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Antitumor sulfonylurea-inhibited NADH oxidase of cultured HeLa cells shed into media

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Abstract

Conditioned culture media of HeLa S cells contain a soluble NADH oxidase activity inhibited by the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) similar to that associated with the outer surface of the plasma membrane. This activity was absent from media in which cells had not been grown and was present in conditioned culture media from which cells had been removed by centrifugation both for serum-containing and serum-free media. The K_m with respect to NADH and response to thiol reagents were similar to those of the corresponding activity of the plasma membrane of HeLa cells. The conditioned HeLa culture media bound [^{3}H]LY181984 with high affinity. Both antitumor sulfonylurea-inhibited and -resistant forms of the NADH oxidase were isolated by free-flow electrophoresis. The antitumor sulfonylurea-inhibited activity was purified to apparent homogeneity and was identified with a 33.5 kDa protein with an isoelectric point of about pH 4.5. The 33.5 kDa protein from conditioned HeLa culture medium both bound [^{3}H]LY181984 and retained an LY181984-inhibited NADH oxidase activity. A polyclonal antisera was raised in rabbits to the purified 33.5 kDa constituent from conditioned HeLa culture medium. The antisera blocked the activity of the LY181984-inhibited NADH oxidase activity, immunoprecipitated the activity and reacted with a 33.5 kDa protein on Western blots while preimmune sera did not. Also inhibited and immunoprecipitated was NADH oxidase activity from HeLa plasma membranes. The findings are consistent with the 33.5 kDa drug-inhibited NADH oxidase activity of the culture media being a shed form of the corresponding native 34 kDa antitumor sulfonylurea-inhibited NADH oxidase activity of the HeLa cell plasma membrane.

Keywords: Diarylsulfonylurea; Sulfonylurea; Antitumor drug; NADH oxidase; Plasma membrane; HeLa cell

1. Introduction

Plasma membranes isolated from cultured HeLa cells bound the active antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) with high affinity [1]. The molecular mass of a putative binding protein of the HeLa plasma membrane was determined to be approximately 34 kDa [2]. Also associated with the plasma membrane of cultured HeLa cells, but not with that of rat liver, was an NADH oxidase activity that was sulfonylurea-inhibited [3]. A second characteristic that appeared to distinguish the NADH oxidase of HeLa plasma membranes from that of liver plasma membrane, for example, was an ability to be inhibited by thiol reagents such as *N*-ethylmaleimide or *p*-chloromercuribenzoate [4]. This activity was inhibited by sulfonylurea only with NADH supplied to the external plasma membrane surface suggesting that the sulfonylurea-inhibited activity was an ectoprotein of the plasma membrane [5]. As such, the activity might be shed from the cell surface. Shedding into the medium is a characteristic of many ectoenzymes of the plasma membrane including galactosyltransferases [6,7], sialyltransferases [8,9], 5'-nucleotidase [10] and dipeptidylpeptidase IV [11,12]. In view of these findings, conditioned culture media of HeLa cells were examined for the presence of an antitumor sulfonylurea-inhibited NADH oxidase activity. This report concerns the characterization of a secreted form of the NADH oxidase activity of

Abbreviations: LY181984, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; LY181985, *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea; PCMB, *p*-chloromercuribenzoic acid; NEM, *N*-ethylmaleimide.

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cultured HeLa cells and a comparison of the activity present in the conditioned culture medium with that of HeLa plasma membranes.

2. Materials and methods

2.1. Growth of cells

HeLa S cells with serum were grown in Jolik-modified minimal essential medium (S-MEM) supplemented with glutamine (244 mg/l), phosphate (1.3 g/l Na_2HPO_4), gentamicin sulfate (50 g/l), sodium bicarbonate (2 g/l) and 5% horse serum. For HeLa S cells grown on a defined medium, a reduced calcium (100 μM $CaCl_2$) modification of HB Pro (Irvine Scientific) supplemented with 1 μg/ml Nucellin (Eli Lilly) and 0.2% EX-CYTE VLE (Bayer) was used. Cells were collected by centrifugation for 15 min at 2500 rpm.

2.2. Purification of plasma membranes

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 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 40 s at 10000 rpm using an ST-10 probe and 7 ml aliquots to achieve at least 90% cell breakage without breakage of nuclei. Cell breakage was monitored by light microscopy.

The homogenates were centrifuged for 10 min at $175 \times g$ to remove unbroken cells and nuclei. The supernatant was centrifuged a second time at $1.4 \cdot 10^6 \times g$ min to prepare a plasma membrane-enriched fraction. Plasma membrane-enriched pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of 1 ml per pellet from approximately $5 \cdot 10^8$ cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis and consisting of 6.6% (w/w) Dextran T-500 (Pharmacia), 6.6% (w/w) Polyethylene Glycol 3350 (Fisher) in 5 mM potassium phosphate buffer (pH 7.2) [13]. The resuspended microsomal pellet (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C) and the phases separated by centrifugation at $750 \times g$ for 5 min. The upper phases were withdrawn with a Pasteur pipette, diluted with 5 volumes of cold 1 mM $NaHCO_3$ and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was about 2 mg plasma membrane protein from 10^9 cells.

2.3. Spectrophotometric assay

NADH oxidase activity was determined at 37°C as the disappearance of NADH measured at 340 nm. Activity was measured using a Hitachi U3210 with stirring and continuous recording over 5 min intervals. The reaction mixture contained 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN and 150 μM NADH. A millimolar extinction coefficient of 6.22 was used to calculate the rate of NADH disappearance.

2.4. Free-flow electrophoresis

Proteins of culture filtrates were concentrated by ammonium sulfate precipitation (0–70% of saturation) and resuspended in 1% Triton X-100.

Free-flow electrophoresis separations were as described [14]. The equipment was a VAP-22 continuous free-flow electrophoresis unit (Bender and Hobein, Munich, Germany). The electrophoresis conditions are given with the figures. The separation profiles were monitored by collecting individual fractions and monitoring absorbance at 280 nm as well as measuring the rate of NADH oxidation and response of NADH oxidation rate to the antitumor sulfonylurea LY181984.

