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A cholesterol-binding and transporting protein from rat liver mitochondria

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Abstract

In this communication, we present results indicating a protein isolated from rat liver mitochondrial intermembrane space that is capable of binding cholesterol and transporting it between the inner and outer mitochondrial membranes. This protein has a molecular weight of 57.5 kDa by SDS-PAGE; however, under native conditions, there is cholesterol-binding capability only as a 115 kDa dimer. Our data show that this dimeric protein may play a role in the regulation of mitochondrial membrane cholesterol levels, a perquisite for the optimal activity of inner mitochondrial membrane-associated enzyme complexes. In addition, it appears that this protein is largely responsible for the differences in membrane cholesterol levels observed in normal and hepatoma mitochondria, a discrepancy which may help to explain the lack of energy production via oxidative phosporylation in malignant tumor mitochondria.

Keywords: Cholesterol-binding protein; Adenine nucleotide transporter; Mitochondrial membrane

1. Introduction

The enzyme complex responsible for ATP synthesis is located on the inner mitochondrial membrane [1]. A limiting factor in this process is the export of ATP from the mitochondrial matrix space in exchange for import of ADP from the intermembrane space. The transport of these highly charged molecules is carried out by the membrane-bound adenine nucleotide translocase in a 1:1 exchange of ADP and ATP [2]. In previous studies, it was found that in tumor cells, the ATP/ADP exchange mechanism malfunctioned in that ADP was exchanged not only with ATP, but also with ADP in a futile transport cycle [3]. This prevented efficient ATP efflux from the matrix into the cytosol. This can explain in part the lack of oxidative phosphorylation and dependence on higher aerobic glycolysis for energy production in certain tumor cells [4]. An Arrhenius plot of adenine nucleotide translocase activity in normal mitochondria and tumor mitochondria revealed a 4–5 °C difference in phase transition point. This discrepancy could be explained by a membrane composition difference, particularly due to cholesterol [5].

Cholesterol is a critical component of membranes that influences the fluidity of the lipid bilayer [6]. Specific levels of cholesterol and other lipids are required for optimal activity of membrane channels and transporters such as adenine nucleotide translocase [7]. This restriction of function to a particular membrane environment points to the necessity of a regulatory mechanism capable of sustaining cholesterol composition that is favorable for optimal enzyme performance. The importance of such a process for the mitochondrion is enhanced by its major role in the production of ATP for the rest of the cell. Since cholesterol is not produced in the mitochondrion [8], there must be a transport mechanism to; (a) deliver cholesterol to, and (b) maintain cholesterol composition of the inner membrane, where key oxidative phosphorylation enzymes are located. To determine the mechanism of this transport, mitochondria were fractionated into mitoplasts (containing intact inner membranes), outer membranes and intermembrane space proteins. When cholesterol transport was investigated, it was found that transport between the two membranes was greatly facilitated by intermembrane space constituents. In this paper, we identify a mitochondrial cholesterol-binding protein present in rat liver mitochondria. The molecular

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weight of 57.5 kDa and localization excludes this protein from other sterol binding proteins such as steroidogenic acute regulatory protein (StAR), a 37 kDa steroidogenic tissue mitochondrial protein that facilitates the delivery of cholesterol to cytochrome P450 on the inner membrane [9–11]; metastatic lymph node protein, MLN64 [12], a StAR homologue and late endosomal cholesterol-binding protein; SCP, an intracellular sterol carrier protein [13]; and other cholesterol-binding proteins [14,15]. The protein studied in this communication is capable of binding cholesterol in the 115 kDa molecular weight region, indicating a probable dimer. In addition, the cholesterol-binding function of this protein was further confirmed by the fact that in our isolation and purification procedures this protein was purified in the final step by the use of cholesterol affinity chromatography.

2. Materials and methods

2.1. Isolation of rat liver mitochondria

Male Sprague–Dawley rats were sacrificed and the livers were promptly excised, weighed and placed in a 5 × volume of homogenizing buffer (2 mM HEPES, 220 mM mannitol, 70 mM sucrose, and 1 mM EDTA at pH 7.4) at 4 °C [2,3]. The tissue was briefly blended and homogenized using a Dounce motorized homogenizer. The homogenate was centrifuged at a low speed (3000 × *g*) to separate the unbroken cells, red blood cells, cell membranes, nuclei, etc. The pellet was re-suspended in homogenizing medium and homogenized and centrifuged at 3000 × *g* two more times to quantitatively remove all mitochondria. The combined supernatants were centrifuged at 9000 × *g* for 15 min. The pellet was saved and washed with 5 volumes of homogenization buffer and centrifuged at 13,000 × *g* for 10 min. This pellet was again washed with homogenization buffer and centrifuged at 3000 × *g* for 5 min to remove contaminants as before. This supernatant was centrifuged at 13,000 × *g* for 20 min. The final pellet was re-suspended in 2–3 volumes of 0.25 M sucrose, pH 7.4. The isolated mitochondria were washed repeatedly after each step in order to obtain pure mitochondria without contaminants from other cellular components [2], which has been proven to be negligible by Hovius et al. [16]. Hepatoma mitochondria were similarly isolated as previously described [5].

2.2. Isolation of intermembrane space proteins

To avoid the disruption of the lipid composition of outer membranes by digitonin, subfractionation of mitochondria carried out by the swell–shrink–sonicate procedure of Hovius et al. [16]. Mitochondria were centrifuged at 13,000 × *g* for 15 min and the pellet was suspended in 2 volumes of an isotonic 0.25 M sucrose/EDTA/HEPES buffer. To this suspension, doubly distilled water was added to dilute the sucrose concentration to 0.15 M to swell the mitochondrial membranes. The mixture was mildly sonicated for 20 s (intervals of 6–7 s using a Bronson sonicator) to promote the breakage of the outer mitochondrial membrane. After sonication, 1.0 M sucrose was added to obtain a final concentration of 0.25 M sucrose. The suspension was then centrifuged at 13,000 × *g* for 15 min. The pellet contains the mitoplasts, while the supernatant has fragments of the outer membrane and the intermembrane space contents. The supernatant was centrifuged at 100,000 × *g* for 1 h. The supernatant from this spin contains the soluble intermembrane space proteins.

