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Purification and functional reconstitution of intact *ral*-binding GTPase activating protein, RLIP76, in artificial liposomes✪

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We have recently shown that RLIP76, a *ral*-binding GTPase activating protein, mediates ATP-dependent transport of glutathione-conjugates (GS-E) and doxorubicin (DOX) (S. Awasthi *et al.*, *Biochemistry* 39, 9327, 2000). Transport function of RLIP76 was found to be intact despite considerable proteolytic fragmentation in preparations used for those studies, suggesting either that the residual intact RLIP76 was responsible for transport activity, or that the transport activity could be reconstituted by fragments of RLIP76. If the former were true, intact RLIP76 would have a much higher specific activity for ATP-hydrolysis than the fragmented protein. We have addressed this question by comparing transport properties of recombinant RLIP76 and human erythrocyte membrane RLIP76 purified in buffers treated with either 100 or 500 µM

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Abbreviations: RLIP76, recombinant or human tissue purified ral-interacting-protein (chromosome 18p), homologous with *ral*BP1 in rat; GS-E, glutathione-electrophile conjugates; DOX, doxorubicin (adriamycin); DNP-SG, *S*-(2,4-dinitrophenyl) glutathione; DNP-SG ATPase, the protein fraction characterized by the presence of a 38 kDa band purified by DNP-SG affinity chromatography from human tissues; SDS/PAGE, reduced denaturing polyacrylamide gel electrophoresis; GAP, GTPase activating protein; Pgp, P-glycoprotein; MRP, multi-drug resistance associated protein; COL, colchicine; LTC4, leukotriene C4; DAU, daunorubicin; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; BHT, butylated hydroxytoluene; PMSF, phenylmethylsulfonyl fluoride; $C_{12}E_9$, polidocanol.

serine protease inhibitor, PMSF. The purity and identity of recombinant and human erythrocyte RLIP76 was established by SDS/PAGE and Western-blot analysis. These studies confirmed the origin of the 38 kDa protein, previously referred to as DNP-SG ATPase, from RLIP76. Higher PMSF concentration resulted in lower yield of the 38 kDa band and higher yield of intact RLIP76 from both human and recombinant source. In contrast, the substrate-stimulated ATPase activity in presence of DNP-SG, doxorubicin, daunorubicin, or colchicine were unaffected by increased PMSF; similarly, ATP-dependent transport of doxorubicin in proteoliposomes reconstituted with RLIP76 was unaffected by higher PMSF. These results indicated that limited proteolysis by serine proteases does not abrogate the transport function of RLIP76. Comparison of transport kinetics for daunorubicin between recombinant *vs* human erythrocyte RLIP76 revealed higher specific activity of transport for tissue purified RLIP76, indicating that additional factors present in tissue purified RLIP76 can modulate its transport activity.

We have shown that RLIP76, a *ral*-binding GAP, can be reconstituted in artificial liposomes to function as an energy dependent, multi-specific transporter with broad substrate specificity including un-metabolized amphiphilic xenobiotic toxins, glutathionylated-metabolites of xenobiotic electrophiles, and endogenous electrophiles generated from oxidative metabolism of lipids (i.e. leukotrienes) [1–7]. These findings demonstrated that RLIP76 is a novel link between the *ras*-linked cellular signaling pathways [8–15] and transport mediated drug resistance, and suggests that amphiphilic drug substrates could exert toxic or therapeutic effects through competitive inhibition of efflux of physiologic glutathione-conjugates. Thus, elucidation of RLIP76 function at a molecular level could offer mechanistic insight into a diverse array of incompletely understood biological, toxicological and pharmacological phenomena in which physiologic glutathione-conjugates participate [16–19].

RLIP76 was initially cloned by Jullien-Flores *et al.* [8] using a two-hybrid screen for *ral*-binding proteins [8–11, 20, 21]. RLIP76 binds to *ral*-GTP but does not display GAP activity towards it. However, it does display GAP activity towards *cdc*42, a *ras*-family protein in the *rho/rac*-pathway thought to regulate membrane plasticity [8, 9, 12, 13]. Recent studies by Jullien-Flores *et al.* [22] suggest that RLIP76 participates in receptor mediated endocytosis by binding AP2, a clathrin adaptor protein, and *ral*-GTP inhibits clathrin mediated endocytosis by binding to RLIP76. These authors also proposed that RLIP76 functions as a modular protein, an assertion supported by our finding that RLIP76 gives rise to a number of fragments [1].

