

## VDAC1 Is a Transplasma Membrane NADH-Ferricyanide Reductase\*

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**Porin isoform 1 or VDAC (voltage-dependent anion-selective channel) 1 is the predominant protein in the outer mitochondrial membrane. We demonstrated previously that a plasma membrane NADH-ferricyanide reductase activity becomes up-regulated upon mitochondrial perturbation, and therefore suggested that it functions as a cellular redox sensor. VDAC1 is known to be expressed in the plasma membrane; however, its function there remained a mystery. Here we show that VDAC1, when expressed in the plasma membrane, functions as a NADH-ferricyanide reductase. VDAC1 preparations purified from both plasma membrane and mitochondria fractions exhibit NADH-ferricyanide reductase activity, which can be immunoprecipitated with poly- and monoclonal antibodies directed against VDAC1. Transfecting cells with pl-VDAC1-GFP, which carries an N-terminal signal peptide, directs VDAC1 to the plasma membrane, as shown by confocal microscopy and FACS analysis, and significantly increases the plasma membrane NADH-ferricyanide reductase activity of the transfected cells. This novel enzymatic activity of the well known VDAC1 molecule may provide an explanation for its role in the plasma membrane. Our data suggest that a major function of VDAC1 in the plasma membrane is that of a NADH-ferricyanide reductase that may be involved in the maintenance of cellular redox homeostasis.**

A number of electron transport systems have been shown to exist within cell membranes. The mitochondrial and endoplasmic reticulum electron transport chains are perhaps two of the best characterized systems in mammalian cells. However, little interest has been shown for the characterization of the members of the plasma membrane redox system (1). Evidence to support the existence of a plasma membrane NADH-oxidoreductase (PMOR)<sup>1</sup> or redox system arises from reduction of two artificial impermeable electron acceptors (potassium ferri-

cyanide and dichlorophenolindophenol) by whole cells (2). Reduction of ferricyanide causes concomitant oxidation of cytosolic NADH (3), suggesting the presence of an NADH-ferricyanide reductase in the plasma membrane. The importance of this plasma membrane redox system is exemplified by the fact that, during generation of human Namalwa  $\rho^0$  cells (which lack a functional mitochondrial respiratory chain), the rate of whole cell ferricyanide reduction increases ~4-fold (2). Importantly, these cells remain viable when ferricyanide is added to the media (4), suggesting a major role for the plasma membrane NADH-ferricyanide reductase in cell signaling and survival (5). However, little headway has been made in the identification and characterization of the proteins involved in plasma membrane redox function. Two redox enzymes have been suggested to be involved in plasma membrane electron transport from NADH to ferricyanide; however, both NADH-cytochrome *b<sub>5</sub>* reductase flavoprotein (6) and glyceraldehyde-3-phosphate dehydrogenase isozyme (7) cannot account for transmembraneous electron transfer, because both enzymes are only associated with the inner side of the plasma membrane (8).

VDAC1 is a small, 30–35-kDa protein, predominantly found in the outer membrane of mitochondria (9), where it constitutes the major protein (10). Predicted structural studies of human VDAC1 suggest the protein is a  $\beta$ -barrel, consisting of either 13 (11) or 16 (12) membrane-spanning strands. VDAC1 has been shown to co-immunoprecipitate with the anti-apoptotic protein Bcl-2 and has been suggested to be involved in forming the pore that releases cytochrome *c* during apoptosis (13). However, a recent report showed Bax-induced pore formation in yeast lacking VDAC (14).

Although VDAC1 is predominantly expressed in the outer mitochondrial membrane, recently, it has been demonstrated by several groups that VDAC1 can also be expressed in the plasma membrane (15–21). Consistent with these observations, several patch clamp techniques have documented the presence of plasma membrane channels with physiological properties similar to VDAC1 (22, 23). Furthermore, immunocytochemical studies using antibodies raised against the N terminus of VDAC1 block a high conductance anion channel found in the plasma membrane of bovine astrocytes (16). These antibodies also appear to specifically label the plasma membrane in human B-lymphocytes. *In vivo*, VDAC1 harbors two alternative first exons, which leads to the expression of VDAC1 isoforms with different N-terminal sequences. One of these isoforms carries a leader sequence that directs the protein via the Golgi apparatus into the secretory pathway (19). The other VDAC1 isoform, devoid of any cleavable pre-sequence, is targeted to the mitochondria, where it is efficiently inserted into the outer membrane (19).

The presence of VDAC1 in the plasma membrane leads to the question of its role there. In this report we establish that

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<sup>1</sup> The abbreviations used are: PMOR, plasma membrane NADH-oxidoreductase; VDAC, voltage-dependent anion-selective channel; GFP, green fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; HTP, hydroxylapatite; FACS, fluorescence-activated cell sorting; TBS, Tris-buffered saline; FITC, fluorescein isothiocyanate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

TABLE I  
Enrichment of plasma membranes by two-phase separation

Enzyme assays were performed as described under "Materials and Methods."

Enzyme (organelle)	Crude extract	Specific activity	After two-phase separation	Specific activity	Enrichment factor
	$\mu\text{kat}$	$\text{nkat/mg}$	$\mu\text{kat}$	$\text{nkat/mg}$	
Succinate dehydrogenase (mitochondria)	44.61	557.6 $\pm$ 46.1	1.21	121.2 $\pm$ 18.2	0.22
Alkaline phosphatase (plasma membrane)	11.64	145.5 $\pm$ 26.5	4.16	415.8 $\pm$ 36.4	2.86
Succinate dehydrogenase: alkaline phosphatase ratio		1.00		0.08	

VDAC1 at the level of the plasma membrane can function as a redox enzyme, capable of reducing extracellular ferricyanide in the presence of intracellular NADH.

#### MATERIALS AND METHODS

**Cell Culture**—Namalwa cells were cultured as previously described (2). COS7 cells were detached by pretreating with cell dissociation solution (Sigma, Castle Hill, New South Wales, Australia) and incubated for 5 min prior to gentle agitation of the flasks. Cell concentrations were determined as previously described (2).

