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Novel Function of Human RLIP76: ATP-Dependent Transport of Glutathione Conjugates and Doxorubicin†

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ABSTRACT: Active transport of conjugated and unconjugated electrophiles out of cells is essential for cellular homeostasis. We have previously identified in human tissues a transporter, DNP-SG [*S*-(2,4-dinitrophenyl)-glutathione] ATPase, capable of carrying out this function [Awasthi et al. (1998) *Biochemistry 37*, 5231−5238, 5239−5248]. We now report the cloning of DNP-SG ATPase. The sequence of the cDNA clone was identical to that of human RLIP76, a known Ral-binding protein. RLIP76 expressed in *E. coli* was purified by DNP-SG affinity chromatography. Purified recombinant RLIP76: (1) had ATPase activity stimulated by DNP-SG or doxorubicin (DOX), and the K_m values of RLIP76 for ATP, DOX, and DNP-SG were similar to those reported for DNP-SG ATPase; (2) upon reconstitution with asolectin as well as with defined lipids, catalyzed ATP-dependent transport of DNP-SG and DOX with kinetic parameters similar to those of DNP-SG ATPase; (3) when transfected into K562 cells, resulted in increased resistance to DOX, and increased ATP-dependent transport of DNP-SG and DOX by inside-out membrane vesicles from transfected cells; (4) direct uptake of purified RLIP76 protein into mammalian cells from donor proteoliposomes confers DOX resistance. These results indicate that RLIP76, in addition to its role in signal transduction, can catalyze transport of glutathione conjugates and xenobiotics, and may contribute to the multidrug resistance phenomenon.

Glutathione (GSH),[1] a sulfhydryl-containing tripeptide, is the chief soluble nucleophile in cells and serves to protect nucleophilic sites on DNA bases by scavenging mutagenic electrophilic chemicals. Glutathione *S*-transferase (GST) catalyzes the formation of conjugates of electrophiles with GSH (GS-E). Although GS-E are generally less reactive than the parent electrophiles, activation to more reactive species is well documented and appears to be an underlying mechanism for organ-specific and idiosyncratic toxicities of many drugs and toxins (*1, 2*). GS-E derived from arachidonic acid, leukotrienes, are crucial mediators of cell motility and chemotaxis (*3*). GS-E have been shown to be potent inhibitors of GSH-linked enzymes such as GSTs and glutathione reductase (*4, 5*). Metabolism of GS-E to mercapturic acids, as well as through active efflux cells by incompletely defined membrane transporters, protects cells from adverse effects of their accumulation. We originally reported the existence of a unique ATP-dependent GS-E transport activity (*6, 7*) and designated it as dinitrophenyl *S*-glutathione (DNP-SG) ATPase because it stimulated ATP hydrolysis in the presence of DNP-SG. We later showed that DNP-SG ATPase had extraordinarily wide substrate specificity including various anionic conjugates as well as weakly cationic amphiphilic chemotherapy drugs such as doxorubicin (Adriamycin, DOX) and vinblastine (*8*), classical substrates for ATP-dependent transport by P-glycoprotein (Pgp) (*9*). These studies extended the potential role of GS-E to include competitive inhibition of the efflux of chemotherapeutic drugs, and proposed the function of GS-E transporters as potential mediators of multi-drug resistance (MDR). Subsequent studies by other investigators showing that MDR-associated protein (MRP) could also mediate the transport of GS-E and amphiphilic chemotherapy drugs (*10, 11*) substantiated our hypothesis. We have recently reported successful purification and functional reconstitution of DNP-SG ATPase into artificial liposomes to demonstrate ATP-dependent transport of both GS-E, DOX and colchicine (*12−14*). The molecular identity of DNP-SG ATPase, however, remained elusive due to poor yields from purification, aberrant behavior in SDS−PAGE, and rapid degradation of the purified protein (*12, 15*). This prompted us to clone the cDNA encoding DNP-SG ATPase using antibodies raised against the purified protein, and to use heterologously expressed DNP-SG ATPase for further characterization.