RLIP76 contains numerous serine-protease cleavage sites, particularly in the N-terminal region [23]. Despite considerable proteolytic fragmentation, we observed ATP-hydrolysis and ATP-dependent transport activity in liposomes reconstituted with highly purified total RLIP76 [1, 23]. If intact RLIP76 were an absolute requirement for transport, ATP-hydrolysis and transport activities would be expected to increase significantly in preparations enriched for intact RLIP76. If the opposite were true, we could conclude that fragments of RLIP76 can reconstitute a functional transporter in artificial membranes. In present studies we have compared the ATPase and transport properties of RLIP76 purified in buffers treated with either 100 or 500 μM PMSF.

MATERIALS AND METHODS

Reagents. Blood was collected according to a protocol approved by the Institutional Review Board from a healthy adult male. CNBr-activated Sepharose 4B, CDNB, GSH, ATP, PMSF, BHT, EGTA, EDTA, soybean asolectin, cholesterol, $C_{12}E_9$, and horseradish-peroxidase-coupled goat-anti-rabbit antibodies were purchased from Sigma Chemical

Company (St. Louis, MO). Reagents for SDS/PAGE and SM-2 BioBeads were purchased from Bio-Rad Laboratories (Hercules, CA). DAU was obtained from Wyeth Laboratories (Philadelphia, PA). Rabbit anti-human antibodies toward RLIP76 and erythrocytes DNP-SG-ATPase used in these studies were the same as described previously [1, 2, 4, 6]. $[^{14}C]$doxorubicin (specific activity 57 mCi/mmol) was purchased from Amersham corporation (Arlington Heights, IL). $[^{3}H]$daunorubicin (specific activity 1.7 Ci/mmol) and $[^{32}P]$ATP (specific activity 3000 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA).

Cloning and prokaryotic expression of RLIP76. The 1968 bp full length open reading frame cDNA of human RLIP76, cloned from a λgt11 human bone marrow library by immunoscreening using anti-DNP-SG ATPase antibodies, was subcloned into the prokaryotic expression plasmid, pET-30a(+) (Novagen), creating the pET30-RLIP76 plasmid free of extraneous sequences. This plasmid was transfected into *Escherichia coli* BL21(DE3). Details of experimental procedures for cloning of RLIP76 full-length cDNA and its expression in *E. coli* have been provided previously [1].

Purification of RLIP76. Purifications were monitored by measuring DNP-SG ATPase activity defined as ATPase activities measured in the absence from that observed in the presence of 120 μM DNP-SG, 10 μM DAU, 10 μM DOX, and 10 μM COL [1, 4]. Since polidocanol interfered with Bradford reagent, protein was estimated by the method of Minamide & Bamburg [24]. Western blot analysis was performed by the method of Towbin *et al.* [25]. SDS/PAGE was carried out in the buffer system described by Laemmli [26]. DNP-SG was synthesized, purified, authenticated, and used for preparing DNP-SG-Sepharose 4B-affinity resin [2]. Human erythrocyte RLIP76 was purified from peripheral blood collected from one 35 year old male donor using the DNP-SG Sepharose affinity chromatography [4, 6]. This DNP-SG affinity procedure, with minor modifications detailed previously [1], was also used to purify RLIP76 protein from *E. coli* BL21(DE3) transfected with pET30a(+)-RLIP76 plasmid.

Functional reconstitution of RLIP76. Reconstitution of DNP-SG affinity purified DNP-SG ATPase and RLIP76 in proteoliposomes was separately performed using the method previously described by us [1, 5, 6, 27]. The size of reconstituted vesicles was examined by electron microscopy and intra-vesicular volume was estimated by $[^{14}C]$inulin trapping [4, 5]. Control vesicles were prepared using an equal amount of crude protein from *E. coli* not expressing RLIP76 or crude erythrocyte ghost protein.

Daunorubicin transport by RLIP76. DAU transport measurements were carried out as previously described for DOX, DNP-SG, and COL transport [2, 5, 6]. Reconstituted proteoliposomes were diluted 2–20-fold in 90 μl transport-buffer containing $[^{3}H]$DAU (1.2 $\times$ 10^4 c.p.m./nmol) radiolabeled substrate and incubated for 15 min at 37°C. Measurement of ATP-dependent transport was initiated by addition of 10 μl ATP prepared in transport buffer to the experimental group and 10 μl equiosmolar NaCl to the control. The final concentration of ATP was 4 mM. After incubation with ATP for 5 min, aliquots of the reaction mixtures containing 2–400 ng reconstituted protein was filtered using a Millipore Multiscreen 96 well plate vacuum filtration system as described previously [1, 2, 5, 6]. The filtration membranes were individually cut out of the filtration manifold and dissolved in scintillation fluid overnight before radioactivity counting. Background binding of the radiolabeled substrate to the filtration membrane was determined for each experimental condition and subtracted to obtain the vesicular uptake of substrate. The effect of ATPase on substrate uptake by vesicles was studied by comparing uptake with or without ATP by liposomes reconstituted in the absence or presence of RLIP76 or human eryth-

rocyte DNP-SG ATPase fraction. Experimental details are provided in the figure legends.