**Preparation of Plasma Membrane-enriched Fractions from Namalwa Cells**—Unless indicated otherwise, all steps were performed at 4 °C. Approximately 6–8  $\times 10^8$  cells were pelleted and washed twice with ice-cold PBS. Following the second wash, the supernatant was removed and the cells were adjusted to 2  $\times 10^8$  cells/ml using ice cold lysis buffer consisting of 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.2, and 1 mM EGTA. Digitonin (1.4 mg/10<sup>9</sup> cells) was added, and the cells were lysed on ice using 72 strokes in a glass homogenizer (Sigma). After lysis, the homogenate was centrifuged at 2000  $\times g$  for 5 min. The supernatant was removed and kept at –20 °C for later analysis. The pellet was washed twice with buffer A (50 mM Tris-HCl, pH 8.0) and then resuspended in buffer A adjusted to 4  $\times 10^8$  cells/ml. The resuspended pellet was then loaded onto a two-phase system. The two-phase system contained 6.4% (w/w) dextran T-500, 6.4% (w/w) poly(ethylene glycol) 3350, and 0.1 M sucrose. 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 40 times in the cold (4 °C). The phases were separated by centrifugation at 150  $\times g$  in an IEC Centra-7R refrigerated centrifuge. The upper phase was carefully withdrawn, together with the interface, and transferred into 10-ml centrifuge tubes. The sample was then centrifuged at 5000  $\times g$  for 30 min. The fluffy pellet resulting from the spin constituted the plasma membrane-enriched fraction. Purity of plasma membrane preparations was determined by subcellular marker enzyme assays (Refs. 2 and 24; see following section).

Alternatively, after the second wash the cells were resuspended in ice-cold 1 mM NaHCO<sub>3</sub>, 0.2 mM EDTA (dipotassium salt) at an approximate ratio of 1 ml/10<sup>8</sup> cells to osmotically swell the cells (25). The resulting cell suspension was then incubated at 4 °C with gentle shaking for 40 min in a 10-ml disposable centrifuge tube. Following incubation, cells were mechanically disrupted using 110 strokes in a 7-ml all-glass Kontes Dounce homogenizer (Sigma). The extent of cell rupture was monitored by light microscopy and trypan blue exclusion, and was found to be consistently  $\geq 85\%$ . The crude homogenate was centrifuged at 500  $\times g$  for 10 min in Sorvall Evolution centrifuge (SS34 rotor) for 10 min, 4 °C, to remove nuclei and unruptured cells. The resulting supernatant was then centrifuged at 25,000  $\times g$  for 30 min as above at 4 °C. The supernatant was extracted with a plastic transfer pipette and stored at 4 °C for later analysis, whereas the pellet was thoroughly resuspended in 50 mM Tris-HCl, pH 8.0 (buffer A), to a net mass of 8 g. This concentrated suspension was divided into four 2-g aliquots, each of which was then individually loaded onto one of four 16-g aqueous two-phase systems constructed on a weight basis. Each system had a final composition of 6.6% poly(ethylene glycol) 3350 (Sigma) + 6.6% Dextran-500 (Sigma) + 1.9 g of 1 M sucrose solution + 0.4 g of 200 mM K<sub>2</sub>HPO<sub>4</sub> (pH 7.4) + 2 g of resuspended membranes in buffer A. In each case the total mass of the system was brought to 16 g with distilled Milli-Q H<sub>2</sub>O. Each two-phase system was constructed in 15-ml glass Corex centrifuge tubes, sealed with Parafilm, and mixed vigorously with at least 70 inversions at 4 °C. Following the separation of the phases by centrifugation at 1000  $\times g$  for 5 min as above at 4 °C, the upper phase of each system was extracted (taking care not to disturb the material collected at the interface) and repartitioned against a fresh lower phase of a second set of two-phase systems

constructed in parallel, except containing 2 g of buffer A in place of the crude membrane suspension. The material from the second set of upper phases was collected, diluted 1:5 in buffer A, pelleted at 25,000  $\times g$  for 30 min (as above) at 4 °C, and then resuspended in fresh buffer A. All preparations used were highly enriched in plasma membranes and showed minimal mitochondrial contaminations (Table I).

**Enzyme Assays**—All enzyme assays and subcellular enzyme markers have been used as described elsewhere (24), with a slight modification. Alkaline phosphatase was assayed for as the plasma membrane marker, using an adaptation of a previously described method (26). Briefly, 50  $\mu\text{g}$  of protein were added to 1 ml of 50 mM Tris, pH 10.5, 2 mM MgCl<sub>2</sub>. The reaction was started with the addition of 4 mM *p*-nitrophenyl phosphate. The increase in absorbance at 420 nm was measured at 37 °C for 1 h. Rates of activity are expressed in nanokatals/mg of protein using the extinction coefficient of *p*-nitrophenol in alkaline solution of 9620 M<sup>-1</sup> cm<sup>-1</sup>. Succinate dehydrogenase activity was determined via the spectrophotometric measurement of the initial linear rates of the reduction of the artificial electron acceptor, dichlorophenolindophenol, at 600 nm (decrease in absorbance) in the presence of succinate. Each 1 ml of reaction medium contained 50 mM dipotassium phosphate, pH 7.4, 40  $\mu\text{M}$  dichlorophenolindophenol, and with the reaction being started by the addition of 20 mM sodium succinate. Rates of activity are expressed in nanokatals/mg of protein using the extinction coefficient of dichlorophenolindophenol at physiological pH of 5500 M<sup>-1</sup> cm<sup>-1</sup>. NADH-ferricyanide reductase activity was measured in a Beckman DU 7500 spectrophotometer. Reduction of ferricyanide to ferrocyanide was ascertained in 1-ml reactions containing 50 mM Tris-HCl, pH 8.0, 3% (v/v) Triton X-100, 750  $\mu\text{l}$  of fraction, 250  $\mu\text{M}$   $\beta$ -NADH. The reaction was initiated by the addition of 250  $\mu\text{M}$  potassium ferricyanide, following a 5-min pre-incubation period at 37 °C. Rates were measured at either 420 or 340 nm at 37 °C for 500 s, with the linear rate of decrease in absorbance being determined from the second 250-s duration. Actual rates of enzymic activity were determined using an extinction coefficient for ferricyanide (420 nm) of 1000 M<sup>-1</sup> cm<sup>-1</sup> or 6220 M<sup>-1</sup> cm<sup>-1</sup> for NADH (340 nm).

**Determination of Protein Concentrations**—Protein concentrations were determined by using the bicinchoninic acid assay (27).

**Purification of the NADH-Ferricyanide Reductase**—The chromatographic steps of DEAE-Sephacel and Blue Sepharose affinity column were carried out using stepwise gradient elution, respectively. Solubilized proteins from the enriched plasma membrane pellet were applied to the DEAE-Sephacel column (5-ml bed volume) pre-equilibrated with buffer B (buffer A + 0.05% (v/v) Triton X-100). The column was washed three times with 20 ml of buffer B. The material bound onto the column was eluted by 10 ml of buffer B containing 50 mM NaCl. Active fractions eluting from the column were pooled and applied to the Blue Sepharose affinity column (5-ml bed volume), which had been pre-equilibrated with buffer B. The column was washed twice with 10 ml of buffer B, then eluted with a linear 10-ml gradient (0–10  $\mu\text{M}$ ) of NADH in buffer B. One-ml fractions were collected and assayed for NADH-ferricyanide reductase activity.