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[1] Abbreviations: GSH, glutathione; DNP-SG, *S*-(2,4-dinitrophenyl)-glutathione; GS-E, glutathione−electrophile conjugate; GST, glutathione *S*-transferase; DOX, doxorubicin (Adriamycin); Pgp, P-glycoprotein; MDR, multi-drug resistance; MRP, MDR-associated protein; PAGE, polyacrylamide gel electrophoresis.

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Screening of a human bone marrow expression library using antibodies against DNP-SG ATPase unexpectedly yielded several independent clones of a known protein, RLIP76. Human RLIP76 and its mouse and rat orthologs, RIP1 and RalBP1, have been originally identified by their ability to bind to Ral-GTP in yeast two-hybrid or phage expression screens (*16*−*18*). Ral is a membrane-associated GTPase of the Ras family which functions downstream from $p21^{ras}$. RLIP76 contains a Ral-binding domain, and a GTPase-activating domain thought to interact with target proteins such as CDC42 and Rac (*19, 20*), signaling proteins involved in the actin cytoskeleton. RalBP1 is able to transmit signals from Ral to downstream effector proteins (*21*), suggesting that its human counterpart, RLIP76, may function in a similar manner. Cytocentrin, another member of this family of proteins, is probably a splice variant of RalBP1 with an extended C-terminus and is involved in the regulation of the mitotic spindle (*22*). Since prior analyses of the RLIP76 sequence have not revealed any features indicating transporter functionality, we carried out a detailed experimental characterization of the protein to determine whether DNP-SG ATPase and RLIP76 are related or indeed identical. The results of studies presented in this paper demonstrate, for the first time, that RLIP76, a Ral-binding protein, mediates ATP-dependent transport of DOX and DNP-SG in a manner similar to that by DNP-SG ATPase. Thus, RLIP76 is a non-ABC transporter of GS-E and DOX. Furthermore, we demonstrate that K562 human erythroleukemia cells transfected with RLIP76 acquire resistance to DOX, implicating RLIP76 in the mechanisms of drug resistance in cancer chemotherapy.

EXPERIMENTAL PROCEDURES

Reagents. Sources of all chemicals used in these studies have been described in refs *8, 12*−*14*.

Cloning and Prokaryotic Expression of RLIP76. Polyclonal antibodies against DNP-SG ATPase were raised in rabbit (*7*), and purified by passing through a column of immobilized total *E. coli* Y1090 lysate. The specificity of the antibodies was confirmed through Western blotting: a 38 kDa band was recognized in erythrocyte ghosts and in membrane fractions from human cell lines H69 (small cell lung cancer), K562 (erythroleukemia), and HL60 (promyelocytic leukemia), but no reaction was observed with *E. coli* Y1090 lysates. The antibodies were used for immunoscreening of 2.5×10^7 plaques from a human bone marrow cDNA library in λgt11 (Clontech), yielding one full-length and two partial RLIP76 clones. The 1968 bp open reading frame of the full-length clone was identical to the published sequence of RLIP76 (*16*).

Prokaryotic Expression of RLIP76. DNA was isolated from the RLIP76 λgt11 clone, and was used as the template for PCR amplification of the RLIP76 coding sequence. PCR primers were designed to introduce a *Bam*HI restriction site immediately upstream of the initiator codon, and a *Xho*I site immediately downstream of the stop codon of the RLIP76 open reading frame. The PCR product was subcloned, and its identity confirmed by sequencing. For expression of RLIP76 protein carrying a 6xHis tag at its N-terminus which was used in initial experiments, the *Bam*HI−*Xho*I fragment was inserted between the same sites of pET-30a(+) (Novagen), creating the pET30-His-RLIP76 plasmid. All work reported in the present paper was carried out with RLIP76 free of extraneous sequences. For this purpose, an *Nde*I site spanning nucleotides −3 to +3 relative to the initiator ATG was introduced in the pET30-His-RLIP76 plasmid by site-directed mutagenesis, the *Nde*I−*Nde*I fragment was excised, and the plasmid was religated. Protein was expressed in *E. coli* BL21(DE3) grown at 30 °C after induction with 0.4 mM IPTG.

