Functional reconstitution of the mitochondrial Ca^{2+}/H^+ antiporter Letm1

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The leucine zipper, EF hand–containing transmembrane protein 1 (*Letm1*) gene encodes a mitochondrial inner membrane protein, whose depletion severely perturbs mitochondrial Ca^{2+} and K^+ homeostasis. Here we expressed, purified, and reconstituted human Letm1 protein in liposomes. Using Ca^{2+} fluorophore and $^{45}Ca^{2+}$-based assays, we demonstrate directly that Letm1 is a Ca^{2+} transporter, with apparent affinities of cations in the sequence of $Ca^{2+} \approx Mn^{2+} > Gd^{3+} \approx La^{3+} > Sr^{2+} >> Ba^{2+}$, Mg^{2+}, K^+, Na^+. Kinetic analysis yields a Letm1 turnover rate of 2 Ca^{2+}/s and a K_m of ~25 µM. Further experiments show that Letm1 mediates electroneutral 1 Ca^{2+}/2 H^+ antiport. Letm1 is insensitive to ruthenium red, an inhibitor of the mitochondrial calcium uniporter, and CGP-37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger. Functional properties of Letm1 described here are remarkably similar to those of the H^+-dependent Ca^{2+} transport mechanism identified in intact mitochondria.

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INTRODUCTION

The leucine zipper, EF hand–containing transmembrane protein 1 (*Letm1*) gene, located on the short arm of human chromosome 4, was initially cloned because of its immediate proximity to a 165-kb critical region deleted in all patients with the Wolf–Hirschhorn syndrome (Endele et al., 1999). It encodes a mitochondrial inner membrane protein with one or two predicted transmembrane segments and a large soluble portion containing a 14-3-3–like domain, two coiled-coil domains, and two EF hand Ca^{2+}-binding motifs (Schlickum et al., 2004; Lupo et al., 2011). *Letm1* is conserved in all eukaryotes; down-regulation of the gene product in yeast (Nowikovsky et al., 2004) and protozoa (Hashimi et al., 2013) drastically alters mitochondrial morphology, and in Drosophila (McQuibban et al., 2010) and mice (Jiang et al., 2013) it causes embryonic lethality.

The role of Letm1 in mitochondrial physiology has been a subject of intensive investigation. Letm1 was initially proposed to mediate K^+/H^+ exchange (KHE) across the mitochondrial inner membrane, as the yeast mitochondrial phenotype resulting from Letm1 knockout is reverted by nigericin, a monovalent cation exchange ionophore (Nowikovsky et al., 2012). On the other hand, later work showed that Letm1 affects mitochondrial Ca^{2+} dynamics (Jiang et al., 2009; Waldeck-Weiermair et al., 2011; Alam et al., 2012). Using a fluorescent protein, Pericam, to follow changes of [Ca^{2+}] and pH in the matrix, a previous study demonstrated that silencing *Letm1* in *Drosophila* S2 and in HeLa cells impairs Ca^{2+}/H^+ exchange (Jiang et al., 2009). These observations are not contradictory. In a complex physiological system such as the intact mitochondrion, it is not surprising that perturbing the homeostasis of one ion species could lead to profound effects on that of other ions.

To understand how Letm1 regulates ion homeostasis in mitochondria, it is essential to establish the protein's primary transport function, which can be directly revealed in a reduced, reconstituted system. Purified human Letm1 has previously been reconstituted into liposomes (Jiang et al., 2009), but two technical ambiguities have undermined the interpretability of those results. First, the homogeneity of the purified protein, which may be examined by size exclusion chromatography, remains unclear. Second, Letm1 was reconstituted at extremely low protein density (0.02 µg protein/mg lipid), where most liposomes would be devoid of protein, and transport would arise from a minuscule fraction of the liposome population. We now rigorously establish a purification of functionally competent human Letm1 and a reconstituted liposome system in which ion transport mediated by the protein may be quantified. The results demonstrate directly that Letm1 catalyzes electroneutral Ca^{2+}/H^+ antiport independently of K^+.

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Abbreviations used in this paper: KHE, K^+/H^+ exchange; *Letm1*, leucine zipper, EF hand–containing transmembrane protein 1; SEC, size-exclusion column; Vln, valinomycin.

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MATERIALS AND METHODS

Reagents

All detergents were purchased from Affymetrix, and lipids, including 1-palmitoyl, 2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl, 2-oleoylphosphatidylglycerol (POPG) were from Avanti Polar Lipids, Inc. $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ were obtained from PerkinElmer, and Ca^{2+} fluorophores were from Invitrogen. The following inhibitors were used: RR from Sigma-Aldrich, Ru360 from EMD Millipore, and CGP-37157 from Santa Cruz Biotechnology, Inc. Anti-His tag antibody was from QIAGEN (no. 34660).