**Solubilization of Plasma Membrane Proteins**—The plasma membrane-enriched fraction was centrifuged at 25,000  $\times g$  as above for 30 min at 4 °C. The resulting creamy white pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 3% (v/v) Triton-X-100 to an approximate concentration of 5 mg/ml and stirred for 1 h at 4 °C. The solubilized fraction was obtained by pelleting the insoluble material at 100,000  $\times g$  for 40 min in a Beckman TLX optima ultracentrifuge (TLA-100.3 rotor) at 4 °C.

**Purification of VDAC1**—VDAC1 was purified according to Ref. 28 with a slight modification from either Namalwa cell plasma membrane or rat liver mitochondria preparations. For the preparation of mitochondria, Wistar rats were decapitated, and their livers excised and immediately washed twice in lysis buffer. Following the second wash, the

TABLE II  
Purification of a plasma membrane NADH-ferricyanide reductase

Enzyme assays were performed as described under "Materials and Methods."

Step	Protein	Volume	Enzyme activity	Specific activity	Purification factor
	mg	ml	$\mu$ kat	$\mu$ kat/mg	
Plasma membrane	25	25	2775	111	1
Solubilization	10	25	2775	277.5	2.5
DEAE	0.16	3	364.5	2278.1	20.6
a. Blue Sepharose	0.01	1	250	25,000	225
b. 2', 5'-ADP	0.001	1	27.7	27,700	249.5

livers were diced into 2-mm<sup>3</sup> pieces and diluted 1:3 (w/w) with ice-cold lysis buffer (as outlined for Namalwa cells). The diced livers were then homogenized in a 50-ml glass Teflon homogenizer (Sigma) at 1500 rpm, for five strokes. The homogenate was centrifuged at 600 × *g* for 5 min to remove nuclei and unbroken cells. The supernatant was decanted, and a second centrifugation was performed at 600 × *g* for 5 min to remove any contaminating nuclei. Following the second spin, the supernatant was carefully aspirated so as not to disturb the pellet. The supernatant was then centrifuged at 30,000 × *g* for 30 min to pellet the mitochondria. The supernatant was removed, and the pellet was resuspended in lysis buffer 2 (225 mM mannitol, 25 mM sucrose, 2 mM MOPS, pH 7.4, 1 mM EGTA). A second centrifugation was performed at 30,000 × *g* for 30 min to wash the mitochondria. Following the second spin, the mitochondrial pellet was taken and diluted to 5 mg/ml in buffer A. After incubating in buffer A for 1 h, the sample was centrifuged at 30,000 × *g* for 30 min and the pellet, consisting mainly of mitochondrial membranes, was used further. The membranes were then solubilized in buffer A + 5% (v/v) Triton X-100. Following constant stirring for 1 h, the sample was centrifuged at 100,000 × *g* for 30 min, and the supernatant was taken. The supernatant was then applied onto a DEAE column, which had been pre-equilibrated with buffer B. The flow through was taken and applied to a dry 2:1 (w/w) hydroxylapatite:celite (HTP:celite) column. The flow through from the HTP:celite column was collected, which consisted of purified VDAC1.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**—SDS-PAGE and silver staining were performed as described elsewhere (29, 30).

**Western Blotting**—Following transfer of proteins to nitrocellulose, the membrane was blocked in 5% (w/v) skim milk powder (Bio-Rad, Regents Park, New South Wales, Australia) in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) for 1 h at 37 °C. Following three washes with washing buffer (0.05% (v/v) Tween 20 in TBS) for 20 min, the membrane was hybridized overnight at 4 °C with primary antibody at 1/1000 dilution. The membrane was then washed three times with TBS and incubated with a dilution of 1/1250 horseradish peroxidase-labeled secondary antibody (Amersham Biosciences, Castle Hill, New South Wales, Australia), for 1 h at 37 °C. Following three washes, ECL was used as previously described (31).

**Immunoprecipitation**—Approximately 1–2 μg of rabbit anti-porin IgG antibody was added to the 10-ml purified fraction and allowed to agitate at 4 °C overnight. Following this, 50 μl of a 50% protein G bead slurry was added and the mixture was agitated for another 2 h. The sample was spun at 10,000 × *g* for 15 s, and the supernatant containing the unbound material was removed and kept for later analyses. The protein G-antibody-antigen complex was further washed three times with buffer A + 0.5 M NaCl and once with buffer B. The immunopellet was then resuspended in 1 ml of buffer B.

**Precipitation of Protein from Detergents**—Proteins were precipitated according to the method of Wessel and Flügge (32).

**FACS Analysis**—Approximately 1 × 10<sup>7</sup> cells were harvested, pelleted (800 × *g*, 5 min) and washed twice in 1% fetal calf serum in PBS. The cells were suspended in 4 ml of PBS and incubated for 40 min with goat anti-mouse IgG F<sub>c</sub> fragment (Bethyl Laboratories, Montgomery, TX). The cells were then washed and divided equally into four tubes. Tubes 2 and 4 were incubated for 40 min at 4 °C with anti-VDAC1 antibody at 1/1000 dilution. The cells were pelleted (800 × *g*, 5 min) and the supernatant aspirated. The pellet was then washed twice with 1% fetal calf serum in PBS. Following the second wash, tubes 3 and 4 were incubated with FITC-conjugated anti-mouse antibody for 40 min. FITC histograms were obtained by analyzing 15,000 cells with the MODfit program (Becton-Dickinson, Wheelers Hill, Victoria, Australia). The amount of viable cells was simultaneously obtained by staining intracellular DNA with propidium iodide, and the fluorescence was measured using the same program.

**Growth and Maintenance of Escherichia coli**—Transformed *E. coli* bearing the recombinant plasmids were grown in liquid Luria Broth supplemented with kanamycin. Cells were incubated at 37 °C overnight for growth.

**DNA Plasmid Amplification**—pl-VDAC1-GFP was a kind gift from Prof. Dermietzel (19). Approximately 1 μg of DNA was taken and used to inoculate 10<sup>7</sup> viable *E. coli* cells. The cells were grown on solid Luria Broth medium with kanamycin to select for transformed cells. Plasmid DNA was purified according to the instructions from the manufacturer (Clontech, East Meadow Circle, PA). As a control, the commercially available pEGFP vector was used (Clontech).