Purification of RLIP76 by GS-E Affinity Chromatography. Bacteria were lysed in the presence of 1% (w/v) $C_{12}E_9$ (polyoxyethylene-9 lauryl ether or polidocanol, Sigma) in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 1.4 mM 2-mercaptoethanol, 100 μM EDTA, 50 μM butylated hydroxytoluene (BHT), and 100 μM PMSF. After incubation in the above buffer for 16 h with gentle shaking, sonication, and centrifugation at 27000*g* for 30 min, the supernatant was reconstituted into proteoliposomes (*13*). RLIP76 was purified by binding the resulting membranes to DNP-SG Sepharose followed by removal of contaminating proteins and elution with 10 mM ATP, 10 mM $MgCl_2$, as described previously (*12*).

DNP-SG ATPase Activity Assay. DNP-SG ATPase activity was measured as previously described by us (*12*). Aliquots of protein fraction containing 1−50 μg of protein were added to a 0.5 mL reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 0.8 mM sodium phosphate, 2.8 mM β-mercaptoethanol, and 1 mM oubain and incubated for 5 min at 37 °C. The reaction was started by addition of 1.6 mM [γ-^{32}P]ATP without or with 0.12 mM DNP-SG or 0.01 mM DOX. After incubation for 60 min at 37 °C, the reaction was terminated by addition of 2.5 mL of a cold mixture of 1 M perchloric acid and 5% ammonium molybdate in water (4:1), and extracted with a 2.5 mL mixture of isobutanol−benzene (1:1). Radioactivity was quantified in the organic phase to determine cleaved terminal phosphate. ATPase activity was calculated by subtracting background counts obtained in the absence of protein from those obtained in the presence of protein. DNP-SG ATPase activity was calculated by subtracting the ATPase activity seen in the absence of stimulator from that seen in its presence. Each assay was performed in triplicate.

ATPase activity in crude and purified fractions was also determined by measuring formation of ADP using HPLC to separate and quantify nucleotides. Adenine nucleotides were analyzed on a Bondpak reversed phase C_{18} column equilibrated at 1 mL/min with solvent A (100 mM potassium phosphate, 3 mM tetrabutylammonium phosphate) before loading samples, and gradient elution was carried out over 30 min with solvent B (solvent A with 20% methanol) from 0 to 100%. The column eluate was monitored at 254 and 280 nm. ATP, ADP, AMP, and cAMP standards were used to determine retention time (R_T 25.4, 22.2, 17.6, and 42.3 min, respectively) and calibration curves. Reaction mixtures containing purified RLIP76 for ATPase activity assay (*12*) were incubated for 60 min at 37 °C after addition of ATP and filtered over a 0.22 μm nitrocellulose membrane, and the filtrate was injected into HPLC. A prominent ADP peak (R_T 22.2 min) was observed which was absent in controls containing no RLIP76.

Functional Reconstitution of RLIP76 as a Transporter. Reconstitution was carried out according to refs *13* and *23*,