Kinetic analysis of DAU transport. ATP-dependent DAU transport kinetics were evaluated at eight DAU concentrations between 1.5–10 μM with ATP concentration fixed at 4 mM to obtain the K_m for DAU. The K_m for ATP was determined with fixed concentration of [^{3}H]DAU at 5 μM. Triplicate measurements of uptake were performed in the absence and presence of ATP in both control liposomes and proteoliposomes at each of eight concentrations of DAU and seven concentrations of ATP.

RESULTS

Purification of intact RLIP76

We purified recombinant human RLIP76 expressed in *E. coli*, and human RLIP76 from erythrocytes in the presence of either 100 or 500 μM PMSF by the present method as described above. Western-blot analysis of DNP-SG-Sepharose 4B affinity purified RLIP76 fraction is presented (Fig. 1). Intact RLIP76 was observed as a band at 95 kDa, confirmed by N-terminal sequencing. This anomalous behavior of RLIP76 in SDS/PAGE has been described by us [1] and shown by other investigators for Ral-BP1, the rat homolog of RLIP76 [21]. No significant contaminant protein bands were observed in SDS/PAGE and the pattern of bands in Western-blot was nearly identical to that seen in SDS/PAGE. We have previously shown that the present purification method in the presence of 100 μM PMSF yields RLIP76 which is >95% pure as determined by amino acid analysis [1]. Purity of RLIP76 protein obtained in presence of buffers containing either 100 or 500 μM PMSF by amino acid analysis indicated that the higher concentration of PMSF did not significantly increase purity.

Exclusion of PMSF from buffers during purification of RLIP76 from erythrocyte mem-

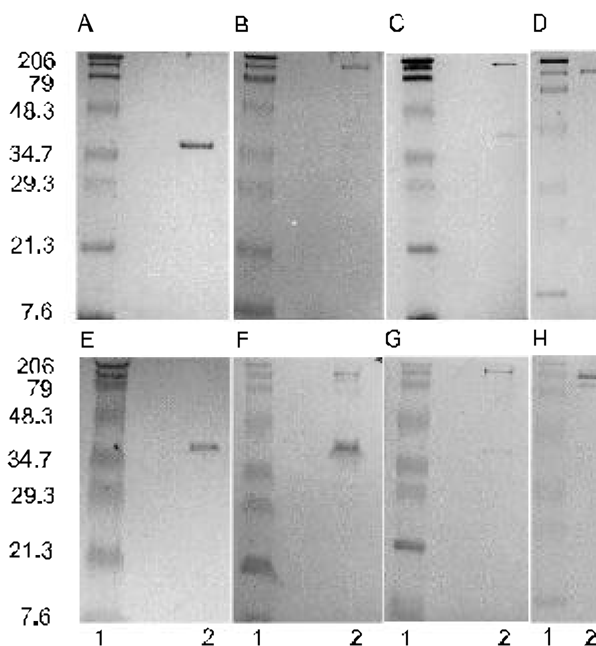

Figure 1. SDS/PAGE and Western-blot analysis of purified human erythrocyte and recombinant RLIP76.

DNP-SG-Sepharose-4B affinity was used to purify human erythrocyte and recombinant human RLIP76 in the presence of buffers treated with either 100 or 500 μM PMSF. Coomassie-stained SDS/PAGE of 5 μg protein aliquots from human erythrocyte RLIP76 purified in presence of 100 or 500 μM PMSF (panels A and B, respectively), and recombinant human RLIP76 purified in presence of 100 or 500 μM PMSF (panels C and D, respectively) are presented. Western-blot analysis was carried out against polyclonal anti-RLIP76 antibodies raised in rabbit. Horseradish peroxidase-linked goat anti-rabbit antibodies were used as secondary antibodies and the blot was developed using 4-chloro-1-naphthol. Results of Western-blot analysis of 5 μg protein aliquots from human erythrocyte RLIP76 purified in presence of 100 or 500 μM PMSF (panels E and F, respectively), and recombinant human RLIP 76 purified in presence of 100 or 500 μM PMSF (panels G and H, respectively) are presented. Lane 1 in all panels are broad-range pre-stained molecular mass markers (Bio-Rad).