**Electroporation of COS7 Cells**—Cells to be electroporated were grown logarithmically (70% confluent). Each transfection required 2 × 10<sup>6</sup> cells to yield a reasonable number of transfectants. One ml of trypsin was added for 5 min per 10-cm culture dish, and the cells were collected and harvested by centrifuging for 5 min at 1200 rpm and then resuspending in 400 ml of ice-cold medium and placed in a 0.8-cm cuvette (Bio-Rad). The cells were then electroporated at 200 V in a Gene-Pulser electroporator (Bio-Rad) with the capacitor set at 960 microfarads. The time constants obtained ranged from 15 to 45 ms. The cells were then transferred to growth medium and allowed to grow for 48 h.

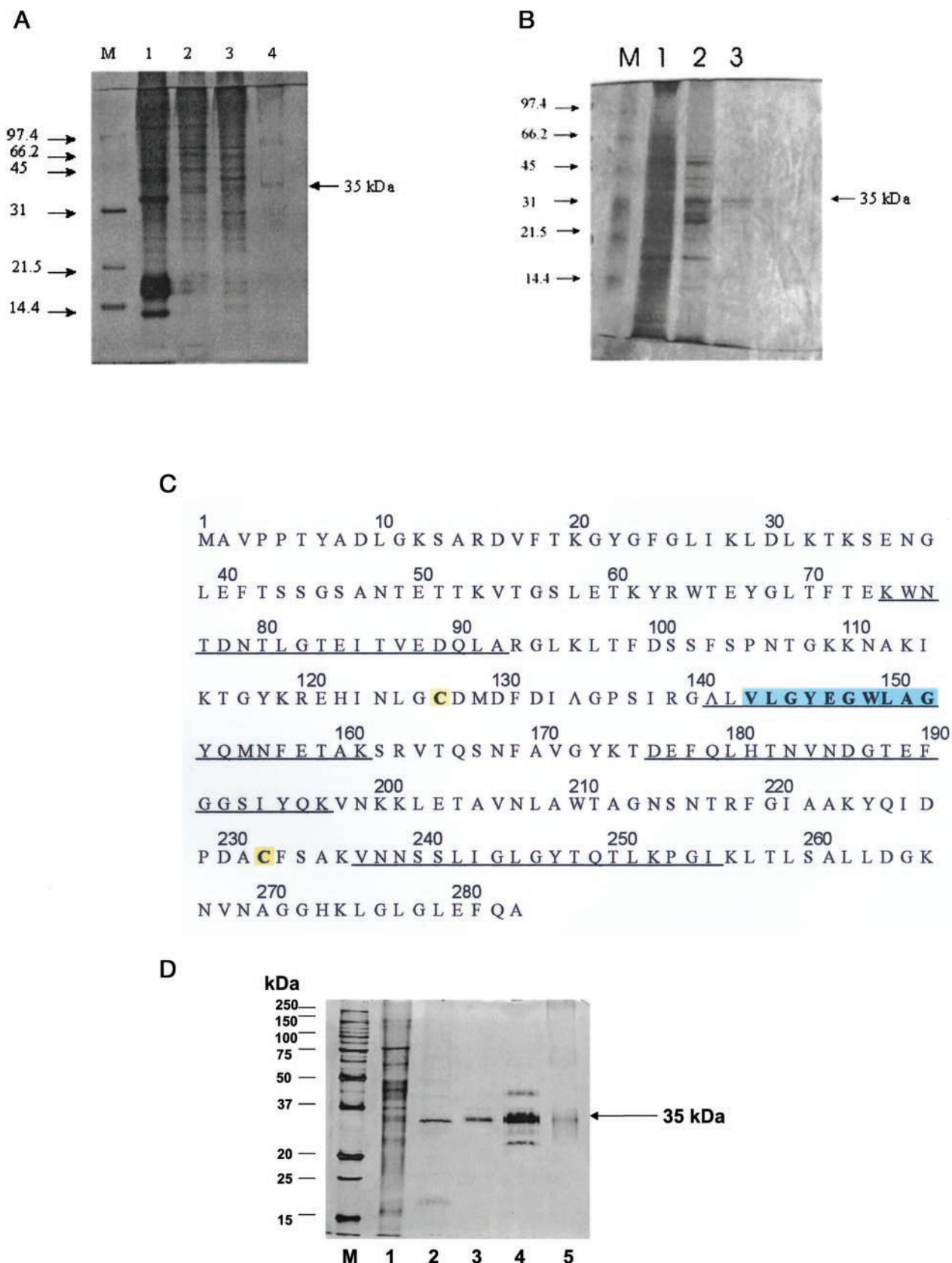
## RESULTS

**Purification of a Plasma Membrane NADH-Ferricyanide Reductase**—Human Namalwa cells were harvested and washed, and the plasma membrane extracted to a purity of >90% as described under "Materials and Methods." Triton X-100 (3% (v/v)) was the best detergent for solubilizing maximal enzyme activity (data not shown). To further purify the enzyme, the solubilized membrane proteins were applied onto a DEAE-Sephacel column. Active enzyme was eluted with 50–100 mM NaCl (data not shown).

Those fractions with NADH-ferricyanide reductase activity eluting from the DEAE-Sephacel column were pooled and applied to a Blue Sepharose, a 2', 5'-ADP affinity, or an HTP:celite (1:2) column. Following washing with 5 column volumes, elution of the enzyme from the former two columns was achieved with a 10-ml gradient of 0–50 μM NADH. One-ml fractions were collected and assayed for enzyme activity. Those fractions with NADH-ferricyanide reductase activity were then precipitated and run in an SDS-PAGE. A 35-kDa band correlated with enzyme activity, suggesting it might be the NADH-ferricyanide reductase (data not shown). Both purification protocols yielded more than 200-fold enrichment from the plasma membrane enriched fractions of the enzyme activity (Table II) and the purification of a major protein band of ~35 kDa (Fig. 1, A and B).

The 35-kDa band was excised from the gel and subjected to a MALDI-TOF analysis. Four resulting peptides with their predicted amino acid sequences were used to search the BLAST and TREMBL data bases for sequence homology to known proteins. This search revealed the 35-kDa band to be identical to human VDAC1 (Fig. 1C).