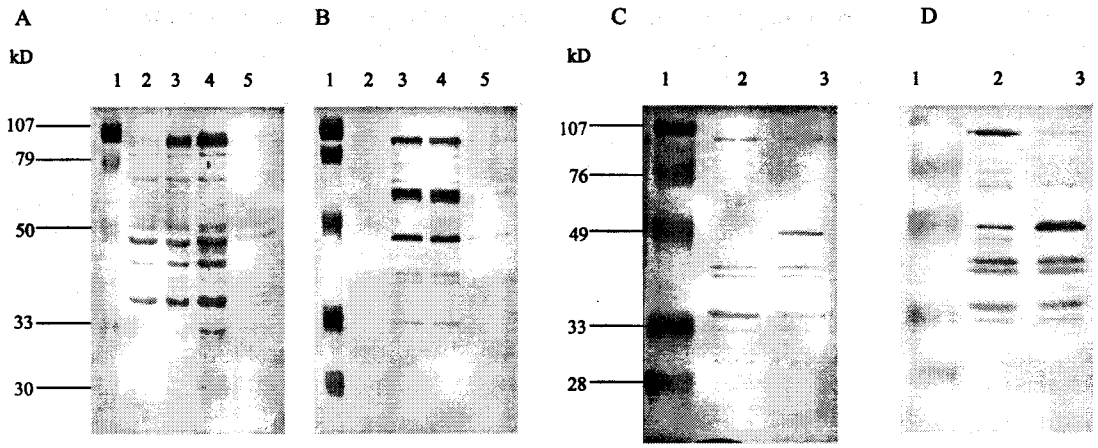

FIGURE 1: Expression and purification of human RLIP76. *E. coli* BL21(DE3) transfected with full-length RLIP76 cDNA [BL21(DE3)-RLIP76] was inoculated in 30 mL cultures to which either 0.4 mM IPTG (induced) or diluent alone (un-induced) was added and allowed to incubate for 12 h. The bacterial pellet was collected by centrifugation at 1400*g* and lysed by sonication in 1.5 mL of lysis buffer without $C_{12}E_9$. This total homogenate fraction (5 μL) from un-induced (lane 2) and IPTG-induced (lane 3) BL21(DE3)-RLIP76 was analyzed by Coomassie-stained SDS−PAGE (panel A) and Western blot against anti-DNP-SG ATPase antibodies (panel B) to demonstrate the effect of IPTG induction. The 28000*g* supernatant fraction of the homogenate of IPTG-induced BL21(DE3)-RLIP76 (10 μL) was applied to lane 5. The pellet of the 28000*g* centrifugation was solubilized in 1.5 mL of lysis buffer containing 1% (w/v) $C_{12}E_9$ and centrifuged at 28000*g*, and the supernatant was applied in lane 4 (10 μL). Since lysis followed by detergent extraction offered no additional advantage, the lysis and detergent extraction steps were combined for the purification of RLIP76 from BL21(DE3)-RLIP76 by directly dissolving the bacterial pellet in lysis buffer containing $C_{12}E_9$. The effect of detergent concentration on yield of peptides from DNP-SG affinity purification was tested by performing parallel purification from 28000*g* supernatants of bacterial pellets solubilized directly with either 1% (panels C and D, lane 2) or 2% $C_{12}E_9$ (panels C and D, lane 3). DNP-SG−Sepharose affinity chromatography was as described under Experimental Procedures. SDS−PAGE (panel C) and anti-DNP-SG ATPase Western blot analysis (panel D) of the DNP-SG−Sepharose affinity-purified fraction (10 μg) are presented. Lane 1 in all panels was loaded with prestained, low molecular weight standards obtained from BIO-RAD.

except that 50 μg of RLIP76 protein and 5 mg of asolectin−cholesterol (4:1) were used per milliliter of the reconstitution mixture. The resulting vesicles had a mean radius of 0.25 μm (determined by electron microscopy) and an average intravesicular volume of 18 μL/ml vesicles (as estimated by [^{14}C]inulin trapping). Control vesicles were prepared using an equal amount of crude protein from *E. coli* not expressing RLIP76. Transport activities were carried out as previously described by us (*13, 14*). Experimental details are included in the legend for Figure 2. Kinetic parameters of ATPase and transport activities were determined by nonlinear regression fitting of a Michaelis−Menten hyperbola to the experimental data.

Eukaryotic Expression of RLIP76. RLIP76 cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen), and used to transfect K562 cells. Clonal stable transfectant lines were obtained by plating in soft agarose or by sequential dilution in the presence of 400 μg of G418/mL. The cells expressed RLIP76, as demonstrated by Northern blotting, PCR, and Western blotting. Purification of RLIP76 from transfected K562 cells yielded a pattern of bands in SDS−PAGE similar to that of RLIP76 preparations obtained from *E. coli* overexpressing the protein. Unlike the expression of cytocentrin (*22*), transfection with RLIP76 did not cause any obvious disturbances of cell growth or division.