Letm1 expression, purification, and reconstitution

The coding sequence of the human *Letm1* gene (GenBank accession no. AF061025) with an appended C-terminal hexahistidine (His_6) tag was cloned into the pET21 expression vector. Transformed Rosetta 2 (DE3) cells (EMD Millipore) were grown in Terrific Broth (BD) at 37°C to A_{600} of 0.8–1.0 and induced with 0.5 mM IPTG for 2.5 h. Cells were pelleted, incubated at 4°C for 12–16 h, and resuspended in breaking buffer (BB; 100 mM NaCl, 2 mM TCEP, and 50 mM Tris-HCl, pH 8.0) supplemented with protease inhibitors (1 µg/ml leupeptin/pepstatin and 1 mM phenylmethanesulfonyl fluoride), DNase, RNase, and lysozyme. The cell suspension was incubated on ice for 15 min and then sonicated. After this step, all of the procedures were performed at 4°C, as Letm1 is extremely susceptible to proteolysis. The cell lysate was centrifuged at 15,000 *g* for 40 min to remove cell debris, and the membrane fraction was harvested at 200,000 *g* for 2 h. The membrane pellet was resuspended in BB containing leupeptin/pepstatin and extracted with 50 mM decylmaltoside (DM) for 3 h. The protein-detergent micelle solution was loaded onto a cobalt affinity column, which was washed with wash buffer (WB; 100 mM NaCl, 10 mM DM, and 20 mM Tris-HCl, pH 7.5), then with 30 mM imidazole in WB, followed by Letm1 elution with 300 mM imidazole in WB. After concentrating the eluate ~10-fold to 0.5–0.7 ml, the sample was loaded onto a Superdex 200 size-exclusion column (SEC) equilibrated with WB. After the elution of a nonprotein component at 8 ml, Letm1 eluting at 11–12.5 ml was collected. To remove trace contaminants, the sample was repurified on SEC. The typical yield of purified Letm1 was ~100 µg/L culture.

Reconstitution was performed immediately after purification, as the protein loses function within a day in detergent micelles at 4°C. Proteoliposomes were formed from a micellar solution containing 40 mM CHAPS in reconstitution buffer (RB; 120 mM KCl and 30 mM HEPES, pH 7.5), 20 mg/ml POPE/POPG (3:1 wt/wt), and 5 µg Letm1/mg lipid by removing detergent with extensive dialysis against RB at 4°C. The dialysis buffer was changed twice every 6–12 h. The proteoliposomes could then be frozen at −80°C without a significant loss of transport activity for at least 2 mo.

Rb^{+} and Ca^{2+} flux assays

Proteoliposomes were loaded with the desired luminal composition by three freeze–thaw cycles in the appropriate solution, followed by light bath sonication to produce unilamellar vesicles. Just before the assay, external medium was exchanged by spinning 100-µl samples through prespun, 1.5-ml Sephadex G-50 columns (GE Healthcare) equilibrated with suitable extraliposomal solution.

To test $^{86}Rb^{+}$ uptake into Letm1 liposomes, a trace amount of $^{86}Rb^{+}$ (~5 µCi/ml) was added to the sample collected from G-50 columns. The uptake reaction was terminated by loading 50-µl samples onto 2.2-ml Dowex 50-X4 columns (The Dow Chemical Company), NMDG form, preequilibrated with RB to remove external $^{86}Rb^{+}$. The column was flushed with 2 ml RB to collect liposomes into scintillation vials for counting. The membrane potential (Ψ) clamped by valinomycin (Vln), a K^{+} ionophore, in the presence of a predefined K^{+} gradient could be estimated from the Nernst equation:

$$\Psi = RT/F * \ln\left(\left[{}^{86}Rb^{+}\right]_{ex}/\left[{}^{86}RB^{+}\right]_{i}\right),$$

where the subscripts "i" and "ex" used here and in the Results refer to the inside and outside of liposomes, respectively.

For fluorophore-based Ca^{2+} transport assays, the internal medium contained either 1 µM Fluo-4 or 0.5 µM Calcium Green 5N (Invitrogen). Liposomes (100 µl) collected from G-50 columns were immediately diluted into 1.9 ml of external medium in a stirred quartz cuvette, which was loaded onto a spectrophotometer (F-4500; Hitachi). Ca^{2+} uptake upon the addition of external Ca^{2+} was followed by Ca^{2+}-dependent fluorescence (Ex/Em: 490/520 nm for Fluo-4 and 506/532 nm for Calcium Green 5N), and the signal was digitized at 2 Hz.

For $^{45}Ca^{2+}$ flux assays, 7.5 mM EGTA was loaded into liposomes to maintain low internal free $[Ca^{2+}]$. The G-50 spin columns were equilibrated with an additional 10 µM EGTA to prevent trace amounts (3–5 µM) of Ca^{2+} loading into proteoliposomes while the sample passed through the column. To initiate $^{45}Ca^{2+}$ uptake, the sample was diluted 10-fold into suitable EGTA-free extraliposomal medium containing 15–30 µM $^{45}Ca^{2+}$ (~5 µCi/ml). The uptake reaction was terminated, as in $^{86}Rb^{+}$ assays, by using Dowex columns.