2.5. Equilibrium binding

Tritium-labeled *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenylurea) [^{3}H]LY181984 (lot 497-1-291) (2898 mCi/mg) was obtained from Lilly Research Laboratories, Indianapolis, IN and diluted with dimethylsulfoxide (DMSO). For binding studies, a multi-cell rotating teflon cell equilibrium dialyzer (Spectrum Equilibrium Dialyzer, Spectrum Medical Instruments, Los Angeles, CA) with a dialyzing volume of 1 ml and a 47 mm diameter membrane (12 to 14 kDa molecular weight cut off) area was used as described [1]. The dialysis membranes were prepared by soaking in water for 30 min followed by 30% ethanol for 30 min and several changes of distilled water. Both sides of the chamber were supplied with 0.5 ml final volume of a buffer solution containing 25 mM Tris, 100 mM potassium chloride, 5 mM magnesium chloride and 1 μM dithiothreitol, pH 7.4. To one side of each chamber was added the [^{3}H]LY181984 in DMSO and the fraction to be evaluated. An equivalent amount of DMSO was added to the opposite side of the chamber and equilibrium established by rotating the cells at about 100 rpm at 25°C 2 h to overnight. A 100 μl sample was withdrawn from each chamber and radioactivity was determined by liquid scintillation spectrometry. Determinations were in triplicate and radioactivity was determined at the 99% confidence level.

2.6. Isolation of a 33.5 kDa protein with LY181984-inhibited NADH oxidase activity from conditioned culture media of HeLa cells

A fraction was prepared by ammonium sulfate-precipitation (55 to 70% of saturation) from approximately 2.3 l of conditioned culture medium. The ammonium sulfate

precipitate was resuspended in about 30 ml of Tris-HCl, pH 7.0, dialyzed overnight against 50 mM Tris-Mes, pH 8.0, and applied to a 150 ml (6.0 × 5.5 cm) DEAE cellulose column equilibrated with 50 mM Tris-Mes, pH 8.0. The column was eluted with a series of buffers of decreasing pH (8, 7.5, 7.0, 6.5, 6, 5.5, 5, 4.5 and 3) and assayed for antitumor sulfonylurea-inhibited NADH oxidase activity.

The pH 5.0 eluate was concentrated to a final volume of approximately 2 ml and applied to a 2.85 × 13.5 cm Sephadex G-200 (Pharmacia) column. The column was eluted with 50 mM Tris-HCl, pH 7.0, at a flow rate of 18 ml/h. Fractions of 1.4 ml each were collected.

The fractions containing the sulfonylurea-inhibited NADH oxidase eluted from the G-200 column were combined, concentrated and further purified by preparative SDS-PAGE using a Bio-Rad preparative gel electrophoresis system (Bio-Rad Column, Model 491 Preparative Cell). The separation gel was 10% acrylamide and the stacking gel was 3.9% acrylamide. Separation was at constant power of 12 W with a flow of 0.37 ml/min. Fractions of 2.5 to 3 ml each were collected. The fraction was diluted 1:1 with sample buffer (0.06 M Tris-HCl, pH 7.0, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.01% bromophenol blue), boiled for 10 min and cooled on ice. The collected fractions were analyzed by SDS-PAGE on 10% acrylamide and assayed for NADH oxidase activity inhibited by sulfonylurea as well as sulfonylurea binding.

2.7. Preparation of antisera

The material eluting at 33.5 kDa with sulfonylurea-inhibited NADH oxidase activity and sulfonylurea binding activity was further purified by preparative SDS-PAGE and stained with Coomassie Blue on 10% acrylamide. The protein with electrophoretic mobility corresponding to 33.5 kDa was excised, diced and macerated in 0.5 μl Freund's complete adjuvant and 0.5 ml phosphate-buffered saline and further homogenized by forcing through an 18 gauge and then a 22 gauge needle. The mixture was used as immunogen for antisera generation in a male, New Zealand white rabbit 6-month-old (Hazelton HRP, Inc., Denver, PA). The rabbit was boosted 3 weeks later with a similar second gel slice corresponding to the first. The rabbit was bled at approximately 1 week intervals and sera prepared.

3. Results

Conditioned culture medium in which HeLa S cells had been grown and from which the cells were removed by centrifugation, contained an NADH oxidase activity that was approximately 50% inhibited by the antitumor sulfonylurea LY181984 at a final concentration of 1 μM (Table 1). Freshly prepared serum-containing medium prior to growing cells, exhibited an activity coming from the donor horse serum but this activity was unaffected by the antitumor sulfonylurea. NADH oxidase activity of conditioned culture medium with serum present also was ca. 50% inhibited by thiol reagents (Table 2). With conditioned serum-free medium, activity also was present after cells were removed by centrifugation. This activity was ca. 90% inhibited by 1 μM LY181984 (Table 1). Defined medium that was serum-free and in which cells had not been grown, lacked an NADH oxidase activity.

Table 1
NADH oxidase activity of conditioned HeLa S cell culture medium and response to 1 μM LY181984

Source	LY181984	nmol/min/100 μl
Conditioned culture medium with 5% horse serum [a]	none	0.8 ± 0.08
	1 μM	0.4 ± 0.16
Culture medium with 5% horse serum	none	0.4 ± 0.08
	1 μM	0.4 ± 0.12
Conditioned defined culture medium (serum-free) [a]	none	0.46 ± 0.11
	1 μM	0.04 ± 0.03
Defined culture medium (serum-free)	none	n.a.
	1 μM	n.a.

Results are based on averages of 10 independent determinations ± S.D.
[a] HeLa S cells were grown to a density of about $2.5 \cdot 10^6$ per ml and removed by centrifugation for 15 min at 2500 rpm.
n.a. = no activity.

The NADH oxidase activity of the conditioned medium was characterized and compared to that of HeLa membranes. Activity was proportional to time and concentration of conditioned medium. Heating for 20 min at 60°C reduced the activity by 50% and heating for 20 min at 80°C destroyed the activity. There was little or no response of the activity to ionic additions (KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$). The rate of oxidation of NADH was proportional to the concentration of NADH (Fig. 1) and yielded a K_m of 18 ± 5 μM compared to 15 ± 10 μM determined in parallel for the corresponding activity from plasma membranes of HeLa cells [4].

Table 2
NADH oxidase activity of conditioned serum-containing culture medium and response to *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide (NEM)

Addition	NADH oxidase activity (nmol/min/100 μl)
None	0.4 ± 0.09 (10)
1 μM LY181984	0.18 ± 0.04 (10)
0.1 μM PCMB	0.10 ± 0.04 (6)
0.1 μM NEM	0.18 ± 0.03 (2)

The number of determinations is given in parentheses.
HeLa S cells were grown to a density of about $1.2 \cdot 10^6$ per ml and removed by centrifugation for 15 min at 2500 rpm.