2.3. Purification with affinity column chromatography

Ammonium sulfate fractionation was performed on the intermembrane soluble proteins for the following saturations: 0–35%, 35–55%, and 55–75%. The 35–55% fraction was frozen, thawed and then re-fractionated to 35–55%. This new fraction was suspended in 20 mM sodium phosphate buffer (pH 7.4) and saved for separation on a gel filtration column. The partially purified protein was placed on an agarose (0.5 M, 200–400 mesh, Bio Rad) gel column (100 × 1.5 cm) and eluted with a 20 mM sodium phosphate buffer (pH 7.4) at 4 °C. Fractions were collected and measured for presence of protein by UV spectrophotometric analysis at 280 nm as well as the BCA method (Pierce) [17] for determining protein concentration. Peaks were plotted and molecular weights were estimated by calculating the average elution values of standard molecular weight proteins (BSA and cytochrome *c*).

Cholesterol affinity chromatography was used to further purify this protein. Cholesteryl-3-hemisuccinate (5-cholesten-3β-ol-3-hemisuccinate) (Sigma) was attached to free amino groups of 1,6-diaminohexyl (EAH) Sepharose 4B (Pharmacia) via the carbodiimide coupling method as described [18]. Twenty-five milliliters of EAH Sepharose 4B was added to 50 ml of 70% dioxane containing 2.43 g cholesteryl-3-hemisuccinate (10 mM), pH 4.5. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added to a final concentration of 0.1 M and the mixture was stirred overnight at 30 °C. The gel slurry was washed repeatedly to remove excess ligand; with 250 ml 1,4 dioxane, 500 ml 80% methanol, 500 ml distilled water. The gel was packed in binding buffer (20 mM sodium phosphate, pH 7.4). Protein fractions from gel filtration were added to the column and first washed with the binding buffer for approximately 25–30 ml until no discernable protein was detected by UV 280 nm. Due to the insolubility of cholesterol in aqueous solution, a competitive elution of proteins with free ligand was not feasible. To overcome this, a change in ionic strength was used to elute proteins retained on the column [19]. A linear gradient of 0–500 mM potassium chloride was applied to elute cholesterol-binding proteins as described in the manner of Kuwada et al. [19] for purification of cytochromes P-450$_{SSC}$. Protein eluted in the region of

100–150 mM KCl which was confirmed by examining fractions for OD 280 nm.

2.4. Binding of [^{3}H] cholesterol and sucrose density gradient

[^{3}H] cholesterol (NENuclear, 47.9 Ci/mmol) was dissolved in chloroform and air-dried in a glass test tube. The gel filtration fraction corresponding to the largest molecular weight peak (115 kDa) was added and the mixture was incubated overnight at 4 °C. This sample was then placed on a sucrose density gradient in a centrifuge tube (a linear gradient from 5% to 25% sucrose in 20 mM sodium phosphate buffer). A standard sample of 5 mg/ml BSA and 2 mg/ml cytochrome *c* was added to another identical tube for direct comparison. The tubes were centrifuged at 120,000 × *g* for 22 h. 0.5 ml fractions were collected starting from the bottom of the centrifuge tube and 0.1 ml aliquots were added to scintillation fluid (Liquiscent, National Diagnostics) for detection of radioactivity by scintillation counting. The absorbance at 280 nm was determined in the fractions to detect the presence of protein and the samples were run on SDS-PAGE to determine the molecular weight and purity of the protein.

2.5. Cholesterol transport assays

Cholesterol transport between mitochondrial membranes was monitored in the following manner: the three compartments on the mitochondrion (mitoplast, outer membrane, and intermembrane space protein) were isolated by differential centrifugation as stated previously. The assays were performed with the mitoplast and outer membranes kept at the same amount of protein (~ 200 μg) as well as varying amounts of membrane protein concentration up to 5-fold more inner membrane to that of outer membrane. The former ratio containing significantly more outer membrane/inner membrane, and the latter ratio resembling a more physiological relationship. The two membrane fractions were separated by differential centrifugation and suspended in 5 volumes of 0.25 M sucrose/EDTA/HEPES. The tubes were centrifuged at 3000 × *g* for 5 min to wash the membranes. [^{3}H] Cholesterol was added to the mitoplast fraction and incubated at 4 °C for 1 h. This is centrifuged at 3000 × *g* to remove all unbound cholesterol and the pellet is resuspended. For experiments examining the effect of protein concentration on cholesterol transfer, labeled mitoplasts were incubated with outer membranes and the intermembrane space proteins ranging from 0 to 240 μg for 40 min. For experiments on the effect of time; the labeled mitoplasts were incubated with outer membranes and 120 μg of the intermembrane space protein for times ranging from 0 to 60 min. The tubes were then centrifuged at 3000 × *g* again and the pellet (mitoplast) is removed. The supernatant, consisting of the intermembrane space proteins and the outer membrane is centrifuged by a Beckman Airfuge (85,000 rpm) for 30 s. The pellet from the high-speed spin contains the outer membrane. The radioactivity present in the outer membrane fraction shows the magnitude of cholesterol transfer from the inner membrane to the outer membrane dependent on time or amount of intermembrane space protein. This assay was also similarly performed by initially labeling the outer membrane to monitor transfer from the outer to inner membrane. To determine the efficiency of the membrane separation and purity of the two membranes, the activities of two specific enzymes, monoamine oxidase (MAO) from the outer membrane, and cytochrome oxidase from the inner membrane were measured in each membrane fraction. Briefly, the oxidative deamination of ^{14}C-phenylethylamine was monitored to determine the level of MAO activity present in the inner membrane fractions after the transport assay was performed [20]. Any activity present in this inner membrane fraction was compared with that of the outer membrane fraction as a gauge for determining the level of contamination of the mitoplasts by the outer membranes. Conversely, cytochrome oxidase activity [21] was measured in the outer membrane fraction and compared to that of the inner membrane fraction to determine the extent of inner membrane contamination during the transport assay. From these determinations, the membrane cross-contamination was generally in the range of 3.97–5.66% of outer membrane in the inner membrane fraction and up to 6.2–8.32% of inner membrane in the outer membrane fraction.