The results from $^{86}Rb^{+}$ and $^{45}Ca^{2+}$ assays are frequently reported as fractional uptake, defined as the fraction of total radioactivity that is trapped within the liposomes. Statistics are presented as average ± standard error, typically of three independent determinations.

RESULTS

To establish a robust Letm1-reconstituted system, hexahistidine (His_6)-tagged human Letm1 was expressed in *Escherichia coli* and purified by cobalt affinity and gel filtration. The final Letm1 preparation runs as a symmetrical, monodisperse peak on an SEC (Fig. 1 A) and as a single band on SDS gel (Fig. 1 B) at a position expected from its polypeptide sequence (83.5 kD). The identity of the protein was further confirmed by Western blotting using an anti-His tag antibody (Fig. 1 B). Proteoliposomes containing Letm1 at a density (5 µg Letm1/mg lipid) high enough so that each liposome would contain multiple copies of protein (Walden et al., 2007) were made by detergent dialysis. Although the purified

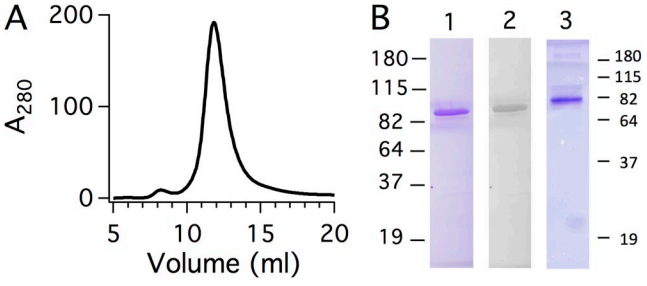

Figure 1. Purification of human Letm1. (A) Gel-filtration profile of Letm1 in the final purification step. (B) Purified Letm1 protein was analyzed using SDS-PAGE (lane 1) and Western blot with anti-His tag antibody (lane 2). After Letm1 reconstitution, protein integrity was confirmed by running SDS-solubilized proteoliposomes on protein gels (lane 3).

protein degraded after a day in detergent solution, no significant degradation was detected after reconstitution, as shown by SDS-PAGE (Fig. 1 B).

Ca^{2+} transport mediated by Letm1

Because Letm1 was suggested to regulate KHE in mitochondria, we first tested whether Letm1 directly transports K^+, using a flux assay based on $^{86}Rb^+$, a faithful K^+ surrogate in K^+-specific transporters, pumps, and channels (Heginbotham et al., 1998). Letm1-containing liposomes with a 1,000-fold outward K^+ gradient were exposed to a trace amount of $^{86}Rb^+$. If the liposome contains a K^+ pathway, $^{86}Rb^+$ will accumulate in the liposomes at a concentration much higher than that in the external medium, as demanded by the equilibrium condition:

$$\left[{}^{86}\mathrm{Rb}^+\right]_i \Big/ \left[{}^{86}\mathrm{Rb}^+\right]_{ex} = \left[\mathrm{K}^+\right]_i \Big/ \left[\mathrm{K}^+\right]_{ex}.$$

Letm1-containing liposomes, like protein-free controls, fail to take up $^{86}Rb^+$, even after 30 min (Fig. 2 A), whereas the addition of either Vln, a K^+-selective ionophore, or nigericin evokes rapid and massive $^{86}Rb^+$ uptake, with a steady state reached within 1 min. This experiment strongly refutes the idea that Letm1 catalyzes K^+ transport.

We next tested Letm1 for Ca^{2+} transport. Ca^{2+} was applied externally to proteoliposomes loaded with EGTA, and the subsequent Ca^{2+} uptake, driven by the large inward Ca^{2+} gradient, was monitored by the preloaded Ca^{2+} indicator Fluo-4. There is no detectable Ca^{2+} influx at a $[Ca^{2+}]_{ex}$ of 0.5 µM. In contrast, raising $[Ca^{2+}]_{ex}$ to 30 µM produces an immediate rise of Ca^{2+} fluorescence, representing the response of leaked Fluo-4 to the sharp increase of external Ca^{2+}, and then a slower time course reflecting Ca^2 movement into the liposomes (Fig. 2 B, black trace). This Ca^{2+} influx is not seen in empty liposomes nor in liposomes similarly reconstituted with heat-treated Letm1 or the glutamate transporter GadC (not depicted). We note that the 120 mM KCl present in the liposome suspension does not interfere with Ca^{2+} transport, as replacing KCl with Na_2SO_4 barely affects the transport, and as adding KCl back to the KCl-free system during Ca^{2+} uptake does not alter the flux (Fig. 2 B, red trace).