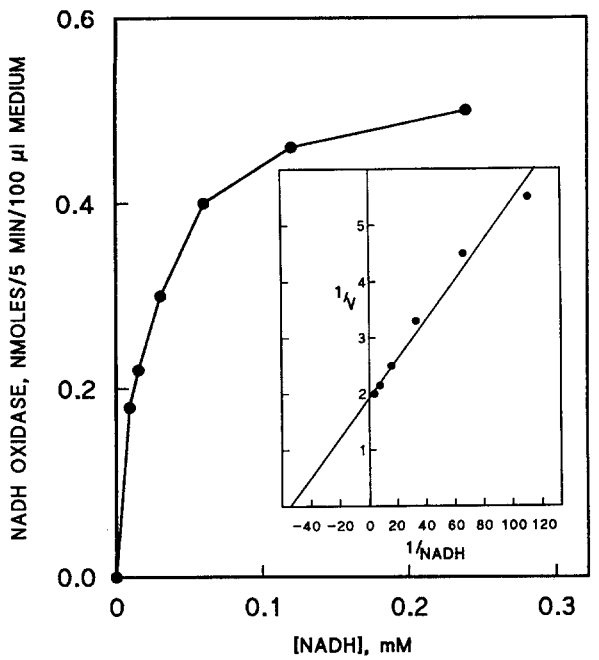

Fig. 1. NADH oxidase activity of conditioned, defined HeLa S culture medium assayed in the presence of 100 mM Tris-HCl (pH 7.4) with varying concentrations of NADH. Cells were grown as for Table 4. The inset shows the double reciprocal plot calculated from the experimental means.

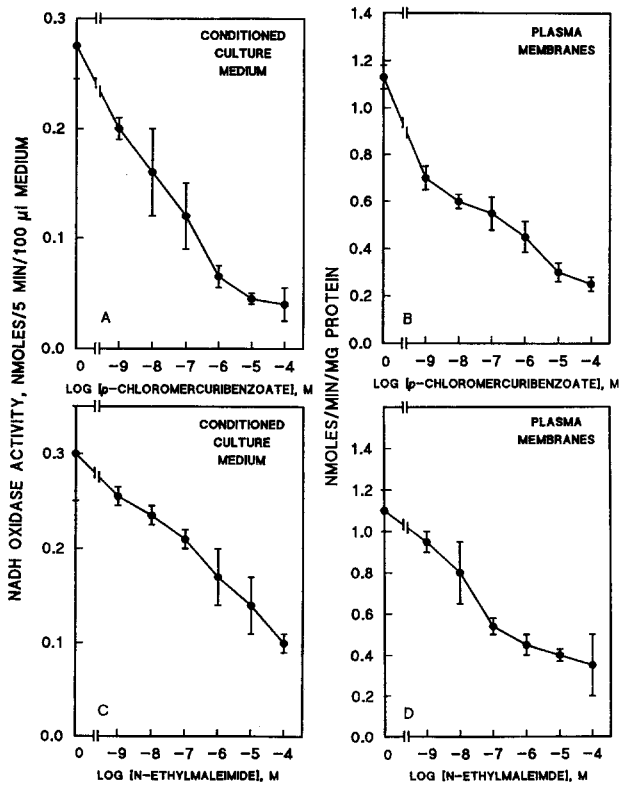

Fig. 2. Dose-response of inhibition of NADH oxidase activity. (A,B) *p*-Chloromercuribenzoate (PCMB). (A) Conditioned, defined HeLa cell culture medium. Cells were grown as for Table 4. (B) HeLa S plasma membranes. (C,D) *N*-Ethylmaleimide (NEM). (C) Conditioned defined HeLa S cell culture medium. Cells were grown as for Table 4. (D) HeLa plasma membranes.

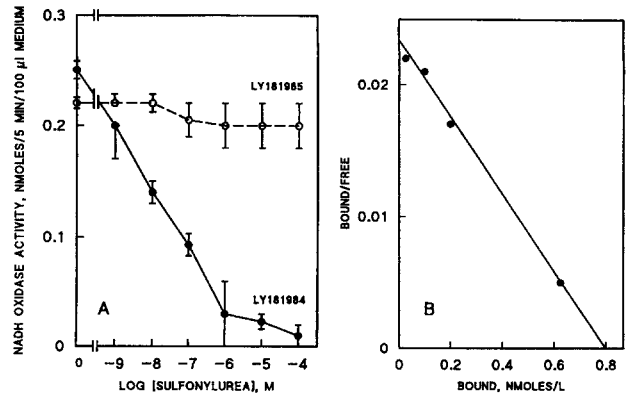

Fig. 3. NADH oxidase activity and sulfonylurea binding. (A) Dose-response of NADH oxidase activity of defined culture medium conditioned by growth of HeLa S cells to the logarithm of sulfonylurea concentration comparing an active antitumor sulfonylurea, LY181984, and an inactive sulfonylurea, LY181985, that differ only in the presence (LY181984) or absence (LY181985) of a chlorine on the B ring. Cells were grown as for Table 4. (B) Scatchard analysis of high affinity [^{3}H]LY181984 binding by conditioned, defined (serum-free) culture medium of HeLa cells. Cells were grown as for Table 3. The K_d was estimated to be about 30 nM. Results were confirmed in three independent experiments.

One characteristic of the NADH oxidase activity of HeLa and rat hepatoma plasma membranes to distinguish the activity from that of rat liver plasma membrane, was based on sensitivity to thiol reagents [4]. The activity of HeLa and hepatoma plasma membranes was inhibited but that of rat liver plasma membranes was not inhibited.

The dose response of the NADH oxidase activity of conditioned serum-free (defined) culture medium and of HeLa cell plasma membrane to *p*-chloromercuribenzoate (PCMB) (Fig. 2A,B) and to *N*-ethylmaleimide (NEM) (Fig. 2C,D) were similar. NADH oxidase activity of conditioned defined HeLa culture medium and of HeLa cell plasma membrane was inhibited by PCMB with an EC_{50} of ca. 10 nM and by *N*-ethylmaleimide with an EC_{50} of 0.1 to 1 μM.