2.6. Electrophoretic analysis

Fractions from the gel filtration column and sucrose density gradient run were examined on denaturing and non-denaturing polyacrylamide gel systems. A 12% SDS polyacrylamide gel was prepared by the method of Laemmli [22]. To visualize the functional native form of the isolated protein, a non-denaturing, native, electrophoresis procedure [23,24] was used as well. Native PAGE gels were made at varying percentages of acrylamide from 4% to 10%. The upper chamber buffer used was 42.6 mM Tris and 46.4 mM glycine at pH 8.4. The lower chamber buffer was 239 mM Tris at pH 8.1. All native gels were run at 60 V for several hours according to percentage of acrylamide in a 4 °C room to maintain the native form of the proteins.

To permit visualizing protein dimers on a native gel as monomers on a denaturing SDS gel, two-dimensional gel electrophoresis was carried out by excising a single gel lane from a native gel and placing it horizontally along the stacking gel of a 12% SDS-PAGE gel. Gels were stained with Coomassie Brilliant Blue and Bio Rad Silver stained according to protocol (Bio Rad).

2.7. Colorimetric determination

Cholesterol content of membranes was expressed in terms of micrograms of cholesterol per milligram of protein.

Total amounts of cholesterol were determined by the method of Zlatkis and Zak [25]. Lipids were extracted from inner and outer membrane fractions by addition of 0.5 ml methanol and 0.5 ml chloroform to a sample volume of 0.2–0.3 ml followed by vortexing for 1 min. One milliliter of distilled water was added followed by vortexing for 2 min. The mixture is centrifuged in a tabletop centrifuge at maximum speed for 5 min and the aqueous layer is removed. The chloroform layer is separated into 0.2 and 0.3 ml fractions and dried under N_2 gas. The dried lipids were dissolved in 0.1 ml glacial acetic acid and 2 ml of the cholesterol reagent *o*-phthaldialdehyde (0.5 mg/ml acetic acid) was added. After mixing well, 1 ml concentrated sulfuric acid was added and the tubes were vortexed and incubated at room temperature for 10 min. The OD 550 was recorded and compared to a standard curve of 10–100 μg cholesterol. Protein concentration was determined by the BCA method [17].

3. Results

3.1. Cholesterol contents

To investigate the differences in ATP translocation between normal and tumor mitochondria, the membrane cholesterol composition of mitochondria from both normal and tumor cells was compared. There were generally higher levels of total cholesterol (μg cholesterol/mg protein) in tumor mitochondria (Morris hepatomas 7800, 16, 5123D, and 7777) than in normal mitochondria (Table 1). Moreover, the elevated levels of cholesterol seem to be correlated to the degree of malignancy of the tumor in question. The most malignant tumors, Morris hepatoma 7777, have nearly 5-fold more cholesterol than normal mitochondria whereas less malignant tumors, such as Morris hepatomas 16 and 7800, only show an increase of approximately 2-fold. Intermediate growth rate tumors (Morris hepatoma 5123D)

Table1
Morris hepatoma mitochondrial cholesterol contents (μg cholesterol/mg protein)

	Whole mitochondria	Outer membrane	Inner membrane
Liver	8.74 ± 1.0	36.6 ± 4.3	10.1 ± 2.6
Hepatoma 16	12.84 ± 4.0	37.4 ± 2.4	18.8 ± 3.8
Hepatoma 7800	17.5 ± 1.5	27.5	17.25
Hepatoma 5123D	25.5 ± 5.2	ND	ND
Hepatoma 7777	43.1 ± 9.5	43.0	28.75

Cholesterol content in membranes of normal (liver) and various Morris hepatoma cell lines expressed in micrograms of cholesterol per milligram of protein. Tumor cell lines are listed in order of increasing malignancy (hepatoma 16 is the slowest growing cell line, hepatomas 5123D and 7800 are intermediate-growing cell lines, and hepatoma 7777 is the most malignant cell line). Results are averages (± standard errors) from four determinations for normal liver mitochondria, and duplicates for hepatoma mitochondria. (ND, not determined).