brane resulted in decreased yield, stability and activity of the 38 kDa band (data not presented). This finding was attributed to high concentration of serine protease concentration of blood, and their activation during blood collection and storage. In order to overcome this problem, we included PMSF in

blood collection buffer, resulting in significantly increased yield and activity of purified protein from blood [4, 6] as compared with our previous result [2]. Consistent with our previous findings [4, 6], DNP-SG affinity purification from human erythrocyte in buffers treated with 100 μM PMSF yielded a major band near 38 kDa in Coomassie-stained SDS/PAGE (Fig. 1A, lane 2) and this band was recognized by polyclonal rabbit-anti-human-RLIP76 antibodies (Fig. 1E, lane 2). In contrast, SDS/PAGE revealed a predominant 95 kDa band from the purification of human erythrocyte RLIP76 in the presence of 500 μM PMSF (Fig. 1B, lane 2). Western-blot analysis revealed that the 95 kDa band was recognized by anti-RLIP76 antibodies, and that the 38 kDa protein could still be detected in this preparation (Fig. 1F, lane 2). The higher PMSF concentration, thus, partially protected RLIP76 from cleavage to yield the 38 kDa band.

Purification of recombinant human RLIP76 in buffers treated with 100 μM PMSF with present procedures showed much less fragmentation than we have observed previously [1]. The 38 kDa, however, could be detected by both SDS/PAGE (Fig. 1C, lane 2) and Western-blot analysis (Fig. 1G, lane 2). Purification of recombinant human RLIP76 in the presence of 500 μM PMSF showed virtual disappearance of the 38 kDa band from the preparation as shown by both SDS/PAGE (Fig. 1D, lane 2) and Western-blot analysis (Fig. 1H, lane 2). These results show that we can enrich for intact RLIP76 by increasing the concentration of PMSF used to prepare purification buffers and shortening overall purification time.

The effect of PMSF on recombinant-RLIP76 ATPase activity and drug transport

In order to determine whether this increased yield of intact RLIP76 activity affected its activity, we compared substrate-stimulated ATPase activity of recombinant human RLIP76 purified with either 100 or 500 μM PMSF (Fig. 2). Surprisingly, no effect was

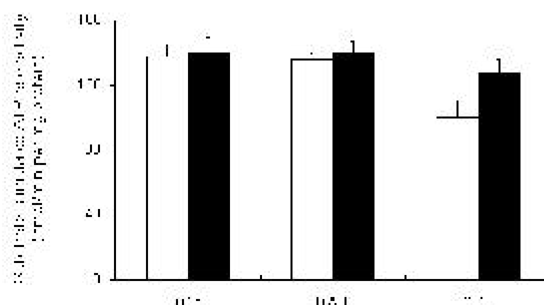

Figure 2. Effect of PMSF on ATPase activity of recombinant RLIP76.

The ATPase activity of recombinant RLIP76 purified in presence of either 100 (open bars) or 500 (closed bars) μM PMSF. ATPase activity was measured by determining the cleavage of [γ-^{32}P]ATP as previously described in the absence or presence 10 μM DOX, DAU and COL [1]. Substrate stimulated activity was calculated by subtracting ATPase activity observed in the absence of substrate. Mean values ±S.D. for three experiments are shown.

found on the DOX, DAU or COL stimulated activity of RLIP76 with either 100 or 500 μM PMSF. These finding strongly suggested that the ATPase activity of RLIP76 remains despite limited proteolytic cleavage. Similarly, ATP-dependent transport of DOX was in proteoliposomes reconstituted with RLIP76 was found not be significantly affected by PMSF (18.8 ± 1.3 *vs.* 19.5 ± 1.5 nmol/min per mg, ± S.D., n = 6, with 100 and 500 μM PMSF, respectively).