A further defining characteristic of the NADH oxidase activity of the plasma membrane of HeLa cells was its inhibition by the antitumor sulfonylurea LY181984 [3]. The NADH oxidase activity of plasma membranes of rat liver, for example, was not inhibited by LY181984. The EC_{50} of inhibition of the NADH oxidase activity of HeLa plasma membranes was about 30 nM [3]. A similar dose-response was seen with the conditioned defined culture media in which HeLa cells had been grown (Fig. 3A). The EC_{50} was about 30 nM and nearly complete inhibition was observed at 1 μM. The antitumor-inactive sulfonylurea LY181985 was without effect both on the NADH oxidase activity of the conditioned defined HeLa culture medium and on the NADH oxidase activity of plasma membranes from HeLa cells [3]. LY181985 differs from LY181984 by the absence of a chlorine from the B ring with LY181985 that is present in LY181984 [15].

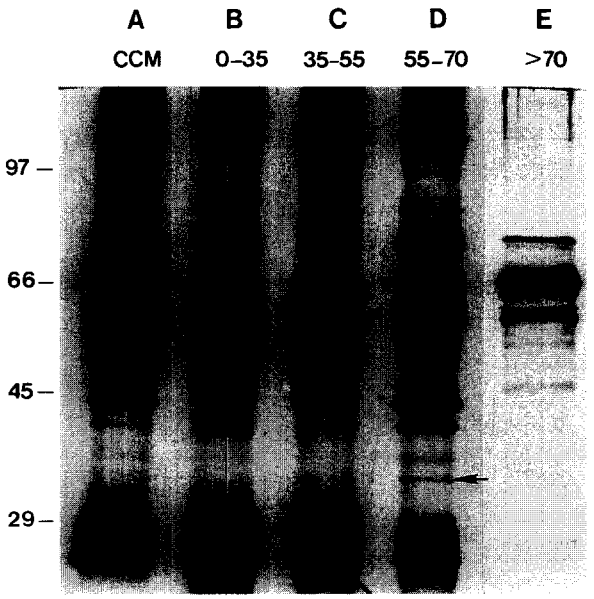

Fig. 4. SDS-PAGE analyses (10% acrylamide) of ammonium sulfate fractions (as percent of saturation) of conditioned HeLa S culture medium. Cells were grown as for Table 3. Each lane was loaded with 1 μg protein. Lane A: starting conditioned culture medium (CCM). Lanes B–E: fractions precipitated by ammonium sulfate over the percent of saturation ranges indicated.

Table 3
Ammonium sulfate precipitation of NADH oxidase activity of cell-free conditioned HeLa S cell culture medium and response of different fractions to 1 μM LY181984

Fraction	LY181984	nmol/min/100 μl
Cell-free conditioned culture medium	none	0.76 ± 0.07
	1 μM	0.50 ± 0.14
Percent of saturation of ammonium sulfate:		
0–30%	none	0.11 ± 0.02
	1 μM	0.05 ± 0.01
31–50%	none	0.22 ± 0.04
	1 μM	0.12 ± 0.01
51–70%	none	0.09 ± 0.02
	1 μM	0.05 ± 0.01
71–100%	none	0.22 ± 0.01
	1 μM	0.15 ± 0.02
Total recovered	none	0.64
	1 μM	0.36

Results are from 3 experiments ± S.D.
HeLa S cells were grown to a density of about $2.5 \cdot 10^6$ per ml and removed by centrifugation for 15 min at 2500 rpm.

Conditioned culture medium, both with and without serum, bound [^{3}H]LY181984. Analysis of binding with serum-containing medium was complicated by the albumin present from the donor horse serum which appeared to contribute significantly to sulfonylurea binding. However, with conditioned serum-free defined medium, high affinity binding was observed comparable to that of the HeLa cell plasma membrane [1] with a K_d of ca. 30 nM (Fig. 3B).

Because the NADH oxidase activity of the conditioned medium was relatively weak, ammonium sulfate precipitation was employed in an attempt to concentrate the active fraction. While ammonium sulfate did precipitate the bulk of the activity (ca. 90% recovery, Table 3), there was no obvious enrichment of the sulfonylurea-inhibited NADH oxidase activity in any one fraction (Table 3). However, the protein distribution pattern of bands, especially those migrating at or near the 34 kDa component tentatively associated with sulfonylurea binding activity [2], appeared to be most favorable for further fractionation with the 55 to 70% of saturation fraction (Lane D, Fig. 4). Therefore, since ammonium sulfate did concentrate the material without substantial losses in activity, further experiments were with protein fractions precipitated by either 0 to 70% (free-flow electrophoresis) or 55 to 70% (DEAE cellulose/G-200) of saturation of ammonium sulfate.

Because the putative 34 kDa sulfonylurea-binding protein of HeLa plasma membranes exhibited an apparent acidic isoelectric point, free-flow isoelectric focusing in the presence of 10 mM ammonium acetate, pH 5, containing 0.05% Triton X-100 to disrupt protein–protein interactions was used in initial attempts to purify the NADH oxidase from conditioned, serum-containing media and to determine the approximate molecular mass of the sulfonylurea-inhibited NADH oxidase activity. The bulk of the NADH oxidase activity separated well ahead of the bulk of the material absorbing at 280 nm at an isoelectric focusing

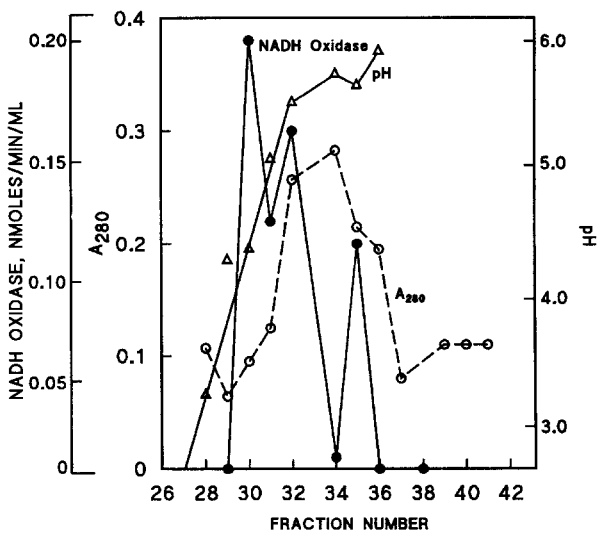

Fig. 5. Free-flow isoelectric focusing separation of a 0–70% ammonium sulfate precipitate of conditioned HeLa S culture medium. Cells were grown as for Table 3. The dotted lines give A_{280} and the open triangles give the pH. Solid symbols are NADH oxidase activity. The chamber buffer consisted of 0.5% Resolyte, pH 4 to 8 and 0.2% hydroxypropylmethyl-cellulose (HPMC). The anodal stabilization medium was 100 mM phosphoric acid plus 0.2% HPMC. The cathodal stabilization medium was 50 mM NaOH ± 0.2% HPMC. Electrophoresis was at 1300 V with a current of 13 MA and a transit time of 15 min using a VAP-22 free-flow electrophoresis instrument.