Using Fluo-4 to follow Ca^{2+} transport offers the advantage of real-time recording, but its nonlinear response to Ca^{2+} and pH sensitivity weaken its utility for quantifying Ca^{2+} transport kinetics. Accordingly, we used $^{45}Ca^{2+}$ in an alternative flux assay to determine the kinetic parameters of Letm1. In a typical experiment, proteoliposomes were loaded with EGTA to buffer internal Ca^{2+} at a near-zero level. The addition of micromolar $^{45}Ca^{2+}$ to the external solution produces an inwardly directed Ca^{2+} gradient and evokes robust uptake. The initial rate of Ca^{2+} influx (Fig. 2 C) increases along a Michaelis–Menten curve (Fig. 2 D), with a K_m of 27 µM, a V_{max} of 21 pmol/µg protein/s, and a k_{cat} of 2 s^{-1}.

Ionic selectivity and Mn^{2+} transport of Letm1

The results above show that Letm1-mediated Ca^{2+} transport is negligibly affected by K^+ or Na^+ at 0.1-M levels. To further characterize Letm1's ionic selectivity, we measured the initial rate of $^{45}Ca^{2+}$ uptake in the presence of various divalent or trivalent cations (Fig. 3 A). Unsurprisingly, Mg^{2+}, which has a smaller ionic radius than Ca^{2+} and a much larger hydration shell, fails to slow Ca^{2+} flux, even at ~300-fold excess. In contrast, Sr^{2+} and Mn^{2+}, with chemical properties mimicking Ca^{2+}, behave as competitors. Lanthanides, including Gd^{3+} and La^{3+}, having ionic radiuses similar to Ca^{2+} but higher charge

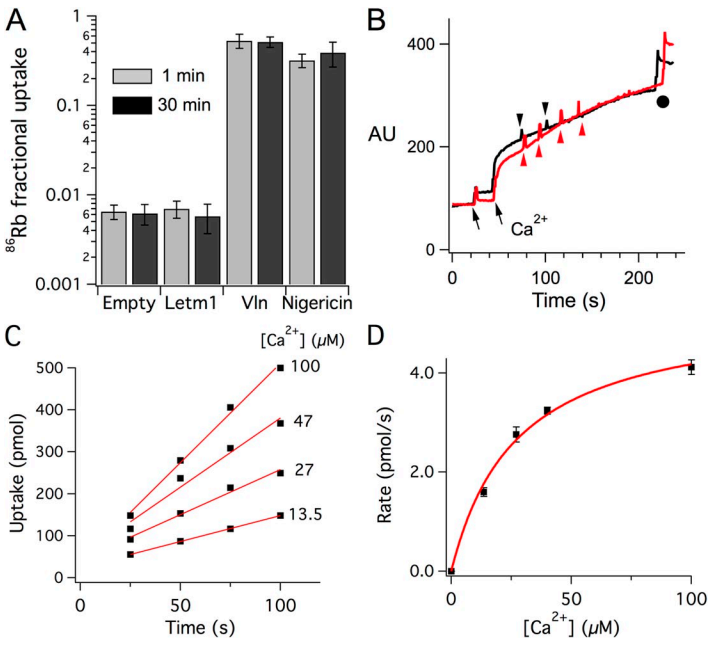

Figure 2. Letm1-mediated Ca^{2+} transport. (A) $^{86}Rb^+$ uptake into empty liposomes or Letm1 proteoliposomes in the presence or absence of Vln or nigericin. Intraliposomal solution: 100 mM KCl and 30 mM HEPES, pH 7.5; external solution: 0.1 mM KCl, 100 mM NMDG-Cl, and 30 mM HEPES, pH 7.5. Vln and nigericin concentration: 1 µg/ml. (B) Fluo-4–based Ca^{2+} flux assay. 120 mM KCl (black trace) or 80 mM Na_2SO_4 (red trace) is present on both sides of liposomes; intraliposomal solution also contains 2 mM EGTA, 1 µM Fluo-4, and 30 mM HEPES, pH 7.5, and external solution also contains 1 mM EGTA and 30 mM HEPES, pH 7.5. Two doses of $CaCl_2$ (for KCl system) or Ca acetate (for Na_2SO_4 system) were added to bring external free $[Ca^{2+}]$ to ~500 nM (left arrow) and subsequently to ~30 µM (right arrow). During Ca^{2+} uptake, two shots of 10 mM Na_2SO_4 (black arrowheads) were added to the KCl-containing system, and four shots of 10 mM KCl (red arrowheads) were applied to the KCl-free liposomes. At the end of the uptake time course, proteoliposomes were treated with 1 µg/ml A23187 (black circle) to equilibrate free Ca^{2+} in internal and external solutions. (C) Initial $^{45}Ca^{2+}$ uptake at indicated external free $[Ca^{2+}]$. Red lines represent linear fit to calculate initial rate of uptake. (D) Michaelis–Menten analysis of Ca^{2+} transport from data as in C, with K_m = 27 µM and V_{max} = 21 pmol/µg protein/s (red curve).