**VDAC1 Is an NADH-Ferricyanide Reductase**—Co-elution of VDAC1 with enzyme activity from various columns suggested that VDAC1 is an NADH-ferricyanide reductase. However, because the eluants were not homogeneous, further evidence was sought to confirm the function of VDAC1 as a redox enzyme.



**FIG. 1. Identification of VDAC1 as a plasma membrane NADH-ferricyanide reductase.** *A* and *B*, active enzyme pools from Blue-Sepharose (*A*) or 2',5'-ADP-Sepharose (*B*) elutions (lane 1 in panels *A* and *B*, 15  $\mu$ g of protein; lane 2 in panel *A*, 13  $\mu$ g of protein; lane 2 in panel *B*, 3  $\mu$ g of protein; lane 4 in panel *A* and lane 3 in panel *B*, 0.5  $\mu$ g of protein) from the various purification steps according to Table II were precipitated (MeOH/CHCl<sub>3</sub>), resuspended in loading buffer, and subjected to 12% polyacrylamide gel electrophoresis. The gels were silver-stained as described under "Materials and Methods." The positions of the molecular mass markers are shown on the left-hand side. The arrow on the right-hand side indicates the purified 35-kDa protein. *C*, the predicted amino acid sequence of four resulting peptides (underlined) from the MALDI-TOF analysis matched to VDAC1. The amino acid sequence of VDAC1 contains an hypothesized NADH binding domain (blue) and 2 cysteine residues (yellow), which is consistent with the function of an NADH dehydrogenase. *D*, following DEAE extraction of a solubilized

The involvement of NADH in the regulation of VDAC has been well documented (33–35). We identified a putative NAD<sup>+</sup> binding motif on residues 143–152 of human VDAC1. This amino acid sequence (VLGXXGXXXG, where X represents any amino acid) (Fig. 1C) is very similar to that of the NADH binding domain in alcohol dehydrogenase (VLGXXGXXXG) (36), with the exception that VDAC1 contains an extra amino acid between the first and second glycine residue.

Further investigations into the amino acid sequence of human VDAC1, identified the existence of two cysteine residues (Fig. 1C). Cysteine residues appear to be essential for electron transfer by the NADH-ferricyanide reductase, because addition of sulfhydryl binding reagents inhibits ferricyanide reduction in whole cells (24). The identification of a putative NADH binding site and two cysteine residues within the amino acid sequence of VDAC1 is consistent with a function as an NADH-reductase.

To further confirm that VDAC1 can function as an NADH-ferricyanide reductase, two different antibodies were used to immunoprecipitate the protein. These antibodies included a polyclonal antiserum, raised against the entire VDAC protein, and a monoclonal antibody, raised against the N-terminal portion of VDAC1. The specificity of these antibodies was established via Western blot analysis (Refs. 18 and 36–41; data not shown). Both antibodies recognize a band in whole cell homogenates of ~35 kDa in size. To further demonstrate the identity of our enzyme with VDAC1, the plasma membrane NADH-ferricyanide reductase was purified and 1  $\mu$ g of either antibody was added to active fractions eluting from the Blue Sepharose column. Upon addition of the polyclonal (Fig. 2A) or monoclonal (Fig. 2B) antibody, no direct inhibition of the original activity was observed (lane 2). In both cases, however, following immunoprecipitation, most of the NADH-ferricyanide reductase activity was present in the immunopellet fraction (lane 4). Conversely, very little enzyme activity was left in the immunodepleted supernatant (lane 3). The addition of protein G beads only, as a control, did not result in any enzyme activity being precipitated (lane 5). The ability of both antibodies to immunoprecipitate enzyme activity, together with the co-elution of VDAC1 and the reductase activity using different purification protocols, strongly suggests that VDAC1 can function as a redox protein. One could, however, still argue that VDAC1 co-immunoprecipitates with another protein, which may be responsible for the enzyme activity and may constitute an impurity in both purification protocols.

To exclude this, we analyzed whether authentic VDAC1 also exhibits redox activity. Mitochondrial VDAC1 and plasma membrane VDAC1 possess the same amino acid sequence (except for the signal peptide); thus, mitochondria would be a reliable source of material to demonstrate that VDAC1 contains intrinsic NADH-ferricyanide activity. Therefore, VDAC1 was purified from rat liver mitochondria, based on a known purification procedure (28, 34) with a slight modification (see "Materials and Methods"). The resultant purified fraction contained a single 32-kDa band (Fig. 3A) that co-migrated with the ferricyanide reductase activities purified from plasma membrane fractions (Fig. 1D). This fraction also contained NADH-ferricyanide reductase activity (Fig. 3B), suggesting that VDAC1 alone can function as an NADH-

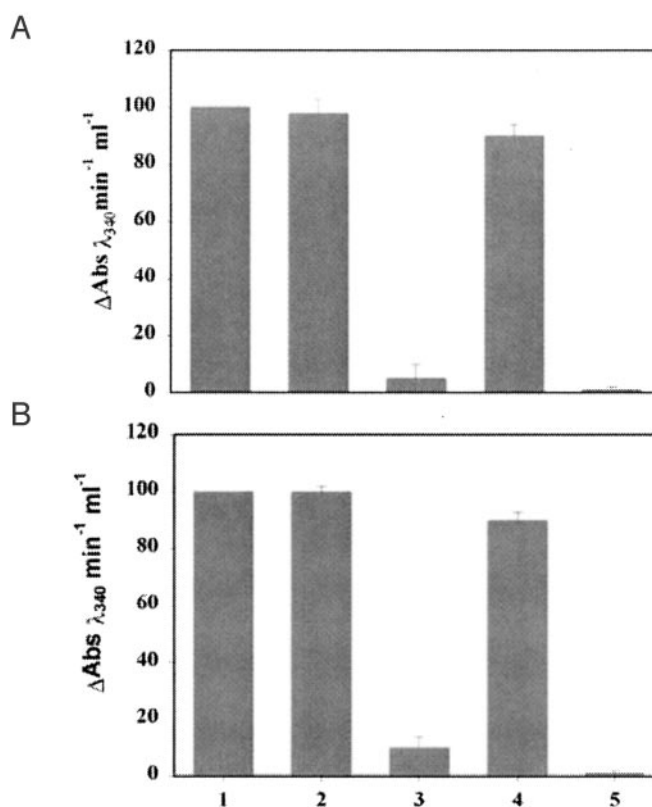


FIG. 2. Immunoprecipitation of NADH-ferricyanide reductase activity with anti-VDAC(1) antibodies. An active fraction eluting from the Blue Sepharose column (5 × 1 cm) was used as starting material for the immunoprecipitation (lane 1). Total enzyme activity was set to 100%. Three micrograms of monoclonal (A) or polyclonal (B) antibody were added (lane 2), following which 60  $\mu$ l of a 50% slurry of protein G were added. The sample was centrifuged (30 s, 10,000 × g), and the unbound supernatant (lane 3) and immunopellet (lane 4) were measured for NADH-ferricyanide reductase activity. The control shows the addition of protein G only (lane 5).

ferricyanide reductase and that the observed activity was not the result of VDAC1 activating (or co-purifying with) a specific plasma membrane NADH-ferricyanide reductase. Furthermore, we used this procedure, established for the purification of VDAC1 from mitochondria, to purify the protein from enriched Namalwa plasma membranes. Again we obtained a single band of ~35 kDa exhibiting a similar specific NADH-ferricyanide reductase activity (Table III), as observed with our previous two purification protocols (Table II).