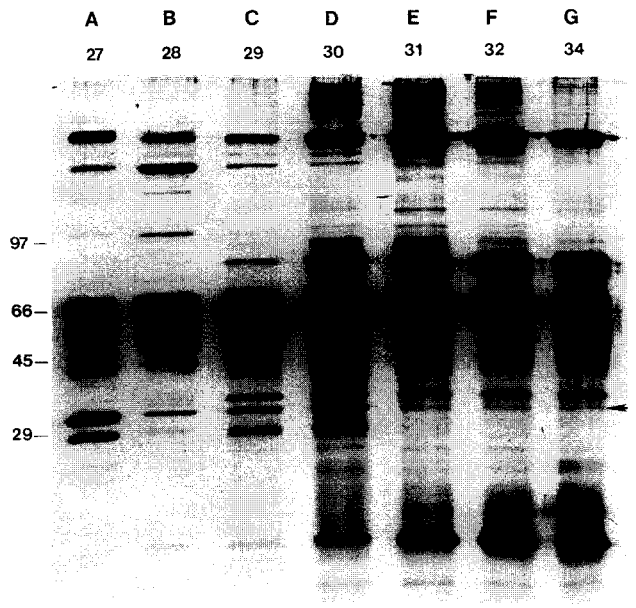

Fig. 6. SDS-PAGE (8% acrylamide) of free-flow electrophoresis fractions corresponding to the separation of Fig. 5. Each lane was loaded with about 1 μg of protein. Lanes A–G: free-flow electrophoresis fractions 27–34 of Fig. 5.

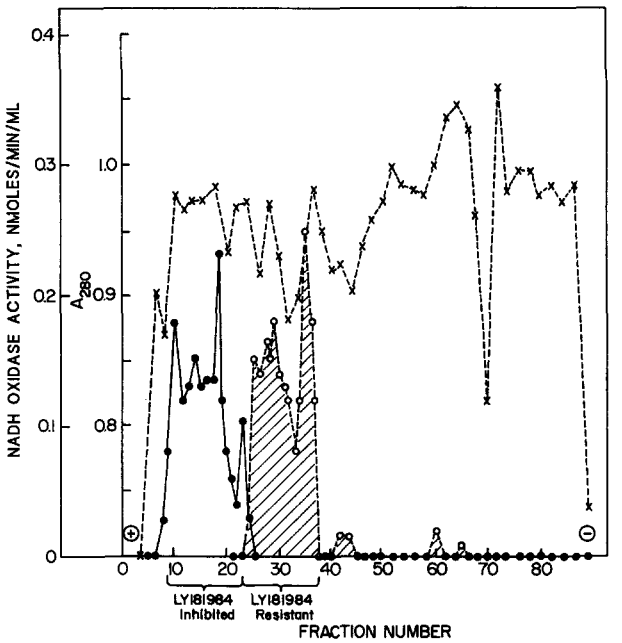

Fig. 7. Separation by zonal free-flow electrophoresis of two NADH oxidase isoforms from culture medium conditioned by growth of HeLa S cells. Cells were grown as for Table 3. The electrophoresis chamber buffer was 10 mM ammonium acetate containing 0.05% Triton X-100, pH 5. Electrophoresis was at 1300 V with a current of 180 mA using a VAP-22 continuous free-flow electrophoresis unit. The buffer flow was 1.7 ml/fraction/h. The sample injection was 2.7 ml/h. NADH oxidase activity with the greatest electrophoretic mobility (solid symbols, unshaded portion) was inhibited completely by sulfonylurea whereas the oxidase activity of lesser electrophoretic mobility (open symbols, shaded portion) was sulfonylurea-resistant. The dashed line with × symbols is A_{280}.

pH of about 4.5 in electrophoretic fraction 30 (Fig. 5). A second peak focused in fraction 32 corresponding to a pH of about 5.4. A third peak remained with the bulk protein in fraction 35. SDS-PAGE analysis revealed fraction 30 and 32 to be complex mixtures of proteins (Fig. 6) although a 34 kDa band (arrow) appeared to be somewhat more abundant in fraction 30.

Improved fractionation, again with conditioned, serum-containing media was achieved by zone electrophoresis in 1% Triton X-100 plus 10 mM ammonium chloride, pH 5. In this experiment, the NADH oxidase activity was present in two fractions of high electrophoretic mobility (Fig. 7). The activity in fractions 10 to 20 of greatest elec-

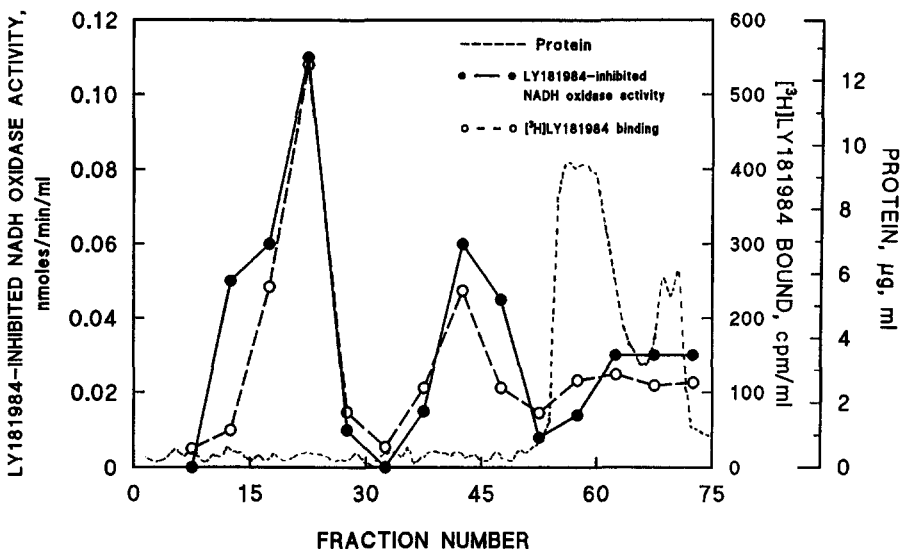

Fig. 8. Correlation between LY181984-inhibited NADH oxidase activity and [^{3}H]LY181984 binding of fractions prepared by zonal free-flow electrophoresis from defined media conditioned by growth of HeLa cells. The cells were grown as for Table 4. The conditions of electrophoresis were as described in Fig. 7.