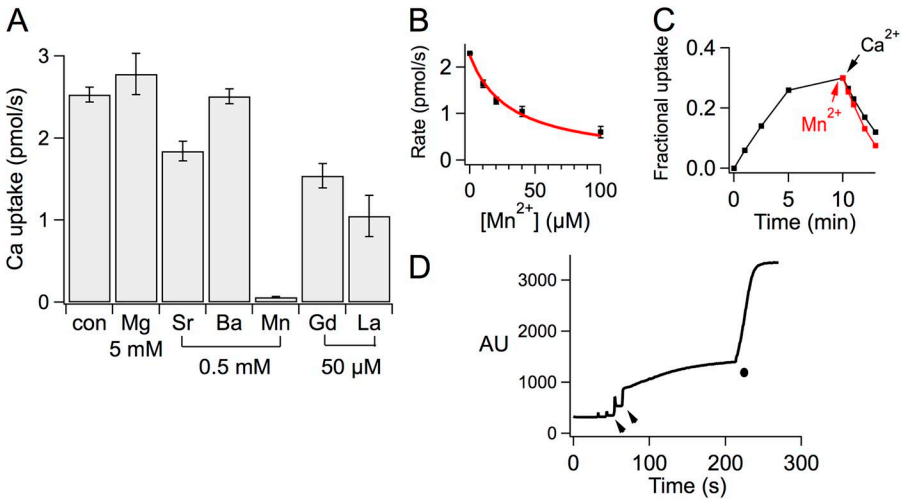

Figure 3. Ion selectivity of Letm1. (A) Initial rate of $^{45}Ca^{2+}$ (15 µM) uptake in the absence (con) or presence of other cations at the indicated concentrations. (B) Plot of $^{45}Ca^{2+}$ (15 µM) uptake rate in the presence of Mn^{2+} at the indicated concentrations. Red curve represents fit using single-site competition model, with K_i = 32 µM. (C) Mn^{2+}/Ca^{2+} exchange. $^{45}Ca^{2+}$ (15 µM) uptake into liposomes was run to approach steady state, and then 150 µM of nonradioactive Ca^{2+} (black arrow and squares) or Mn^{2+} (red arrow and squares) was added to liposome outside. (D) Mn^{2+} transport by Letm1. Liposomes were in symmetrical 120 mM KCl and 30 mM HEPES, pH 7.5. Internal solution also contains 0.5 µM Calcium Green 5N and 5 mM EGTA, and external solution contains 1 mM EGTA. Two doses of $MnCl_2$ (arrows) bring free Mn^{2+} to ~50 µM. A23187 was added at the black circle.

densities, strongly inhibit Ca^{2+} transport. Collectively, these results establish that Letm1 has an ion selectivity pattern characteristic of many Ca^{2+}-binding or transport proteins (Hille, 2003).

Among all the tested cations, Mn^{2+} exerts the strongest inhibition on Ca^{2+} transport, with an apparent K_i of 32 µM (Fig. 3 B). To differentiate if Mn^{2+} is a transported substrate or a blocker that clogs the ion translocation pathway, we tested whether Letm1 could catalyze Ca^{2+}/Mn^{2+} exchange. After accumulation of $^{45}Ca^{2+}$ to a steady state, nonradioactive Mn^{2+} or Ca^{2+} was added to the external solution at 10-fold excess. In either case, rapid $^{45}Ca^{2+}$ efflux ensued (Fig. 3 C), demonstrating Mn^{2+} import in exchange for internal $^{45}Ca^{2+}$. To further confirm Mn^{2+} transport, we show that an inward Mn^{2+} gradient drives Mn^{2+} into Letm1 liposomes, as reflected by the increased fluorescence of the preloaded Mn^{2+}-responsive fluorophore (Fig. 3 D). The Mn^{2+} turnover is similar to that of Ca^{2+}, ~1 s^{-1}.

Electrogenicity of Ca^{2+} transport

A basic property of any ion-transporting protein is its electrogenicity. Any mechanism that moves net charge across the membrane will be sensitive to membrane potential, whereas electroneutral transport will not. To gauge the electrogenicity of Letm1, we prepared Fluo-4–loaded proteoliposomes holding 1,000-fold inward or outward K^+ gradients. The application of Vln "clamps" large positive