*VDAC1 Is Present in the Plasma Membrane of Namalwa Cells*—Using immunohistochemical and many other approaches, VDAC1 has been previously documented to be present in the plasma membrane (22, 23, 38–41). However, criticism of these experiments has arisen, based on the possibility that antibodies raised against VDAC1 can nonspecifically interact with the plasma membrane (42, 43). To further prove that VDAC1 is present in the plasma membrane of the Namalwa cells used in our studies, a set of FACS analyses was performed. To overcome any nonspecific interactions of the antibody with the plasma membrane, cells were pre-incubated

plasma membrane-enriched fraction, and HTP:celite (1:2) (lane 2), 2',5'-ADP-Sepharose (lane 3), or Blue Sepharose (lane 4) column chromatography of the resulting DEAE flow-through (1  $\mu$ g, lane 1), equalized quantities of NADH-ferricyanide reductase activity (~30 nanokatals) were precipitated (MeOH/CHCl<sub>3</sub>), resuspended in Magic Mix, and subjected to 15% SDS-PAGE (reducing conditions). The displayed gel was silver-stained to a detection threshold of ~20 ng, as described under "Materials and Methods." The positions of the molecular mass Bio-Rad Precision Plus Protein™ standards (unstained) are shown in the far left lane (M). The arrow on the right-hand side indicates the purified 35-kDa band common to lanes 2–4 that co-migrates with acetone-precipitated VDAC1/porin purified from bovine heart mitochondria (BHP) (50 ng, lane 5).

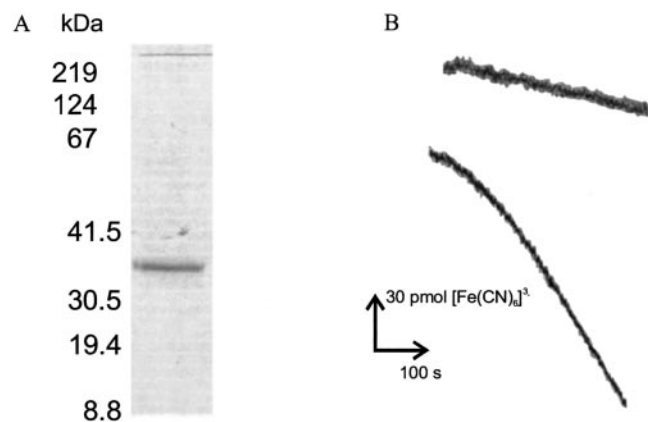


FIG. 3. **Purification of rat liver mitochondrial VDAC1.** Rat liver mitochondrial VDAC1 was purified as described under "Materials and Methods." 30  $\mu$ l of the material eluting from the HTP:celite column was precipitated (MeOH/CHCl<sub>3</sub>) and loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, only one 35-kDa band could be detected in Coomassie Blue-stained (A) or silver-stained gels. 30  $\mu$ l of material eluting from the HTP:celite column were measured for NADH-ferricyanide reductase activity (B).

with an F<sub>c</sub> fragment as a blocking agent in all experiments, prior to the addition of any antibodies. We observed an increase in fluorescence in cells treated with both the primary anti-VDAC1 monoclonal and secondary FITC-conjugated antibodies, when compared with control cells incubated with only the primary or secondary antibody. The significant shift in fluorescence confirms the presence of VDAC1 in the plasma membrane of Namalwa cells (Fig. 4). Mitochondrial VDAC1 cannot account for the result, because the cells were neither fixed nor permeabilized prior to FACS analysis. Furthermore, the cells were viable, as determined by lack of propidium iodide staining (data not shown), ensuring that no antibody could cross the plasma membrane.

**VDAC1 Functions as a NADH-Ferricyanide Reductase *in Vivo***—To demonstrate that the reductase activity of VDAC1 is not an *in vitro* artifact, COS7 cells were transfected with the pl-VDAC1-GFP plasmid (18). This plasmid encodes mouse VDAC1 (which differs by only 4 amino acids from human VDAC1, although these changes do not affect the residues suggested to be involved in its redox functions), N-terminally tagged with a secretory pathway leader sequence and C-terminally linked to GFP. Cells (2–3  $\times 10^6$ ) were transfected with either 20  $\mu$ g of pl-VDAC1-GFP or the vector control. Approximately 48 h after transfection, the cells were harvested, washed, and subjected to Western blot analysis, which demonstrated the presence of a 52-kDa fusion protein in COS7 cells transfected with pl-VDAC1-GFP (data not shown). On the other hand, in COS7 cells transfected with the vector control only, a 27-kDa product, which is identical to the molecular mass of GFP, was detected (data not shown).

To determine whether the pl-VDAC1-GFP product was being efficiently processed through the secretory pathway, confocal analysis was performed. COS7 cells transfected with pl-VDAC1-GFP were shown to have green fluorescence, confirming that GFP was being expressed (Fig. 5). The pattern of GFP expression is reminiscent of secretory pathway/plasma membrane expression, both in attached cells (Fig. 5, *upper panel*) and in premitotic rounded up cells (Fig. 5, *lower panel*). In these cells the expression pattern closely resembled that described for the teratocarcinoma cell line, PA-1 clone 9117, transfected with the same vector (19). In PA-1 cells, such expression was demonstrated to be in the Golgi and secretory pathway (19). In contrast, COS7 cells transfected with the control vector demonstrated green fluorescence in

the typical cytosolic localization.