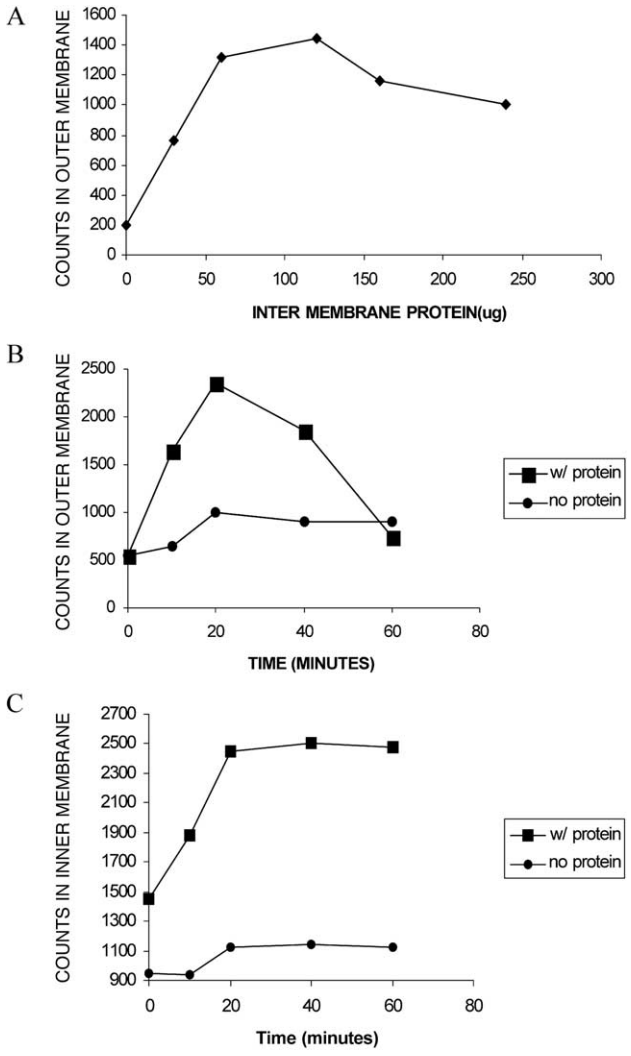

Fig. 1. Cholesterol transport between the inner and outer membranes in the presence of intermembrane space protein. (A) The cholesterol transport is enhanced by the intermembrane space proteins. There is a linear dependence of transport on the amount of protein presence from 0 to 60 μg protein. As the protein is increased further, the slope decreases and becomes negative. This may indicate that at certain levels of protein, cholesterol is removed from the outer membrane. This assay was done over the course of 40 min. (B) Cholesterol transport is dependent on inter-membrane space proteins and time. Cholesterol accumulation in the outer membrane fractions is dependent on protein (■), while there is little difference in transport where no protein is present (●). In addition, as time increases there appears to be removal of cholesterol from the outer membrane in samples with protein. Samples without protein show little difference in cholesterol binding over time. (C) Cholesterol transfer from the outer membrane to the inner membrane is protein- and time-dependent (■). From 0 to 20 min, cholesterol delivery to the inner membrane increases at a near linear rate, but becomes stagnant as the incubation time increases (20–60 min). There is no change in the rate of transfer when no protein is present (●). The quality of separation of membranes was confirmed by measuring the activities of marker enzymes MAO, from the outer membrane, and cytochrome oxidase, from the inner membrane, in both the inner and outer membrane fractions. It was found that the outer membrane contamination to the inner membrane fraction, as determined by MAO activity, was in the range of 3.97–5.66%. Values of 6.2–8.32% were obtained for the contamination of inner membranes to the outer membrane fraction, judged by cytochrome oxidase activity.

have approximately 3-fold higher levels than normal mitochondria. When the membranes were fractionated, it was determined that cholesterol levels per mg membrane protein in the inner membrane fraction increased in tumor membranes, where they exhibited as much as a 3-fold increase over normal inner membranes (Table 1). This is of importance to the potential function of membrane-associated enzymes related to oxidative phosphorylation which are located on the mitochondrial inner membrane. Again, this increase appears to be directly proportional to the relative malignancy of tumor cell lines examined, with the most malignant tumor, hepatoma 7777, containing approximately 1.5-fold more cholesterol than slow-growing tumor cell line inner membranes and 3-fold more cholesterol than in normal mitochondrial inner membranes. Results from outer membranes show only slight increases in tumor mitochondria. These data can support the previously reported differences in ATP/ADP translocation between normal and tumor mitochondria [3] and lead to the pursuit of a mechanism whereby membrane cholesterol composition can be maintained or altered.

3.2. Cholesterol transport between mitochondrial membranes is enhanced by an intermembrane space protein

Cholesterol transport from the inner membrane to the outer membrane was examined as a function of time and amount of protein (Fig. 1A and B). There was a low level of transport observed when no protein was added to the assay, which can be attributed to contact sites formed between the two membranes as a result of the in vitro assay. At small amounts of protein (0–60 μg) cholesterol transport to the outer membrane was linearly dependent on the protein concentration and increased by 7-fold over baseline levels (Fig. 1A). Cholesterol transport leveled and decreased slightly as the intermembrane space protein increased (60–240 μg). This protein-dependent transport mechanism is capable of relocating cholesterol to and, with higher amounts of protein, away from the outer membrane. These results cannot be explained by membrane contamination during the transport assay because only a small percentage of contamination was observed as indicated (Section 2.5). Transfer was also measured as a function of incubation time (Fig. 1B).