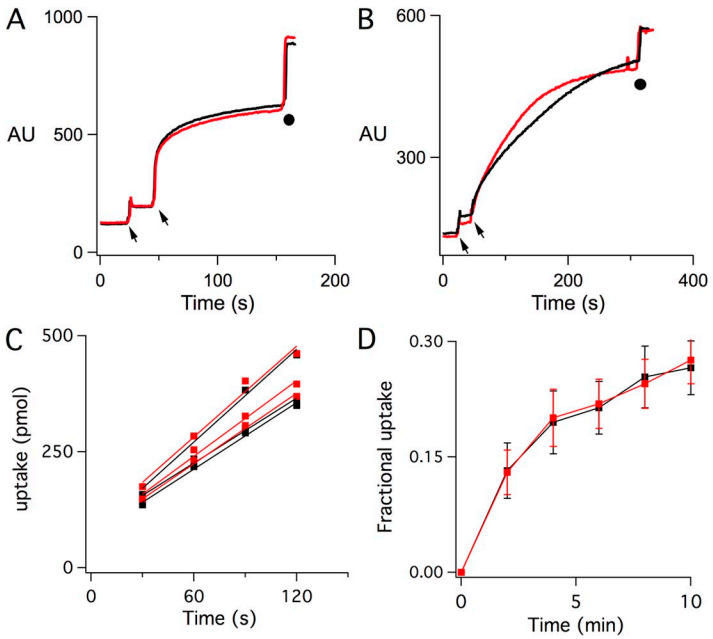

Figure 4. Electroneutral transport by Letm1. (A and B) Liposomes were prepared with 1,000-fold outward (A) or inward (B) K^+ gradient, with 100 mM KCl inside the liposomes and 100 mM NMDG-Cl and 0.1 mM KCl outside, or vice versa. Fluo-4–based Ca^{2+} flux assay was performed in the presence (red) or absence (black) of 1 µg/ml Vln. Left arrow, free $[Ca^{2+}]$ of ~300 nM; right arrow, free $[Ca^{2+}]$ of ~30 µM; black circle, 1 µg/ml A23187. (C and D) Voltage insensitivity of Ca^{2+} uptake. Initial rate (C) and 10-min time course (D) of $^{45}Ca^{2+}$ uptake in liposomes holding a 1,000-fold outward K^+ gradient were examined in the presence (red) or absence (black) of Vln.

(∼70 mV) or negative (approximately −140 mV) membrane potentials across the liposome membranes, as confirmed by Vln-mediated $^{86}Rb^+$ flux. However, Letm1-mediated Ca^{2+} uptake is strikingly unresponsive to these very different membrane potentials (Fig. 4, A and B), indicating that the protein catalyzes strictly electroneutral transport. This observation is further confirmed by $^{45}Ca^{2+}$ uptake experiments, showing that the imposition of negative membrane potential (approximately −140 mV) exerts no effect on Ca^{2+} influx rate (Fig. 4 C) or steady-state Ca^{2+} level (Fig. 4 D). We therefore conclude that in each turnover of Letm1, Ca^{2+} is obligatorily transported along with another ion such that no net charge transfer occurs.

Letm1 is a Ca^{2+}/H^+ antiporter

What is the other ion? In the experiments above, only two abundant inorganic ions are present in the proteoliposome system: K^+ and Cl^-. (We dismiss $NMDG^+$ as a possible substrate, as this is a large "inert" organic cation, biologically absent and hence unlikely to be transported.) That K^+ and Cl^- could be replaced by Na^+ and SO_4^{2-} without affecting Ca^{2+} transport (Fig. 2 B) would seem to rule out Ca^{2+}/Cl^- cotransport or Ca^{2+}/K^+ antiport to explain the electroneutrality of Letm1-mediated Ca^{2+} transport. However, the unlikely possibility remains that Letm1 uses either K^+ or Na^+ (or Cl^-/SO_4^{2-}) interchangeably as the transport partner of Ca^{2+}, and so it is worthwhile to test these ions further by examining if K^+ or Cl^- chemical gradients can drive Ca^{2+} flux, as demanded by coupled transport mechanisms. Accordingly, proteoliposomes with symmetrical 30 µM Ca^{2+} and 100 mM NMDG-Cl were prepared. In the Ca^{2+} fluorophore–based assay, the addition of 10 mM KCl (or NaCl) to the extraliposomal solution produces a large inward K^+ (or Na^+) gradient but fails to drive efflux of Ca^{2+} (Fig. 5 A). Diluting the liposome sample 20-fold into a solution containing NMDG-isethionate creates a 20-fold outward Cl^- gradient but also fails to evoke Ca^{2+} efflux (Fig. 5 B). In contrast, lowering external free Ca^{2+} by EGTA addition produces immediate Ca^{2+} efflux (Fig. 5, A and B). Collectively, these results unambiguously rule out Cl^-/Ca^{2+} cotransport and K^+/Ca^{2+} antiport mechanisms for Letm1.

The only plausible counterion remaining as a transport partner for Ca^{2+} is H^+, which is widely used in mitochondrial-coupled transport systems. Using similar conditions for testing coupled transport of K^+ and Cl^- as above, the extraliposomal solution was acidified to create a two-unit inward pH gradient. This maneuver causes an immediate drop of fluorescence, representing acid quench of leaked fluorophore in the external solution. A second fluorescence decline time course follows (τ of ∼10 s), reflecting rapid efflux of internal Ca^{2+} (Fig. 5 C). This signal is not an artifact caused by internal acidification as a result of inward H^+ leakage, because the liposomes are loaded with a high concentration of pH buffer, and because it is not seen in liposomes containing heat-treated Letm1 (Fig. 5 C). The experiment therefore demonstrates that Letm1 can use a pH gradient to drive Ca^{2+} flux against its chemical gradient. In other words, Letm1 is a Ca^{2+}/H^+ antiporter. Because Ca^{2+} transport is electroneutral, the H^+/Ca^{2+} exchange ratio must be 2:1.