To determine whether the pl-VDAC1-GFP gene product could function as a reductase *in vivo*, transfected COS7 cells were assayed for their ability to reduce ferricyanide. Those cells expressing pl-VDAC1-GFP demonstrated at least 40-fold stimulation in whole cell ferricyanide reduction, compared with the mock transfected cells (Fig. 6, *lane 4*), whole cell ferricyanide reduction of which was almost not measurable at the same or twice the cell concentration used (Fig. 6, *lanes 1 and 2*). When human Daudi or Jurkat cells are stably transfected with the same vector, these cells also show increased ferricyanide reduction (data not shown).

These results demonstrate that overexpression of VDAC1 in the plasma membrane leads to an enhanced rate of whole cell ferricyanide reduction. The data show that VDAC1 can function *in vivo* as a plasma membrane NADH-ferricyanide reductase.

## DISCUSSION

**VDAC1 Is an NADH-Ferricyanide Reductase**—Transplasma membrane electron transport has been established since the 1970s, mainly by the work of Fred Crane (reviewed in Ref. 5). To date, however, only three integral plasma membrane enzymes capable of electron transport across the membrane have been identified on the molecular level (1): the phagocytotic NADPH oxidoreductase (reviewed in Ref. 44), involved in defense against infections, and its relatives; the recently discovered mucosal duodenal cytochrome *b* (Dcytb), involved in uptake of dietary iron (45); and a doxorubicin-inhibited NADH-ferricyanide reductase (46). We sought to identify the plasma membrane enzyme responsible for NADH-ferricyanide reduction. Using several chromatographic procedures, a 35-kDa band was identified as human VDAC1. VDAC1 is one of three isoforms present in human and other mammalian cells. Recently, in mouse the presence of a putative plasma membrane targeting sequence located in frame and just before the exon encoding the VDAC1 starting ATG has been shown (19). The expression of such a sequence gives rise to an alternative VDAC1 protein able to be targeted to the plasma membrane. When the pre-sequence is not expressed, the synthesized protein corresponds to the "classical" porin, devoid of any cleavable pre-sequence. The amino acid sequence of VDAC1 demonstrates characteristics compatible with an NADH reductase. We suggest that residues 143–152 with the sequence VLGXXGXXXG (where X represents any amino acid) form an NAD<sup>+</sup> binding domain. This sequence is very similar to that of the yeast alcohol dehydrogenase NAD<sup>+</sup> binding domain (VLGXXGXXXG) (32), with the exception that VDAC1 contains an extra amino acid between the first and second glycine residue. Another putative NAD<sup>+</sup> binding motif in human VDAC1 was previously suggested (residues 270–278) (47). This sequence (GXXXGXG) is also similar to that of the yeast alcohol dehydrogenase NAD<sup>+</sup> binding domain, but it is inverted.

VDAC1 contains two cysteine residues. Addition of pCMBS to purified VDAC1 resulted in a complete inhibition of its redox activity (data not shown). Because pCMBS binds to free thiol groups, VDAC1 must have (a) cysteine residue(s) at, or near, its active site. The importance of cysteine residues in the PMOR has been well established (24, 48).

To confirm that VDAC1 can function as an NADH-ferricyanide reductase, both monoclonal and polyclonal anti-VDAC(1) antibodies were used to immunoprecipitate the protein. The immunopellet was shown to have NADH-ferricyanide reductase activity. However, this experiment may still be interpreted as the result of the plasma membrane NADH-ferricyanide reductase co-immunoprecipitating

TABLE III  
Purification of VDAC1, a plasma membrane NADH-ferricyanide reductase

Enzyme assays were performed as described under "Materials and Methods."

Step	Protein	Volume	Enzyme activity	Specific activity	Purification factor
	mg	ml	<i>f</i> kat	<i>f</i> kat/mg	
Crude extract	13.6	10	689.8	50.7	1
Plasma membrane	4.52	3	1131.8	250.4	5
Solubilization	0.45	3	727.9	1617.6	32
DEAE-Sephacel	0.17	1.5	697.3	4101.7	81
HTP:celite	0.04	0.75	1402.0	35,050.2	691

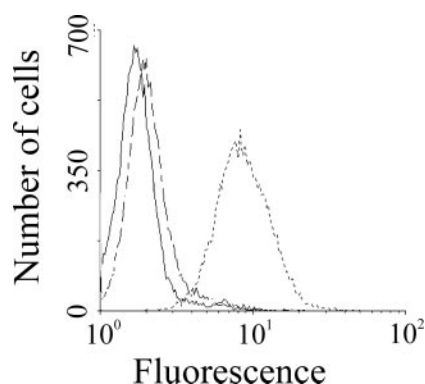


FIG. 4. VDAC1 is present in the plasma membrane of Namalwa cells. Approximately  $1 \times 10^6$  Namalwa cells were harvested, washed and blocked with anti-F<sub>c</sub> for 1 h. The cells were then incubated with monoclonal mouse anti-VDAC (solid line), goat FITC-conjugated anti-mouse (long dashed line), or both antibodies (short dashed line). After washing the cells, FITC expression was analyzed by FACS analysis.

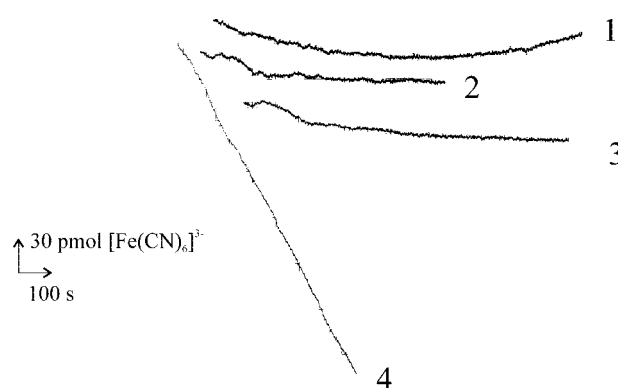


FIG. 6. VDAC1 function as a transplasma membrane NADH-ferricyanide reductase *in vivo*. COS7 cells were transfected with pl-VDAC1-GFP (line 4) or the vector control only (line 3). 48 h after transfection,  $2 \times 10^5$  cells were assayed for whole cell ferricyanide reduction.  $2 \times 10^5$  (line 1) or  $4 \times 10^5$  (line 2) wild type COS7 cells were used as background controls.