Comparison of ATPase activity of human erythrocyte *vs.* recombinant RLIP76

Since higher PMSF did not affect the functional activities of RLIP76, we performed the comparison of human erythrocyte *vs.* recombinant RLIP76 using only RLIP76 purified in the presence of 100 μM PMSF. DNP-SG affinity chromatography was monitored by measurements of basal and substrate-stimulated ATPase activity (Tables 1 and 2). The yield of 24 μg purified DNP-SG ATPase from 20 ml of

Table 1. Purification of RLIP76 from human erythrocyte

Fraction	Total stimulated ATPase activity (mU)		Protein (mg)	Stimulated specific activity (mU/mg protein)		Yield (%)		Purification (fold)	
	DNP-SG	DAU		DNP-SG	DAU	DNP-SG	DAU	DNP-SG	DAU
Detergent solubilized fraction	16.0	8.9	20.0	0.80	0.45	100	100	–	–
DNP-SG affinity chromatography	7.8	5.5	0.024	325*	229*	49	62	406	509

Purification buffers were treated with 100 μM PMSF. Twenty ml human blood was used for purification. One milliunit (mU) of enzyme catalyzed 1 nmol ATP hydrolysis per min at 37°C. DNP-SG and DAU stimulated activity was obtained by subtracting the basal ATPase activities of the fractions determined in the absence of DNP-SG and DAU from the activities determined in the presence of 120 μM DNP-SG and 10 μM DAU. *Basal ATPase activity of this fraction was 191 nmol/min per mg protein, and was increased to 516 and 420 nmol/min per mg, in the presence of 120 μM DNP-SG and 10 μM DAU, respectively. Results presented are from a single purification with activity determinations performed in triplicate. Relative standard deviations were < 8% in all cases.

blood (Table 1) was consistent with our previous studies [4, 6]. Basal ATPase activity of DNP-SG ATPase purified from erythrocyte was 191 nmol/min per mg, which was stimulated 2.7-fold (516 nmol/ min per mg) in the presence of DNP-SG (Table 1). The basal ATPase activity of purified RLIP76 was 150 nmol/min per mg, which was stimulated 2.1-fold (321 nmol/min per mg) by DNP-SG (Table 2). The basal as well as DNP-SG stimulated ATPase activities of DNP-SG ATPase purified from erythrocytes were significantly higher ($P < 0.01$, n = 3) than those of the corresponding activities of recombinant RLIP76.

Comparison of transport activity of human erythrocyte *vs.* recombinant RLIP76

Proteoliposomes were reconstituted with recombinant or human erythrocyte RLIP76 purified in the presence of 100 μM PMSF. Detailed transport kinetics of ATP-dependent DAU-transport were compared. For transport studies with recombinant RLIP76 (Fig. 3), uptake of DAU, with or without ATP, was measured in proteoliposomes reconstituted with purified RLIP76. Control proteoliposomes were reconstituted in the presence of an equal amount of crude *E. coli* protein. Uptake with

Table 2. Purification of recombinant RLIP76 from transformed *E. coli*

Fraction	Total stimulated ATPase activity (mU)		Protein (mg)	Stimulated specific activity (mU/mg protein)		Yield (%)		Purification (fold)	
	DNP-SG	DAU		DNP-SG	DAU	DNP-SG	DAU	DNP-SG	DAU
Detergent solubilized fraction	100.5	76.0	39.5	2.5	1.9	100	100	–	–
DNP-SG affinity chromatography	25.7	20.4	0.15	171*	136*	26	27	67	71

Purification buffers were treated with 100 μM PMSF. Two hundred ml of *E. coli* culture was used for purification. One milliunit (mU) of enzyme catalyzed 1 nmol ATP hydrolysis per min at 37°C. DNP-SG and DAU stimulated activity was obtained by subtracting the basal ATPase activities of the fractions determined in the absence of DNP-SG and DAU from the activities determined in the presence of 120 μM DNP-SG or 10 μM DAU. *Basal ATPase activity of this fraction was 150 nmol/min per mg protein, and was increased to 321and 286 nmol/min per mg, in the presence of 120 μM DNP-SG and 10 μM DAU, respectively. Results presented are from a single purification with activity determinations performed in triplicate. Relative standard deviations were < 8% in all cases.

or without ATP in RLIP76 *vs.* control proteoliposomes are shown for recombinant RLIP76 (Fig. 3 A–F). The presence of ATP caused an increase in uptake of DAU only in liposomes were observed for both ATP (Fig. 3E) and DAU (Fig. 3F).