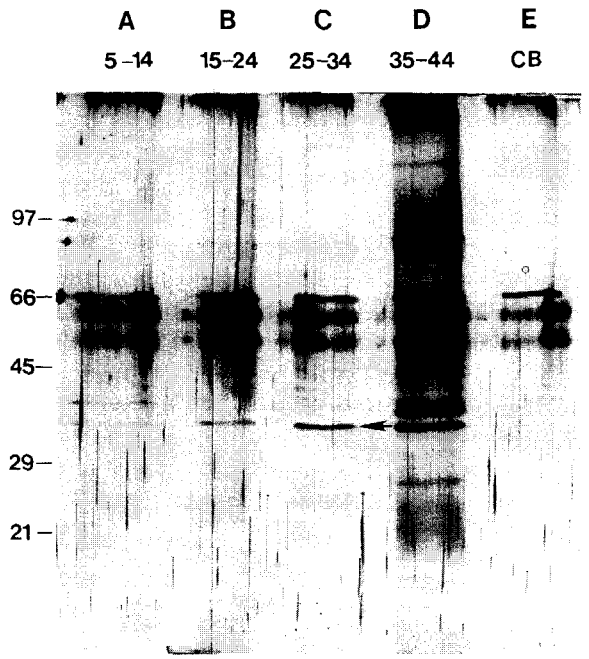

Fig. 9. SDS-PAGE analysis of electrophoresis fractions of Fig. 8. Lane A: pooled fractions 5–14. Lane B: pooled fractions 15–24. Lane C: pooled fractions 25–34. Lane D: pooled fractions 35–44. Lane E: chamber buffer. The bands in the 55 to 65 kDa range were introduced from the electrophoresis chamber buffer (B) (see text).

trophoretic mobility was > 90% inhibited by 1 μM LY181984 whereas the activity contained in fractions 25 to 35 was not inhibited.

The activity was subsequently purified by preparative free-flow electrophoresis to apparent homogeneity as a single polypeptide band from defined (serum-free), conditioned culture media. With defined, conditioned media, the NADH oxidase activity was more nearly completely inhibited by LY181984 and was convincingly separated from the bulk of the material absorbing at 280 nm (Fig. 8). The sulfonylurea-inhibited activity correlated closely with binding of [^{3}H]LY181984 giving two zones of activity, one was between fractions 15 and 24 and a second zone was between 35 and 55. The electrophoretic fractions 15 to 34 contained, after concentration, a single protein band when analyzed by silver-stained analytical PAGE (Fig. 9). Fractions 35–44 also contained a 33.5 kDa band although several other bands were evident as well. The components between 50 and 66 kDa were present in the electrophoretic chamber buffer (lane E marked CB) and were intensified during the 20-fold concentration necessary to visualize the small amount of 33.5 kDa material present in the electrophoretic fractions but absent from the electrophoretic chamber buffer.

In order to further characterize the apparent 33.5 kDa sulfonylurea-binding protein of the conditioned HeLa S culture medium, a Bio-Rad preparative SDS-PAGE column was employed. A 30 to 70% of saturation of ammonium sulfate precipitated fraction was prepared from approximately 800 ml of conditioned culture filtrate. The ammonium sulfate precipitate was resuspended in about 30 ml of Tris-HCl, pH 7.0, and applied to a DEAE cellulose column. Fractions eluting at different pH of elution with 50 mM Tris-Mes were collected and assayed for LY181984-inhibited NADH oxidase activity. The majority of the activity was concentrated in the fractions eluting at pH 5.5 and 6.0. The pH 6.0 eluate was concentrated to a final volume of approximately 1 ml and applied to a G-200 column. Fractions of 25 drops each were collected (1 s per drop) with elution using 50 mM Tris-Mes, pH 7.0, containing 0.02% sodium azide. The LY181984 fractions containing the oxidase activity were collected, combined, concentrated and further purified by preparative SDS-PAGE using a Bio-Rad preparative gel electrophoresis system. The collected fractions were analyzed by SDS-PAGE on 10% acrylamide and fraction 30 was found to contain a 33.5 kDa protein (Fig. 10).

When incubated under reducing conditions (1 μM GSH) in the presence of 0.15 mM NADH, the fraction 30 from the preparative SDS-PAGE column slowly regained NADH oxidase activity. This restored activity was inhibited by 1 μM LY181984 and was largely restricted to fractions 23 to 32 (Fig. 11A). The 33.5 kDa material also bound [^{3}H]LY181984 with high affinity during 12 h of equilibrium dialysis in the presence of 1 μM dithiothreitol (Fig. 11B).

The 33.5 kDa band corresponding to the material shown

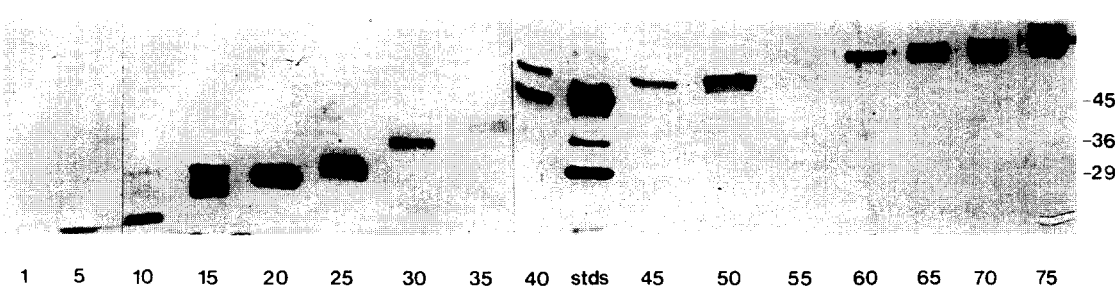

Fig. 10. Analytical SDS-PAGE (8%) of protein fractions separated by preparative SDS-PAGE using a Bio-Rad Preparative Gel Electrophoresis System. Every 5th fraction eluting from the dye front was analyzed up to fraction 75. A protein band with an apparent molecular mass of 33.5 kDa with antitumor sulfonylurea (LY181984)-inhibited NADH oxidase activity (Fig. 11A) and capable of binding [^{3}H]LY181984 (Fig. 11B) was found around fraction 30.