Letm1 responses to inhibitors of mitochondrial calcium transport proteins

How is Letm1 function, as assessed in this biochemically defined system, related to the well-characterized Ca^{2+} uptake and release behavior of whole mitochondria? To approach this question, we examined the sensitivity of Letm1-mediated transport to three mitochondrial transport inhibitors: RR and Ru360, potent blockers of Ca^{2+} uptake by MCU, and CGP-37157, an inhibitor of a Na^+-dependent Ca^{2+} release mechanism (Gunter and Pfeiffer, 1990). The presence of these compounds on both sides of the liposomes at high concentration (10–25 µM) produces only weak or negligible inhibition on $^{45}Ca^{2+}$ flux (Fig. 6, A and B). This result provides a guide toward identifying the role of Letm1 in mitochondria, as discussed below.

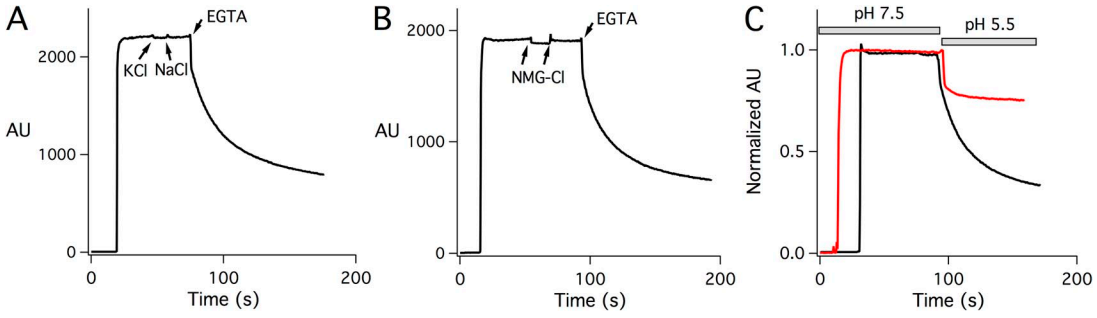

Figure 5. H^+-coupled Ca^{2+} transport. Liposomes loaded with 100 mM NMDG-Cl, 30 µM Ca^{2+}, 0.5 µM Calcium Green 5N, and 50 mM HEPES-NMDG, pH 7.5, were diluted 20-fold into solutions containing 30 µM Ca^{2+}, 20 mM HEPES, pH 7.5, and 100 mM NMDG-Cl (A and C) or 100 mM NMDG-isethionate (B). After the fluorescence recording was stable, 10 mM KCl, NaCl, or NMDG-Cl was added as indicated. To create an inward pH gradient, isethionic acid was used to acidify the extraliposomal solution. Black traces, Letm1 liposomes; red trace, liposomes containing heat-inactivated Letm1.

DISCUSSION

The Letm1 protein plays an essential role in mitochondrial physiology, as its knockdown perturbs KHE across the inner membrane as well as Ca^{2+} homeostasis in the matrix. To understand how Letm1 regulates these processes, its primary transport function must first be ascertained. Functional reconstitution provides a valuable approach to gain such information, as it circumvents the complicated biological context, allowing direct characterization of a protein's membrane–transport behavior. Indeed, it was the field of mitochondrial bioenergetics that long ago felt the usefulness of this reductionist strategy. The molecular character of the high energy connection between the electron transport chain and ATP synthesis—a chemical intermediate (Chance and Williams, 1956) or a transmembrane electrochemical H^+ gradient (Mitchell, 1961)—engaged the field in a drawn-out controversy, as vituperative as it was unresolvable in the biological membrane. To tackle this problem, Efraim Racker and colleagues famously reconstituted the inner membrane ATP synthase in liposomes, demonstrating that ATP is produced at the expense of a transmembrane pH gradient (Racker and Stoeckenius, 1974). These experiments verified the chemiosmotic hypothesis and redirected mitochondrial energy transduction mechanisms toward membrane electrochemical phenomena.

Here, we have used a reconstituted liposome system to address current uncertainties regarding the molecular mechanism of Letm1. Our results establish that Letm1 is a Ca^{2+}/H^+ antiporter that moves these two cations in strict, stoichiometric exchange across the membrane, in a ratio of 1 Ca^{2+} to 2 H^+. We hasten to note that the Ca^{2+}/H^+ antiport mechanism demands that Letm1 also use a Ca^{2+} gradient to drive thermodynamically uphill movement of H^+. However, we failed to obtain convincing recordings of H^+ flux, using either pH electrodes or pH-sensitive dyes. This is likely because the Letm1-mediated H^+ transport (k_{cat} of ~5 H^+/s) is much slower than the rate of proton leakage across the liposome membrane. Finally, we also show that Letm1 does not transport Rb^+, a close K^+ analogue, and nor does K^+ exert any influence on Ca^{2+} transport. From these results we conclude that Letm1 is not a K^+ transport protein, even though it might pose effects upon mitochondrial KHE.