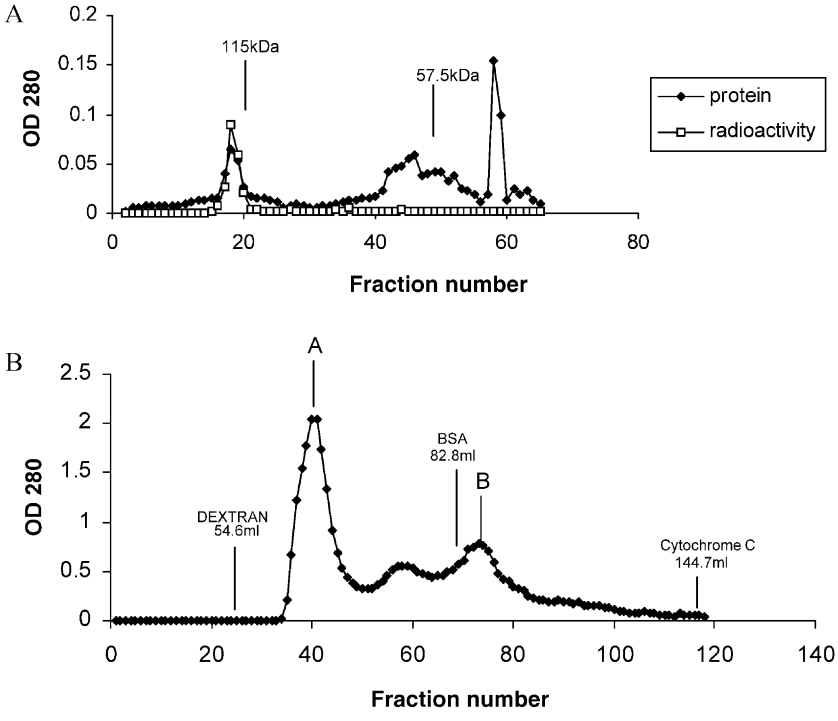

Fig. 2. Identification of a cholesterol-binding protein (A) The 35–55% protein fractions were incubated with [^{3}H] cholesterol and partially purified by gel filtration with Bio gel A-0.5 m 200–400 mesh beads (Bio Rad). Proteins were eluted from the column with 20 mM sodium phosphate buffer (pH 7.4). Fractions from the column were tested for radioactivity (the presence of cholesterol) by scintillation counting and protein by measuring the absorbance at 280 nm. It was found that there was a single radioactive peak (Fraction 18 contained 4.500 counts) which corresponded with a protein peak of approximately 115 kDa. There was only one radioactive peak, indicating that the cholesterol-binding protein was in this 115 kDa fraction. (B) In an effort to purify this protein, gel filtration was repeated in the absence of radioactive cholesterol. Protein was isolated from the intermembrane space of rat liver mitochondria by ammonium sulfate fractionation. The 35–55% fraction was eluted from the gel filtration column with 20 mM sodium phosphate buffer. A duplicate standard run was carried out with Dextran (~ 10^6 kDa), BSA (66 kDa), cytochrome *c* (12.5 kDa), and DNP Alanine (255 Da). Elution volumes for these standard proteins are shown. The average elution volumes were calculated and used to determine the molecular weights of the major peaks. Peak A was determined to be ~ 117 kDa, while peak B was determined to be ~ 58 kDa. Further experiments including affinity chromatography, sucrose density gradient, and two-dimensional electrophoresis were performed using protein from gel filtration peak A.

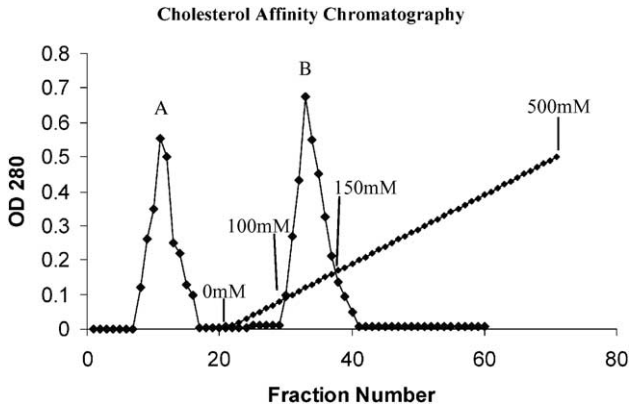

Fig. 3. Cholesterol affinity chromatography. Protein from the major peak of the gel filtration run (Peak A, Fig. 2) was applied to a cholesterol affinity chromatography column. The cholesterol-binding protein was retained on the matrix and all other non-binding proteins were eluted with 20 mM sodium phosphate buffer (pH 7.4) for approximately 30 ml until no significant protein could be detected by the measurement of OD at 280 nm. Once all contaminating proteins had been eluted (Peak A), the cholesterol-binding protein was desorbed from the column by the addition of a linear gradient of 0–500 mM KCl prepared in 20 mM sodium phosphate buffer. The gradient yielded a single protein peak which eluted from approximately 100–150 mM KCl (Peak B). There were no additional peaks observed, which indicates a clean separation and purification of this cholesterol-binding protein.

There was little difference in the amount of cholesterol detected over time (0–60 min) when no protein was added to the assay. After 120 μg protein was added the cholesterol transport increased linearly over the period of 0–20 min exhibiting a 5-fold increase but decreased to near baseline levels as the incubation time increased from 20 to 60 min. These data point to dynamic protein-dependent mechanism by which cholesterol can be transported to the outer membrane and removed from it. Transfer from the outer membrane to the mitoplast was measured as well (Fig. 1C). Although similar to Fig. 1B, cholesterol transfer to the inner membrane was immediate, and increased at a near linear rate for the first 20 min of incubation time with the cholesterol-binding protein. However, as this incubation time increased from 20 to 60 min, there was little difference and transport remained at a plateau. These results indicate the transport of cholesterol mediated by this protein proceeds in both directions and the extent is condition-dependent. The information