All transport studies with recombinant RLIP76 proteoliposomes indicated that DAU-

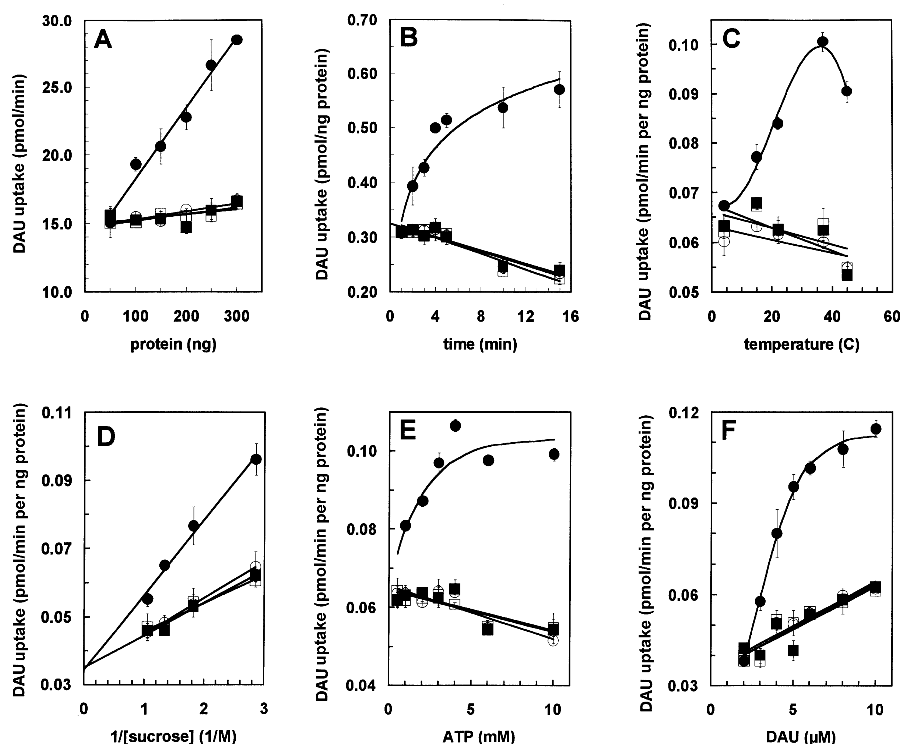

Figure 3. Transport of DAU by recombinant RLIP76.

RLIP76 purified using DNP-SG affinity chromatography was reconstituted in artificial liposomes and uptake was measured in RLIP76 proteoliposomes (circles) and control proteoliposomes containing an equal quantity of crude bacterial protein (squares) in the presence (filled symbols) or absence (empty symbols) of ATP. All transport studies were carried out using 250 ng protein/assay except when protein was varied (panel A). Incubation time was 5 min except for time dependence studies (panel B). Temperature was 37°C except for temperature dependence studies (panel C). External sucrose concentration was 250 mM, except for studies of osmolar dependence studies (panel D). ATP was 4 mM except in ATP-dependence studies (panel E). DAU was 5 μM except in DAU-dependence studies (panel F). Mean values ±S.D. for three experiments are shown.

proteoliposomes reconstituted with RLIP76 and not in control liposomes. ATP-dependent uptake of DAU was linear with respect to the amount of RLIP76 used for reconstitution of vesicles (Fig. 3A). The uptake of DAU by RLIP76 proteoliposomes was time dependent in a manner consistent with uptake by a single compartment (Fig. 3B). The transport was temperature sensitive with an optimal near 37°C (Fig. 3C), and sensitive to osmolarity of extra-vesicular medium (Fig. 3D). Saturable kinetics for the transport by these proteo- uptake was identical in control proteoliposomes in the absence or presence of ATP, and equal to the uptake observed in proteoliposomes containing recombinant RLIP76 in the absence of ATP. Therefore, the calculation of transport rates were unaffected by inclusion or exclusion of uptake results of control liposomes. Because background binding of DAU to the filtration membranes was unaffected by the presence of liposomes or nucleotides, its exclusion had no effect on calculations of ATP-dependent uptake rates (data not

presented). We have previously shown that in identical preparations of RLIP76, estimation of COL [6] or DOX [5] transport rate is unaffected by including or excluding the values for COL or DOX uptake by control artificial liposomes in the presence or absence of ATP.

port studies (Fig. 4A), was time dependent and consistent with uptake into a single compartment (Fig. 4B), sensitive to temperature with an optimal near 37°C (Fig. 4C), and saturable with respect to ATP (Fig. 4D) and DAU (Fig. 4E).

