were significantly different ($P < 0.02$) as determined by two-tailed *t*-test. Results are averages of three determinations $\pm$ S.D. Specific activities were based on plasma membrane protein.

Table 5
Inhibition by the antitumor sulfonylurea LY181984 of the protein disulfide-thiol interchange activity of HeLa plasma membranes assayed by Method II in the presence of 1 μM reduced (GSH) or oxidized (GSSG) glutathione

GSH/GSSG	Antisera	Sulfonylurea inhibition (A/20 min)
None	None	0.011 ± 0.003
	Peptide AB 1472	0.009 ± 0.002
	MAB SPA-891	0.013 ± 0.004
1 μM reduced glutathione (GSH)	None	0.01 ± 0.002
	Peptide AB 1472	0.006 ± 0.004
	MAB SPA-891	0.011
1 μM oxidized glutathione (GSSG)	None	0.003 ± 0.001
	Peptide AB 1472	0.002 ± 0.002
	MAB SPA-891	0.004

Conditions were as for Table 2. Results with anti-PDI peptide antisera 1472 were from three experiments. The values for Anti-PDI MAB (StressGen SPA-891) were from a single experiment of triplicate determinations each. Inhibition of activity by the sulfonylurea in the presence of antisera were not significantly different from those in the absence of antisera.

I (Table 4) or by Method II (Table 5). The peptide antisera and preimmune sera were approximately equal in their effect. With both the antisera to the active site peptide and the monoclonal antibody to protein disulfide isomerase, the component of activity inhibited by LY181984 was unaffected by either antisera as determined using either Method I (Table 4) or using Method II (Table 5) either in the presence or absence of reduced glutathione. Although not significant statistically, the activity in the presence of reduced glutathione was reduced in the presence of antisera compared to no antisera (Table 4).

4. Discussion

Protein disulfide isomerase or protein disulfide isomerase-like enzymes are multifunctional proteins that catalyze disulfide-thiol interchange reactions in protein substrates leading to net protein disulfide reduction, formation or isomerization, or thiol oxidation depending on the initial substrates and the thiol-disulfide potential imposed by the environment (e.g., the redox buffer) [29]. More recently, protein disulfide isomerase-like activity has been reported to be associated with plasma membranes of cultured mammalian cells [30]. In this report, we demonstrate a protein disulfide isomerase-like activity using plasma membrane vesicles isolated from HeLa cells that appears to be distinct from classical protein disulfide isomerases of the endoplasmic reticulum lumen and that is responsive to the antitumor sulfonylureas.

Protein disulfide isomerase activities are normally assayed by the ability of these proteins to catalyze refolding of proteins that contain disulfide bonds [31]. For example, in a glutathione redox buffer, protein disulfide isomerases function catalytically to regenerate native RNAase from the oxidized form at the expense of reducing equivalents from GSH [24].

In the present report, we have utilized two different methods to assay protein disulfide isomerase-like or protein disulfide-thiol interchange activity of HeLa plasma membranes and the response of the activity to sulfonylureas. The active antitumor sulfonylurea LY181984 inhibited the refolding of RNAase depending on the redox buffer and conditions of preincubation. With no preincubation or with membranes preincubated with GSH or a mixture of GSH and GSSG, LY181984 inhibited the activation of scrambled RNAase. The findings suggest that the response to LY181984 was influenced by the imposed thiol-disulfide potential as generated by the surrounding redox buffer. However, even if the redox state was unbalanced, either with GSH or GSSG alone, sulfonylurea inhibitions were still observed.

A response similar to that of the sulfonylurea-inhibited protein disulfide-thiol interchange activity was observed previously with 2,4-D-stimulated NADH oxidase activity and growth [32]. However, the 2,4-D-stimulated activity was blocked in the presence of unbalanced GSH or GSSG.

That the protein disulfide-thiol interchange activity responded to sulfonylurea even in the absence of GSH, GSSG or GSH + GSSG argued against the disulfide-thiol interchange activity being obligatorily driven by reducing equivalents coming from reduced glutathione. Thus the activity inhibited by sulfonylurea appeared to be distinct from the classic protein disulfide isomerase of the endoplasmic reticulum lumen and also distinct from an oxidoreductase activity obligatorily driven by pyridine nucleotide substrates such as glutathione reductase which requires NADPH, for example.