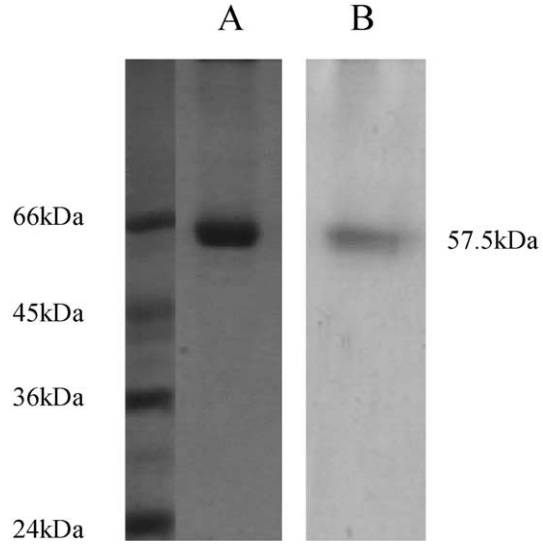

Fig. 4. SDS-PAGE of cholesterol-binding protein. The 115 kDa cholesterol binding fraction from gel filtration was examined on a denaturing electrophoresis system (lane A). The partially purified protein existed as a 57.5 kDa band on SDS gels, indicating the presence of a native dimer form. Cholesterol affinity chromatography was performed to further purify this protein (lane B). Affinity chromatography yielded a single 57.5 kDa band providing a means of purifying this protein to homogeneity.

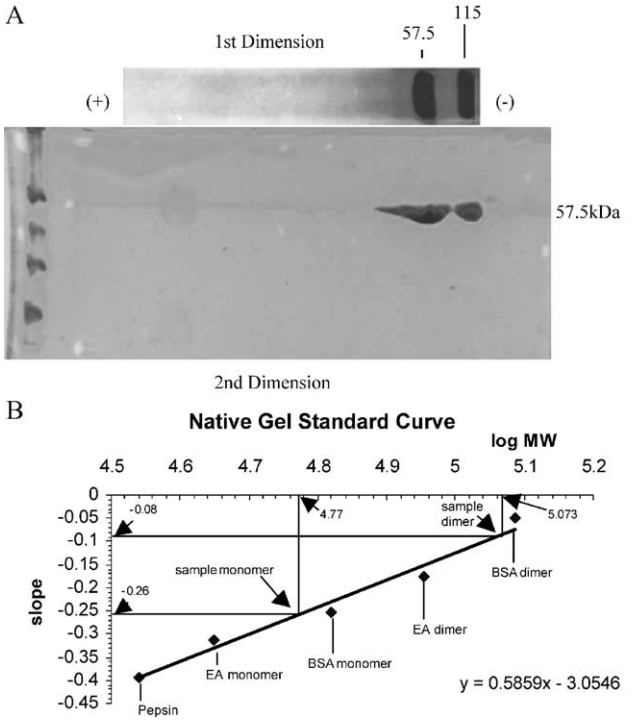

Fig. 5. Two-dimensional electrophoresis. (A) The top lane (first dimension) is cut from a native non-denaturing gel run of the peak (115 kDa) fraction from gel filtration. The two major bands in this lane are the 115 kDa dimer and the 57.5 kDa monomer of the protein. This lane was placed on a SDS-PAGE gel and run under denaturing conditions with a standard protein marker. In the denaturing system, both bands migrate to the same position at 57.5 kDa. This provides evidence that the 115 kDa cholesterol-binding species from gel filtration is in fact a dimer of a 57.5 kDa protein. The native lane is from a 7% acrylamide non-denaturing run. (B) Native gel standard curve. The native gels were run at varying acrylamide percentages (from 5% to 10%). Migration distances of standard proteins BSA (132, 66 kDa), egg albumin, EA, (90, 45 kDa), and pepsin (34.7 kDa) were taken for each gel. The cm/h of each protein species from the different gels (5–10%) was calculated and plotted against the percentage of acrylamide (not shown) to determine the slope (cm/h/% acrylamide) of each protein. This value was plotted against the log of the molecular weight of the known proteins. It was calculated that the cm/h/% acrylamide for the sample protein was -0.260. This value was used to calculate the log MW from the line of best fit, $y = 0.5859x - 3.0546$, to find the molecular weight. The log Mw of the monomer is 4.77, which gives a molecular weight of 58.9 kDa. The cm/h/% of the dimer species is -0.080, which corresponds to a molecular weight of 118.3 kDa.

presented in Fig. 1 shows cholesterol transfer when the membrane proteins of both the inner and outer membranes were kept equal (~ 200 μg). When this ratio was varied up to 1:5 (outer membrane/inner membrane), which more closely resembles in vivo conditions, protein-dependent cholesterol transport was also evident and similar.

3.3. Identification of a cholesterol-binding protein

The intermembrane space contents of mitochondria from male Sprague–Dawley rats were isolated in an effort to identify a cholesterol-binding protein. From 1 g of liver, we isolated 7.3 ± 0.9 mg protein from whole mitochondria, 1.92 ± 0.08 mg protein from the crude intermembrane space fraction, 0.74 ± 0.05 mg protein from the 35% to 55% ammonium sulfate fraction, and 0.160 ± 0.014 mg of the cholesterol-binding protein. To identify the cholesterol-binding protein, proteins were isolated by ammonium sulfate fractionation and incubated with [^{3}H] cholesterol and applied to a gel filtration column. When the fractions were tested for radioactivity, the 35–55% ammonium sulfate fraction of the intermembrane space proteins produced a protein peak that corresponded to the single radioactive peak (Fig. 2A). The size of this protein was estimated to be 115 kDa by running known molecular weight standards (BSA and cytochrome *c*). No other protein peaks in the elution profile correlated with a radioactive peak, which indicates that there was a single 115 kDa species in the intermembrane space that could bind cholesterol. In an effort to purify this protein, the gel filtration run was repeated and the 115 kDa peak (Fig. 2B) was saved for further purification and characterization.