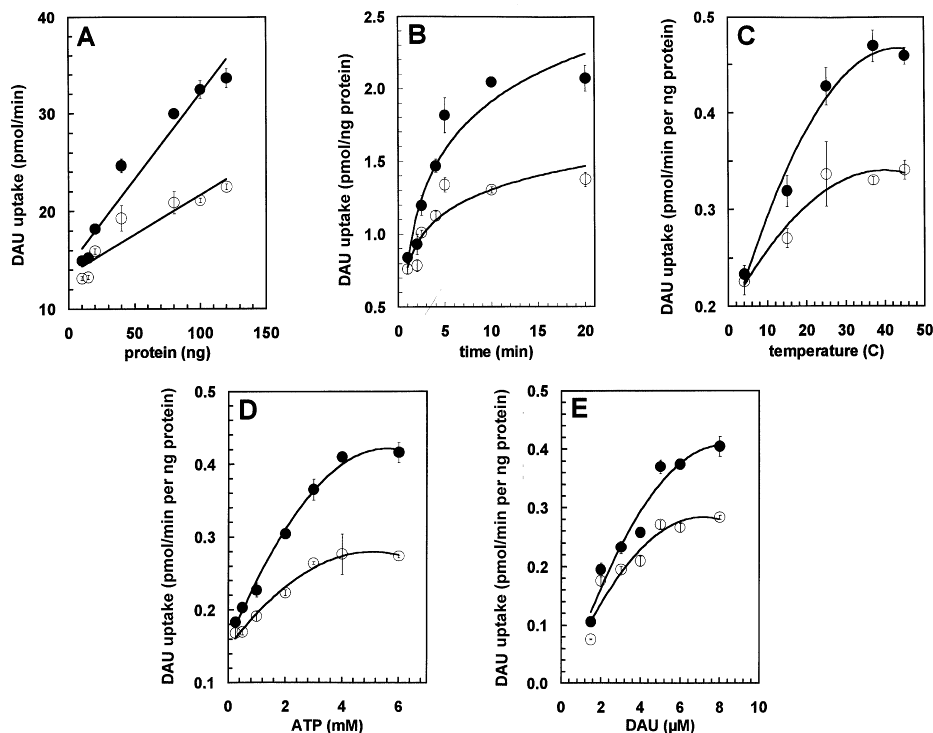

Figure 4. Transport of DAU by human erythrocyte DNP-SG ATPase.

DNP-SG ATPase purified by DNP-SG affinity chromatography was reconstituted in artificial liposomes and uptake was measured in the presence (filled circles) or absence (open circles) of ATP. All transport studies were carried out using proteoliposomes containing 80-ng protein/assay except when protein was varied (panel A). Incubation time after addition of ATP was 5 min except for studies where time dependent uptake was determined (panel B). Temperature was 37°C except for temperature dependence studies (panel C). ATP concentration was fixed at 4 mM except for studies to determine ATP-dependence of transport (panel D). DAU concentration was fixed at 5 μM except for studies to determine DAU-dependence of transport (panel E). Mean values ±S.D. for three experiments are shown.

Thus, DAU-transport with human erythrocyte RLIP76 was measured by subtracting DAU uptake in human-erythrocyte RLIP76 liposomes in the absence of ATP from that observed in its presence (Fig. 4 A–E). Unlike recombinant RLIP76, proteoliposomes reconstituted with human erythrocyte RLIP76 consistently demonstrated ATP-independent DAU-uptake. As with recombinant RLIP76, DAU-transport by human erythrocyte RLIP76 was linearly increased with protein used for trans-

Kinetic properties of DAU-transport by human erythrocyte and recombinant RLIP76 showed interesting similarities and differences (Table 3). ATPase activities of both protein fractions were roughly twice of their maximal transport activity (Table 3). Considering that only half of the transporter is expected to be reconstituted in proteoliposomes a transport competent orientation (outside-to-inside orientation), these results suggest a 1:1 stoichiometry of transport of DAU with

ATP-hydrolysis, consistent with our previous studies DOX and DNP-SG transport [1]. Both transporters had a temperature optimum near 37°C, and the K_m values for ATP and DAU differed slightly, but not significantly. The V_{max} calculated by fitting a two-substrate random bi-bi sequential kinetic model (120 and 70 nmol/min per mg for the erythrocyte and recombinant protein) were expectedly significantly greater than the measured activities, because ATP and DAU concentrations used for routine activity assays are significantly below saturating concentrations. However, the ATPase activity as well as calculated V_{max} for DAU-transport was significantly greater for human erythrocyte as compared with recombinant RLIP76. These observations could be explained by either the presence of additional activating factors in preparations of DNP-SG affinity purified human erythrocyte RLIP76 fraction, potential inhibitors present in the purified recombinant RLIP76 fraction, or lack of some essential post-translational modifications in RLIP76 from the prokaryotic source. Additional studies are needed to address this question.