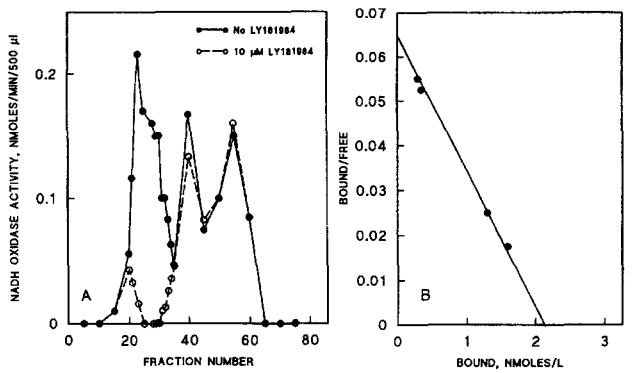

Fig. 11. Analysis of the fractions obtained from the preparative SDS-PAGE of Fig. 10. (A) NADH oxidase activity of 50 μl portions of the column eluate in the absence (solid symbols and line) or presence (open symbols and dotted lines) of 10 μM LY181984. (B) Scatchard analysis of binding of [^{3}H]LY181984 to the 33.5 kDa protein constituent that eluted between fractions 23 and 32. The K_d was estimated to be about 30 nM. The determinations were duplicated with a second separation with similar results. Activity was partially restored for the NADH oxidase by preincubation for 15 min in 0.15 mM NADH followed by incubation in 0.15 mM NADH + 1 μM freshly prepared reduced glutathione for 15 min at 37°C. The [^{3}H]LY181984 binding was in the presence of 1 μM dithiothreitol as described [1].

in Fig. 10 was excised from a preparative SDS-PAGE gel, macerated, combined with Freund's adjuvant and used as antigen to immunize a rabbit. After an appropriate boost with similarly-prepared material, sera was collected and tested for anti-NADH oxidase activity.

The rabbit sera prepared against the 33.5 kDa component of conditioned HeLa S culture medium with LY181984-inhibited NADH oxidase activity and capable of binding [^{3}H]LY181984 blocked the LY181984-inhibited NADH oxidase activity of conditioned, defined HeLa S culture media and of intact vesicles of HeLa S plasma membranes (Table 4A). In the presence of preimmune sera, normal activity remained that was inhibited by 1 μM LY181984. However, in the presence of antisera, activity was reduced to levels found in the presence of 1 μM LY181984 and the addition of 1 μM LY181984 was without effect. The antisera also immunoprecipitated the activity from both sources (Table 4B). Following immunoprecipitation, the supernatants were assayed for LY181984-inhibited NADH oxidase activity. Preimmune sera did not immunoprecipitate. Supernatants exhibited an active NADH oxidase that was LY181984-inhibited. In contrast with antisera, the supernatants lacked activity and addition of 1 μM LY181984 was without effect.

Western blots of 10% SDS-PAGE analyses of the immunoprecipitates revealed an immunoreactive band immunoprecipitated by the rabbit antisera that was absent from the immunoprecipitates with pre-immune antisera (Fig. 12).

4. Discussion

In a previous report, the sulfonylurea-inhibited NADH oxidase of the HeLa plasma membrane was described as being an ectoprotein [5]. The NADH site inhibited by sulfonylurea was demonstrated to respond only to the impermeant substrate, NADH, supplied to the external vesicle surface either to right side-out vesicles or to whole cells. With NADH supplied to inside-out vesicles, the sulfonylurea did not inhibit.

A characteristic feature of ectoproteins of the plasma membrane is that they often are shed from the cell surface into the surrounding medium. Included in this category are galactosyltransferases [6,7], sialyltransferases [8,9], 5′-nucleotidase [10] and dipeptidylpeptidase-IV [11,12].

In this report, we describe a soluble form of the sulfonylurea-inhibited NADH oxidase found in conditioned

Table 4
Antibody blocking and immunoprecipitation of LY181984 (1 μM)-inhibited NADH oxidase activity of conditioned, defined culture media of HeLa S cells and of HeLa S plasma membranes in response to polyclonal rabbit antisera prepared to the putative 33.5 kDa drug-responsive NADH oxidase protein

Sera	HeLa S conditioned, definedmedium (serum-free) (nmol/min/100 μl)		HeLa S plasma membranes (nmol/min/mg protein)	
	−LY181984	+LY181984	−LY181984	+LY181984
(A) Antibody blocking				
Preimmune	0.37 ± 0.02	0.12 ± 0.02	1.92 ± 0.12	0.26 ± 0.03
Rabbit anti 33.5 kDa NOX	0.03 ± 0.02	0.025 ± 0.01	0.26 ± 0.03	0.26 ± 0.10
(B) Immunoprecipitation				
Preimmune	0.3 ± 0.04	0.09 ± 0.02	3.5 ± 0.3	0.4 ± 0.15
Rabbit anti 33.5 kDa NOX	0.12 ± 0.02	0.12 ± 0.02	0.4 ± 0.2	0.4 ± 0.2

Results are from 3 experiments ± S.D.
HeLa S cells were grown to a density of about $2.5 \cdot 10^6$ per ml and removed by centrifugation for 15 min at 2500 rpm.
For the immunoprecipitation, NP-40 at a final concentration of 2% was added to 100 μl of conditioned, defined culture medium from HeLa cells or 100 μg HeLa S plasma membranes. Protein A Sepharose (PAS) was added (3.6 mg in 50 μl) and the mixture was incubated at 4°C for 4 h with shaking. The PAS was removed by centrifugation (Microfuge, 1 min) and 2.5 μl of antisera or preimmune sera were added and incubated overnight at 4°C with shaking. The PAS was collected by centrifugation and the supernatant assayed for NADH oxidase activity. The pellets were washed twice with 0.1% ND-40 and once each with PBS and water and analyzed by 10% PAGE and Western blot (Fig. 12).