To further purify the cholesterol-binding protein, fractions corresponding to the 115 kDa peak from gel filtration were applied to a cholesterol affinity chromatography column. Any contaminating proteins were eluted with 20 mM sodium phosphate buffer and the remaining proteins bound to the gel were desorbed from the column by a linear 0–500 mM KCl gradient. The elution profile shows a single peak eluted between 100 and 150 mM KCl corresponding to the purified cholesterol-binding protein (Fig. 3). The fact that it was possible to elute this protein with increasing salt concentration without the addition of a strong denaturant, or a chaotropic reagent, indicates that this protein binds cholesterol via non-covalent interactions.

3.4. Analysis of cholesterol-binding protein by SDS, native, and two-dimensional electrophoresis

The fraction from gel filtration corresponding to the radioactive peak was examined on a SDS-PAGE gel (Fig. 4, lane A). This fraction determined to be 115 kDa by gel filtration appeared as a 57.5 kDa band on the denaturing gel. SDS gels of the 100–150 mM KCl peak eluted from cholesterol affinity chromatography yielded a single 57.5 kDa band showing the successful purification of a cholesterol-binding protein (Fig. 4, lane B). In addition, the data showing a single 57.5 kDa band present on denaturing gels revealed the possibility that this protein may be a functional dimer. To investigate this further, a two-dimensional gel system using a non-denaturing gel method and SDS-PAGE was used. The protein appeared as a doublet on the native gels with molecular weights 115 and 57 kDa (Fig. 5A, top). The lane was excised from the native gel and carefully

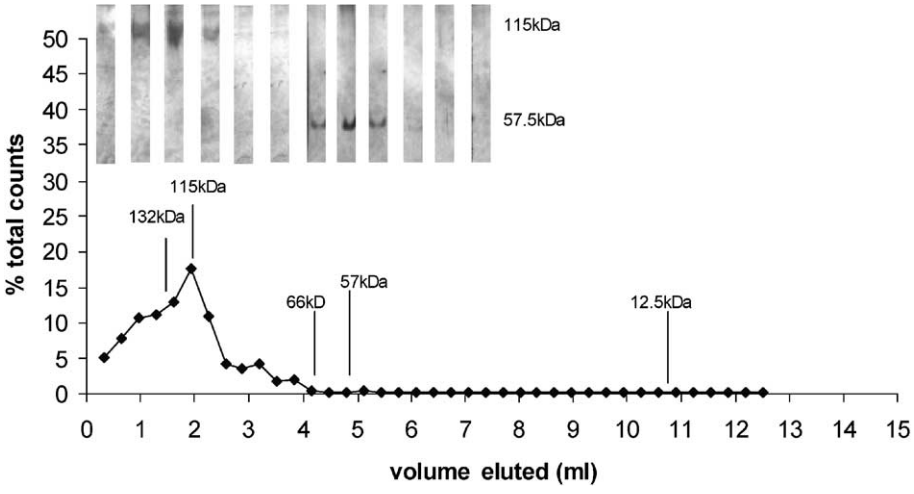

Fig. 6. Radioactive cholesterol binding assay. [^{3}H] cholesterol was added to the peak sample from gel filtration and placed on a 5–25% sucrose density gradient. The tube was centrifuged at 200,000 × *g* for 22 h and the fractions were collected starting from the bottom. Each fraction was measured for radioactivity using a liquid scintillation counter. For comparison, a standard tube was prepared with BSA (132 kDa dimer, 66 kDa monomer) and cytochrome *c* (12.5 kDa) under identical conditions as the sample tube. Points on the graph represent the location of different proteins in the sucrose elution profile. The radioactive peak corresponds to the molecular weight of the cholesterol-binding protein dimer (roughly 120 kDa). There is a lack of cholesterol binding in the 50–60 kDa region, corresponding to the monomer species of the cholesterol-binding protein. This particular protein can only bind cholesterol when present as a high molecular weight dimer.

placed on a SDS gel. Both the high and low molecular weight bands from the native gel appeared as a single 57.5 kDa band on the SDS gel (Fig. 5B, bottom). This proves that this cholesterol-binding protein exists as 115 kDa dimer under native conditions.

3.5. Cholesterol-binding in a high molecular weight region

To further emphasize the nature of cholesterol binding, sucrose density gradient separation was used. The protein peak from gel filtration was incubated with [^{3}H] cholesterol and placed on a 5–25% sucrose density gradient. After centrifugation, the fractions were collected, counted for radioactivity, and examined on SDS gels. The elution profile of cholesterol counts showed a single radioactive peak that corresponds to a molecular weight of ~ 120 kDa (Fig. 6, bottom). PAGE gels of the fractions show a 115 kDa protein band corresponding to this radioactive peak (Fig. 6, top). There is no radioactivity in fractions corresponding to the 57.5 kDa bands. This confirms that cholesterol binding is limited to the high molecular weight dimer (115 kDa) rendering the monomer non-functional.

4. Discussion

We have identified a 115 kDa protein in the intermembrane space of rat liver mitochondria which is capable of binding cholesterol and facilitating the transport of cholesterol between the inner and outer membranes. The purification of this protein by cholesterol affinity chromatography confirms the cholesterol-binding function. We have shown that this protein, with a molecular weight of 57.5 kDa on SDS gels, must be present as a dimer in order for cholesterol binding to occur; there is no apparent cholesterol binding to the monomer.