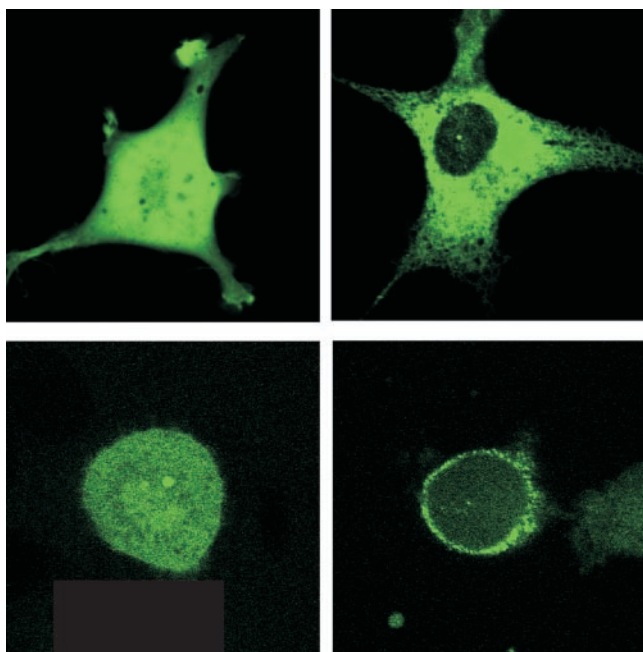


FIG. 5. pl-VDAC1-GFP is expressed in the plasma membrane. COS7 cells were harvested, pelleted, and electroporated with the vector only (left-hand side) or pl-VDAC1-GFP (right-hand side) as described under "Materials and Methods." 48 h after transfection, the cells were visualized using confocal microscopy.

with VDAC1. To ensure this was not the case, VDAC1 was purified using a described method (35) and from rat liver mitochondria. SDS-PAGE analysis demonstrated that VDAC1 was purified to homogeneity (Fig. 3A). The purified sample exhibited NADH-ferricyanide reductase activity. Although it is unlikely that VDAC1 would function as a reduc-

tase in mitochondria, these data show that, in the presence of NADH, VDAC1 can directly catalyze the reduction of ferricyanide.

If VDAC1 was capable of functioning as an NADH-ferricyanide reductase, then overexpression of the protein in the plasma membrane should increase the rate of whole cell ferricyanide reduction *in vivo*. To prove this principle, the plasmid gene pl-VDAC1-GFP was obtained (19). Its gene product is directed into the secretory pathway. COS7 cells transfected with that construct demonstrated green fluorescence in the plasma membrane; Western blot analysis confirmed that the pl-VDAC1-GFP fusion protein was intact. Cells transfected with pl-VDAC1-GFP showed an enhanced rate in whole cell ferricyanide reduction, suggesting that VDAC1 can function *in vivo* as a plasma membrane NADH-ferricyanide reductase. NADH could not be replaced by NADPH (data not shown), nor were VDAC1-transfected cells capable of reducing dichlorophenolindophenol with higher rates than their mock transfected counterparts (data not shown), implying that VDAC1 is a specific NADH-ferricyanide reductase.

**Reductase Activity as a Novel Function of VDAC1**—The ability of VDAC1 to function as a redox enzyme may shed light on some previously unexplained observations. Most of the research done to date on VDAC1 has focused on its anionic conductivity. Further, the presence of porin in the plasma membrane of eukaryotic cells was suggested to Thinnes and co-workers by its co-purification with human transplantation antigens (15, 38). The same group subsequently proved capable of discriminating between isoforms, namely HVDAC1 (*i.e.* porin 31HL) and HVDAC2 (49), thus proving that the plasmallemmatic localization is specific for the VDAC1 isoform. Most evidence for that localization derived from immunodecoration of various intact cultured cells or by tissues histochemistry. Biochemical evidence was presented by two groups that re-

ported the presence of a protein with sequence homology to porin in caveolae-enriched fractions isolated from lung tissue (50). Intact cells were labeled with the membrane-impermeant reagent NH-SS-biotin and demonstrated in two-dimensional PAGE the overlapping of the biotinylated cell proteins with a spot immunodecorated with antiporin antibody (51). Notwithstanding the large amount of data in the literature claiming the presence of VDAC in the plasma membrane (see also Ref. 18), the scientific public is still skeptical about this result, as discussed in Ref. 42. The main reason for this is the conceptual incongruity of the presence of a putative unspecific pore-forming protein in the plasma membrane, a potentially lethal occurrence. The discovery of a new catalytic function of VDAC sheds new light on this debated issue. It is not surprising that VDAC may exert some other function in addition to the "classical" pore formation. Yeast VDAC2, unlike YVDAC1, is unable to form pores in planar bilayers and transport ions across membranes, but its overexpression restores the normal growth in YVDAC1 knockout strains (52). Human and mouse VDAC1 and VDAC2 isoforms show channel-forming activity *in vitro* and can complement the yeast VDAC1 deficiency (52), whereas VDAC3 can only partially complement the defect (53). The problem is to understand how the same polypeptide may function in different ways in two different membranes.

Assuming that VDAC has a transplasma membranous redox activity, two explanations can be offered. (i) The protein conformation of the protein when targeted to the plasma membrane is different than in the outer mitochondrial membrane, and it is more suited for the redox function. This could happen also because some post-translational modification in the Golgi affects somehow the structure. (ii) VDAC in the plasma membrane is controlled by some effector. Against the first assumption stand our data that VDAC1 purified from both plasma membrane and mitochondria exhibit similar specific activities as reductase. In mitochondria the influence of modulator proteins standing in the intermembrane space was proposed (54). A similar situation may be in the plasma membrane. A kind of "stringent control" of the pore forming activity might be exerted in the frame of a multiprotein complex. It is suggestive that the images illustrating the many papers claiming the plasmalemmatic localization of VDAC, similar to our data (Fig. 5), showed a striking punctuate distribution pattern on the surface, which is compatible with a localization in subdomains of the plasma membrane. These images are also consistent with the caveolar localization of this protein. A subdomain localization might be a strategic way to prevent any harmful function of the VDAC protein. In that case the potential modulator protein should thus be looked for in the specific protein set of caveolar domains.

We here describe VDAC1 as a transplasma membrane NADH-ferricyanide reductase. Our results demonstrate that VDAC1 functions as a transmembraneous redox protein and, in doing so, is likely to form part of the PMOR complex. The presence of such a redox enzyme in the plasma membrane is well established in the literature. Although ferricyanide is a nonphysiological substrate, the physiological substrate of the enzyme remains to be established. Preliminary data from our laboratory indicate that (although dichloroindophenol does not act as a substrate for VDAC1) coenzyme Q-10 can act as a substrate for the enzyme, raising the possibility that coenzyme Q-10 may function as (a) physiological substrate. The identification of the physiological substrate(s) of VDAC1 in/at the plasma and identification of proteins that bind to VDAC1 in/at the plasma membrane will now be an important next step.

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