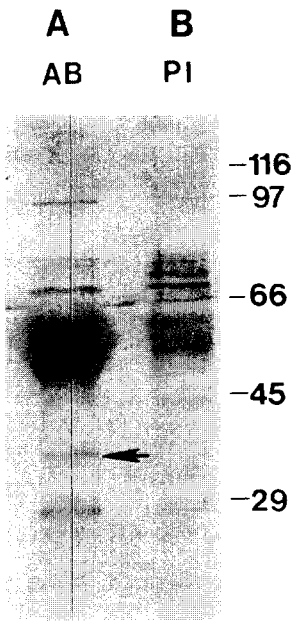

Fig. 12. Western blot of immunoprecipitates. Immunoprecipitates (Table 4B) from defined, conditioned HeLa S culture medium were separated on 10% SDS-PAGE and then transferred by electroblotting onto nitrocellulose. To block unspecific antibody binding sites, the blot was placed in a solution of 5% bovine serum albumin, 10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20 (TBS-T) for 15 min. The blocking solution and the blot were placed in the primary antibody solution (1:1000) overnight at 4°C with shaking. The blots were washed with TBS-T four times for 15 min each after which the blots were placed into secondary antibody solution (Goat anti-rabbit linked to alkaline phosphatase, Jackson ImmunoResearch Laboratories, West Grove, PA, 1:25000 in TBS-T) for 30 min at room temperature with shaking. The blot was washed with TBS-T three times for 15 min each and placed in a mixture of 0.33/mg/ml nitro blue tetrazolium and 0.16 mg/ml of 5-bromo-1-chloro-3-indolyl phosphate prepared in 100 mM Tris, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$ and incubated with shaking until the purple color of positive bands appeared. The color development reaction was stopped by placing the blots in 20 mM Tris, pH 8, containing 5 mM EDTA. Lane A: constituents immunoprecipitated by anti-33.5 kDa serum (Ab). B: constituents immunoprecipitated by preimmune serum (Pre). A band (arrow) at 33.5 kDa was present in the lane containing the immunoprecipitates with the anti-33.5 kDa serum (A) and was absent from the immunoprecipitates prepared with preimmune serum (B).

culture medium of HeLa cells with serum-containing media. The activity was inhibited by approximately 50% by the active antitumor sulfonylurea LY181984 but not by its inactive counterpart LY181985. The two molecules differ only by the presence in LY181984 of a chlorine on the B ring absent from LY181985 [15]. With conditioned, defined medium lacking serum, the activity also was present but now ca. 90% inhibited by 1 μM LY181984. The dose response was similar to that observed with HeLa plasma membranes [3] in that maximum inhibition was observed at about 1 μM LY181984 and the concentration necessary to inhibit 50% of the activity was about 30 nM. These findings are very similar to results obtained with mixtures of right side-out and inside-out vesicles or detergent-solubilized HeLa plasma membranes [5].

A second characteristic of the NADH oxidase activity of HeLa and hepatoma plasma membranes that distinguished it from the plasma membrane NADH oxidase activity of plasma membranes of rat liver, for example, was the response to thiol reagents [4]. Both PCMB and NEM inhibited about 50% of the activity of conditioned cultured medium grown with serum and more with defined, conditioned media.

The explanation given for the ability of the sulfonylurea to inhibit only 50% was based on the premise that the frozen and thawed or detergent-solubilized membrane preparations were a mixture of NADH oxidase activities resistant and susceptible to LY181984 inhibition [5]. A similar explanation can be applied to the findings from the present study. Free-flow separations revealed two zones of activity, one inhibited by sulfonylurea and one resistant to sulfonylurea.

The origin of both the antitumor sulfonylurea- and the thiol reagent-susceptible and -resistant serum-containing culture media is somewhat problematic. Both might be derived from the cells. One could come from the cells and the other from the donor horse serum, for example, that the NADH oxidase activities of conditioned defined medium was more nearly completely inhibited both by LY181984 and by the thiol reagents would support the interpretation of the LY181984-resistant component of the serum was derived from the donor horse serum and the LY181984-susceptible component came from the HeLa cells.

Both the drug-resistant and the drug-susceptible NADH oxidase activities appeared to exhibit a low isoelectric point of about 4.5 and 5.5 and an apparent molecular mass of between 33 and 36 kDa. A putative sulfonylurea binding protein of HeLa S plasma membranes was described previously as having a molecular weight of about 34 kDa based on LY181984 protection of *N*-ethylmaleimide (NEM) binding. The secreted form of the drug-sensitive NADH oxidase also showed a tendency to be protected against NEM inhibition by LY181984 but we have thus far been unable to devise conditions capable of immobilizing the binding protein in order to utilize this characteristic in a similar strategy to mark the secreted form of the putative binding protein.

The conditioned media, both with and without sera, did bind [^{3}H]LY181984 with high efficiency as did a protein constituent from the conditioned media with an average apparent molecular mass of about 33.5 kDa. The binding of [^{3}H]LY181984 to the latter was demonstrated after SDS-PAGE when incubated in the presence of dithiothreitol. Some sulfonylurea binding ability may be retained or restored to the denatured 33.5 kDa constituent under these conditions as reported previously for HeLa plasma membranes [1]. Additionally, some drug-responsive NADH oxidase activity could be restored following electrophoresis in SDS-PAGE by incubation with 1 μM GSH and 0.15 mM NADH to unequivocally associate both the sulfonylurea-binding protein and the drug-responsive NADH oxi-

dase activity with a similar, if not the same, 33.5 kDa protein constituent of the conditioned culture media. Polyclonal antisera raised to the 33.5 kDa constituent of the conditioned culture medium of HeLa cells cross-reacted with a 34 kDa protein constituent of the HeLa plasma membrane. These findings are interpreted as demonstrating that the 33.5 kDa constituent is a released form of the previously described 34 kDa antitumor sulfonylurea-(LY181984) binding constituent of the HeLa cell plasma membrane [2] and further suggest that the antitumor sulfonylurea-binding and sulfonylurea-inhibited NADH oxidase activity reside on the same native 34 kDa or shed 33.5 kDa protein.

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