Many years ago, it was shown that many malignant tissues rely more heavily on glycolysis for production of energy, creating an energy drain on surrounding healthy tissues [26,27]. A contributing factor to this is the function of the mitochondrial ATP/ADP exchanger, the adenine nucleotide translocase. Normal mitochondria exchange adenine nucleotides across the inner mitochondrial membrane in a 1:1 process (one ATP for one ADP) [2]. However, in tumor mitochondria, there is a lack of normal exchange, ATP is not exclusively and efficiently removed from the matrix space, and hence cannot be transported to the rest of the cell [3]. When an Arrhenius plot was constructed for adenine nucleotide transport activity a breakpoint difference of 4–5 °C in tumor mitochondria was observed [3]. This is due to the different lipid compositions of the tumor mitochondrial membranes, particularly an elevated level of cholesterol [5]. To investigate this phenomena, the membrane cholesterol composition of mitochondria from normal cells as well as several tumor cell lines was compared. This communication and others [5,28] show generally higher levels of cholesterol (μg per mg protein) in tumor mitochondria than that of normal mitochondria. The abnormally high levels of cholesterol present in the inner membranes of these tumor cell mitochondria will have a profound affect on the function of membrane-associated enzyme complexes and therefore the energy-producing capacity of mitochondria. This is primarily due to the fact that enzymes integral to the oxidative phosphorylation process such as adenine nucleotide translocase require an optimal level of membrane cholesterol to ensure efficient enzyme activity [7]. When mitochondria are enriched with excess cholesterol in vitro [29], cholesterol levels in normal mitochondria are greatly increased to resemble those of tumor cell mitochondria [5,28]. However, when given oxidizable substrates during incubation, cholesterol was gradually depleted from the inner membrane and transported back to the outer membrane (unpublished results). In contrast, when tumor mitochondria are cholesterol-enriched, cholesterol remains in the inner membrane at high levels. This information and the compartmentalization of cholesterol in the cell lead to the necessity of a regulatory mechanism responsible for maintaining the correct cholesterol composition in the inner mitochondrial membrane.

We have identified a protein from the intermembrane space that when in its native dimer form possesses cholesterol-binding ability and can facilitate cholesterol transfer between the inner and outer mitochondrial membranes. The data presented here indicate that this is a fluid transfer in that cholesterol can be transported from the inner membrane to the outer membrane and, over time, excess amounts can be removed from it. Similarly, when the movement from the outer membrane (OM) to inner membrane (IM) was monitored, cholesterol was transferred to the inner membrane at a relatively rapid rate, and over time, the level of cholesterol remains high. The difference in transfer between both directions (IM to OM, and OM to IM) can be explained in part by the knowledge that the rate of cholesterol transfer is dependent more on the nature of the acceptor membrane rather than the removal of cholesterol from the donor membrane [30]. The inherent dissimilarities between the inner and outer mitochondrial membranes may affect this rate, explaining the slightly different results presented here for each directions of transport. In addition to the biochemical uniqueness of each membrane, there are significant differences in the amount of each membrane present in intact mitochondria. This discrepancy does not have a major effect as cholesterol transfer was observed when the ratio of membrane proteins was kept equal (1:1, outer membrane/inner membrane) and varied to a more in vivo ratio (1:5, outer membrane/inner membrane). Transfer was evident in each case (data not shown). In addition, the dynamic nature of this transfer mechanism appears to support the theory of regulating membrane cholesterol for optimal enzyme function.

Other mitochondrial cholesterol-binding proteins have been identified, such as the peripheral benzodiazepine receptor, PBR [31] and StAR [11]. PBR, an 18 kDa outer

mitochondrial membrane protein, is expressed in most tissues but primarily in those involved in steroid production and has been proven to be most important to the transportation of cholesterol in steroidogenic mitochondria [15] and linked to the mechanism of proliferation of various cancer cell types [32]. PBR has also been shown to exist in elevated levels in liver tumors [33], a fact that, when included with the results of this communication, add to the role of cholesterol transport in the altered cellular function of tumor cells. StAR has been proven to be necessary for delivery of cholesterol to the cholesterol side-chain cleavage complex on the inner mitochondrial membrane during hormone synthesis in steroidogenic tissues [34]. Unconfirmed models of the mechanism of cholesterol transfer include: a cholesterol desorption model in which the outer membrane-associated protein can facilitate transfer of cholesterol to the inner membrane, and a shuttle hypothesis which involves the repeated protein-bound cholesterol transfer from the outer membrane to the target enzyme complex on the inner membrane [35]. This latter model has been shown to be unlikely for StAR due to the fact that truncated forms of the protein (without a mitochondrial targeting presequence) still result in increased steroidogenesis [8] as well as the observation that the imported mature StAR protein is not capable of transporting cholesterol [36]. Although the molecular weight, localization, and preliminary N-terminal sequence data (unpublished results) exclude this protein from other known cholesterol-binding proteins; including SCP-x [37], StAR [9], PBR [15], MLN64 [12], SCP [13], and NCP1 [14], these findings can provide perspectives about the mechanism of action of the protein presented in this communication. The localization of this protein in the intermembrane space of mitochondria leads to the possibility of a carrier model where the protein can shuttle cholesterol from one membrane to the other. It is known, however, that there are membrane contact sites between the two mitochondrial membranes [38]. This protein may bind cholesterol and provide a means to augment these contact sites permitting the transfer of cholesterol between membranes. An important observation on this protein is the fact that only a dimerized form is capable for cholesterol binding, a process which may be itself ATP-dependent. This could lead to the possibility of a lack of dimerization and therefore a lack of a functional cholesterol transporter in tumor cells where high energetic states do not exist. Preliminary unpublished observations show that this protein is also present in tumor mitochondria though proper function in tumor mitochondria seems to be lacking. We are currently studying both cholesterol binding and transportation in tumor mitochondria and whether a correlation exists between the degree of malignancy of the tumors and a lack of normal activity of the protein.

In conclusion, our findings indicate that an intramitochondrial cholesterol-binding protein plays a key role in maintaining proper membrane cholesterol composition that is required for optimal capacity of the oxidative phosphorylation process of energy production in normal liver tissues. The sequencing and molecular cloning of this protein is underway in an effort to further elucidate the mechanism of action of this protein-dependent cholesterol transfer.

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