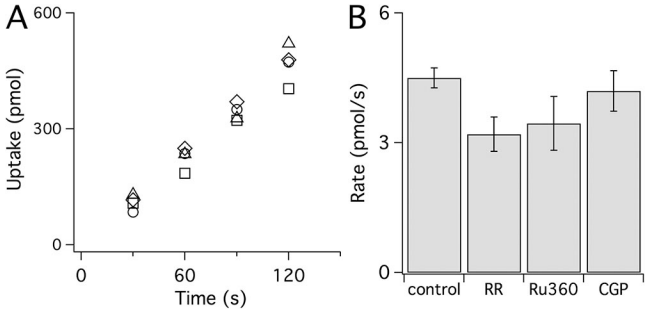

Figure 6. Letm1 response to inhibitors. (A) Uptake time course of 15 µM $^{45}Ca^{2+}$ in the absence (triangles) or presence of 25 µM RR (squares), 10 µM Ru-360 (diamonds), or 10 µM CGP-37157 (circles). (B) Statistics of inhibitor effects on Letm1 transport rate.

The Ca^{2+}/H^+ antiport mechanism inferred here sets an equilibrium condition for Letm1 in the mitochondrial inner membrane:

$$\left(\left[H^+\right]_{im}/\left[H^+\right]_m\right)^2 = \left[Ca^{2+}\right]_{im}/\left[Ca^{2+}\right]_m,$$

where the subscripts "im" and "m" indicate intermembrane space and matrix, respectively. In energized mitochondria, continuous H^+ extrusion fueled by the electron transport chain alkalinizes the matrix, creating an ~1-unit matrix-directed pH gradient (Santo-Domingo and Demaurex, 2012), which would allow Letm1 to reduce matrix Ca^{2+} to a concentration 100-fold lower than in the intermembrane space. Under normal physiological conditions, however, $[Ca^{2+}]$ in the intermembrane space (0.1–1 µM) is ~10-fold lower than that in the matrix (1–10 µM). The equilibrium condition would therefore demand that Letm1 continually expel Ca^{2+} from the matrix.

Intensive studies in the past few decades have established that the Ca^{2+} level in the matrix represents a steady state, regulated by constant uptake and release by three major Ca^{2+} pathways in the inner membrane (Gunter and Pfeiffer, 1990; Santo-Domingo and Demaurex, 2010; Pizzo et al., 2012). First, MCU, an RR-sensitive Ca^{2+} channel, catalyzes passive voltage-driven accumulation of Ca^{2+} into the matrix, becoming active only after cytoplasmic free $[Ca^{2+}]$ rises above a threshold (>3–5 µM). Second, the Ca^{2+}/Na^+ exchanger NCLX, a member of the well-studied NCX protein family, releases Ca^{2+} from the matrix and is inhibited by CGP-37157. Finally, the Ca^{2+}/H^+ exchange system, whose molecular identity has remained unknown, releases mitochondrial Ca^{2+} and has been characterized mainly in isolated mitochondria. The functional properties of Letm1 identified here are remarkably similar to those of the Ca^{2+}/H^+ exchange system. This transport system is (a) electroneutral, (b) insensitive to RR and CGP-37157, (c) inhibited by lanthanides, (d) as competent as a Mn^{2+} transporter, and (e) similar in Ca^{2+} dependence (Km of ~25 µM).

Letm1 deletion has been shown to cause yeast mitochondria to swell, an effect reversed by applying nigericin to facilitate KHE (Nowikovsky et al., 2012). At first glance, it seems difficult to reconcile these observations with our finding that Letm1 is a Ca^{2+}/H^+ antiporter. However, Ca^{2+} regulates numerous mitochondrial processes, some of which could be crucial for K^+ homeostasis. For instance, higher matrix $[Ca^{2+}]$ might stimulate Ca^{2+}-activated mitochondrial K^+ channels (Szabò et al., 2012). Increased K^+ entry into the matrix would overwhelm the capacity of native KHEs to remove matrix K^+ and thus could cause

osmotic swelling. The addition of nigericin would then restore the osmotic balance by enhancing KHE to match the electrophoretic K^+ flux. Previous work also shows that *Letm1* deletion impairs submicromolar Ca^{2+} (0.1–1 µM) uptake into the matrix (Jiang et al., 2009), which seems contradictory to the argument here that Letm1 removes mitochondrial Ca^{2+} under normal conditions. In energy-depleted mitochondria, however, Letm1 might import Ca^{2+} if $[Ca^{2+}]_{im}$ is higher than $[Ca^{2+}]_m$.

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