To become reactivated, the interchain disulfides of the scrambled RNAase must first be reduced under conditions favorable for renaturation and then become reoxidized under renaturing conditions to reform the interchain disulfides in a correct conformation to restore catalytic activity. In the process of protein disulfide-thiol interchange catalyzed by HeLa plasma membranes, the rate of activation of scrambled RNAase appears to follow a sigmoid function with an early accelerating rate and a declining later rate [8]. In the absence of GSH or GSSG, the source of disulfides and thiols to reactivate the RNAase is presumably protein disulfides and thiols of the plasma membrane as the reactivation takes place in the absence of any other external thiol, disulfide or reduced pyridine nucleotide source.

The increment of sulfonylurea inhibition was greatest with GSH present or with a mixture of GSH and GSSG and least with GSSG alone for both Method I and Method II. This agrees with an earlier observation that binding of [^{3}H]LY181984 to HeLa cell plasma membranes was enhanced under reducing and much less under oxidizing conditions [1]. The

enhanced total activity, however, observed as a result of GSH or GSH + GSSG addition (Tables 1 and 2) may not necessarily reflect the sulfonylurea-inhibited activity but may be a contribution coming from a sulfonylurea-resistant activity also associated with the plasma membrane that was enhanced by GSH. The HeLa plasma membranes contain both sulfonylurea-resistant and sulfonylurea-susceptible components of NADH oxidase activity that respond differently to drugs and thiol reagents [4,33]. The constitutive NADH oxidase activity was stimulated by GSH in rat liver, for example, whereas the activity in transformed (e.g. HeLa) cells was not [33]. A similar differential response exhibited by the protein disulfide-thiol interchange activity could account as well for the stimulations by GSH reported here.

The significance of a protein disulfide-thiol interchange activity associated with the plasma membrane and modulated by antitumor sulfonylureas cannot yet be decided with certainty. Specificity is indicated from the observation that the antitumor-inactive sulfonylurea LY181985 that differs from LY181984 by a single chlorine on the B ring [13] was without effect in inhibiting the thiol interchange-catalyzed reactivation of scrambled yeast RNAase. The LY181985 was ineffective at several log orders more sulfonylurea than was necessary to inhibit with LY181984.

In these experiments, a strong correlation exists among the inhibition of protein disulfide-thiol interchange of HeLa cell plasma membranes or whole cells (Fig. 1), of NADH oxidase activity of HeLa cell plasma membranes [4], of diferric transferrin- or ferricyanide-induced proton efflux in intact HeLa cells [34] and of HeLa cell growth in the presence of EGF [35]. All were inhibited with an EC_{50} of about 30 nM which agrees closely with the kDa for binding of [^{3}H]LY181984 to isolated vesicles of HeLa plasma membranes of about 30 nM [1]. Thus a reasonable supposition would be that the sulfonylurea-inhibited protein disulfide-thiol interchange activity and the sulfonylurea-inhibited NADH oxidation may represent two different manifestations of the activity of the same sulfonylurea-binding protein of the HeLa cell plasma membrane.

The sulfonylurea-inhibited activity of the protein disulfide-thiol interchange activity was unaffected by a monoclonal antibody to protein disulfide isomerase purified from bovine liver. The antibody cross-reacted with human protein disulfide isomerase, yet did not block the sulfonylurea-inhibited protein disulfide-like activity of the HeLa plasma membranes. Also, antisera prepared to a synthetic peptide corresponding to the highly conserved consensus region containing the active site of human protein disulfide isomerase [36,37] failed to inhibit. We do not rule out the possibility of an association of protein disulfide isomerase with the mammalian plasma membrane. The component of the protein disulfide-thiol interchange activity stimulated by GSH and resistant to inhibition by LY181984 might represent such an activity. However, the failure of the antiprotein disulfide isomerase antisera to eliminate the sulfonylurea-inhibited activity, taken together with a lack of dependency of activity on the presence of reduced glutathione, suggest the sulfonylurea-inhibited protein disulfide-thiol interchange activity of the HeLa plasma membrane to be an activity distinct from that of a classical protein disulfide isomerase of the endoplasmic reticulum lumen.

If, indeed, the sulfonylurea-inhibited NADH oxidase protein is able to catalyze protein disulfide-thiol interchange, then it follows that the activity may be able to catalyze the net reduction of disulfides or the net oxidation of thiols as well under appropriate conditions of incubation (i.e., function as a disulfide oxidoreductase). As such it could carry out important functions in the membrane related either to protein disulfide-thiol interchange or to net thiol content and obligatorily unrelated either to NADH oxidation or to the operation of a plasma membrane redox chain per se (see also [38]).

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