Table 3. Comparison of ATPase activity and transport by human erythrocyte DNP-SG ATPase and RLIP76[a]

Parameters	Human erythrocyte RLIP76	Recombinant RLIP76
DNP-SG stimulated ATPase activity (nmol/min per mg)	325	171
DAU stimulated ATPase activity (nmol/min per mg)	229	136
V_{max} of DAU transport (nmol/min per mg)	120	70
K_m for ATP (mM)	1.6	2.7
K_m for DAU (μM)	4.4	2.8
Optimal temperature (°C)	37	37

[a] Both proteins were purified with 100 μM PMSF.

DISCUSSION

We have previously demonstrated that DNP-SG-Sepharose 4B affinity chromatography can be used to purify RLIP76 to > 95% homogeneity from *E. coli* expressing recombinant human RLIP76. Initial purification yielded preparations with several fragments that were shown to be derived from RLIP76 and raised questions regarding whether intact RLIP76 or its fragments mediated transport and ATP hydrolysis [1]. Refinement of the purification procedure by shortening the time of purification from 8 to 5 days have resulted in an overall increase in recovery of intact RLIP 76, but persistence of the 38 kDa protein band, which we have observed in previous DNP-SG affinity purification from human erythrocyte and other tissues [2, 4, 6, 28–31]. Present studies were carried out to determine whether treatment of purification buffers with 500 μM PMSF, as opposed to 100 μM PMSF used in all previous studies, would eliminate 38 kDa protein from the purified preparation.

Our results demonstrate that proteolysis is a significant contributor to the previously observed variability in SDS/PAGE patterns of purified DNP-SG ATPase and RLIP76. Using a higher concentration of PMSF than in previous studies, and a shortened duration of purification, we were able to show for the first time that RLIP76 could be purified intact. Despite the greater yield of intact RLIP76 from both recombinant and human erythrocyte source, neither its overall ATPase activity nor transport activity was affected by partial proteolytic degradation. These findings sup-

port a model in which RLIP76 can constitute an ATP-dependent amphiphilic xenobiotic transporter in membranes either as an intact protein or as fragments that can reconstitute ATPase and transport activities. These findings are consistent with the assertion by Jullien-Flores *et al.* [8] that RLIP76 is a modular protein. Their studies have shown that RLIP76 binds AP2, a clathrin binding adaptor protein [22].

Remarkably, fragmentation of RLIP76 appears to occur similarly in both prokaryotic and eukaryotic cells and is inhibited by a serine-protease inhibitor. The specific pattern of proteolytic degradation appears to be a property inherent in its sequence. Analysis of RLIP76 sequence shows that the N-terminal is very rich in serine-protease cleavage sites, whereas the C-terminal has only a few of those sites. This is perhaps the reason that the C-terminal peptide is relatively resistant to proteolysis. The limited proteolytic degradation does not appear to significantly impact overall ATPase or transport activity of purified RLIP76 fractions or human erythrocyte DNP-SG ATPase fractions. This would suggest that RLIP76 is a precursor protein that gives rise to multiple peptides, which can together reconstitute a functional transport complex for structurally diverse amphiphilic compounds. This postulate is supported by our recently reported studies showing that recombinant N-terminal (residues 1–367) and the C-terminal (residues 410–655) when separately reconstituted in proteoliposomes do not function as transporters but when they are incorporated in proteoliposomes together they can mediate ATP-dependent transport of DOX and COL [23]. Further studies are needed to evaluate the significance of the proteolytic cleavage and the role of each fragment of RLIP76 in its transport function.

Present studies validate our earlier suggestion that RLIP76 is identical with DNP-SG ATPase and that both undergo proteolysis to generate various peptides. Subtle differences observed in the fragmentation pattern and ATPase or transport activities of DNP-SG ATPase purified from erythrocyte and RLIP76 purified from transformed *E. coli* may arise from their differential proteolytic processing in eukaryotic and prokaryotic cells. Alternatively, it is possible that essential lipids in erythrocyte membrane co-purified with RLIP76, or the differences in post-transcriptional or post-translational processing of RLIP76 in mammalian cells account for the observed differences. Reasons for these observed differences in the catalytic functions of RLIP76 and DNP-SG ATPase are currently being studied in our laboratory. Since RLIP76 is a protein involved *ras*-linked cellular signaling pathways, our findings imply that GS-E transport functions in signalling within *ras*-linked pathways.

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