Transport Studies in Crude Membrane Vesicles. K562 cells were diluted 40-fold in 0.5 mM sodium phosphate buffer, pH 8.0, incubated overnight at 4 °C, and centrifuged at 28000*g* for 1 h. The pellet was suspended in the same buffer and passed repeatedly through a 27-gauge needle. Inside-out vesicles were purified either by density gradient centrifugation at 105000*g* for 2 h over a dextran barrier (1.03 g/mL), during which the right-side-out vesicles and unsealed ghosts pelleted to the bottom, or by passing mixed vesicles over a wheat germ agglutinin−Sepharose column which selectively retained right-side-out vesicles. Membrane orientation was estimated by comparing acetylcholinesterase activities (*24*).

RESULTS

Purification and Characterization of Recombinant RLIP76. RLIP76 was expressed in *E. coli* BL21(DE3) as described under Experimental Procedures. Figure 1A shows expression of RLIP76 as a 95 kDa band after IPTG induction. To determine whether recombinant RLIP76 is expressed as a soluble protein, the bacteria were lysed in the absence of detergent, and supernatant and particulate fractions of the lysate were obtained by centrifugation at 28000*g* for 30 min. SDS−PAGE followed by Western blotting revealed that the majority of RLIP76 was present in the insoluble fraction (Figure 1B), presumably in an aggregated form. However, if the bacterial cells were lysed in the presence of detergent (1% $C_{12}E_9$), the majority of RLIP76 remained soluble. Therefore, detergent extracts were used as the starting material for further purification of recombinant RLIP76.

Our previous studies on the purification of DNP-SG ATPase have shown that DNP-SG affinity chromatography of detergent extracts obtained from erythrocyte membranes yielded DNP-SG ATPase of low specific activity (*7, 8*). On the other hand, when erythrocyte membrane vesicles were used directly (i.e., without detergent solubilization) for DNP-SG affinity chromatography, the specific activity of the purified DNP-SG ATPase was higher by an order of magnitude (*12, 13*) as compared to that obtained with detergent extracts (*7*). Presumably, the protein was protected by the lipid bilayer, and was purified by progressive fragmentation and removal of membrane constituents which were not bound to the affinity matrix.

To ascertain whether a membrane environment also stabilizes RLIP76, the enzyme, recovered from bacteria by

Table 1: Purification of DNP-SG- and DOX-Stimulating ATPase Activity from RLIP76 Expressing *E. coli*[a]

fraction	protein (mg)	stimulated specific activity [nmol/(mg·min)]		total stimulated activity (nmol/min)		yield (%)	
		DNP-SG	DOX	DNP-SG	DOX	DNP-SG	DOX
detergent extract	34	4	4	141	122	100	100
DNP-SG Sepharose	0.75	62	88	47	66	33	54
anti-DNP-SG ATPase	0.30	157[b]	206[b]	47	62	33	51

[a] 200 mL of *E. coli* culture was used for purification. DOX- and DNP-SG-stimulated activity was obtained by subtracting the basal ATPase activities of the fractions determined in the absence of DNP-SG and DOX from the activities determined in the presence of DOX and DNP-SG. [b] Basal ATPase activity of this fraction was 224 nmol min^{-1} mg^{-1}, and was increased to 430 and 381 nmol min^{-1} mg^{-1}, in the presence of 10 μM DOX or 120 μM DNP-SG, respectively.

Table 2: N-Terminal Sequences of Peptides Seen on SDS Gels for the Purified RLIP Preparations[a]

apparent M_r of peptides seen in SDS gels (kDa)	N-terminal amino acid sequence															corresponding RLIP76 sequences[b]	predicted molecular mass (kDa)
95	T	E	C	F	L	P	P	T	S	P	S	E	H			2−14	76
41	L	N	C	L	H	R	D	L	Q	G	G	I	K	D	L	410−423	28.5
38	S	K	E	E	R	L	W	E	V	Q	R	I	L	T	A	424−438	27.2
35	M	N	E	N	E	E	V	I	N	I	L	L	A	Q	E	472−486	21.4

[a] Bands from SDS gels were transblotted on Immobilon-P PVDF membranes (*32*), excised with a razor blade, and used directly for micro sequence analysis on Applied Biosynthesis equipment with on-line model 120 micro bore HPLC phenyl thiohydantoin analyzer and model 900 data processor. [b] Residue number includes initiator methionine.

detergent extraction, was reconstituted into proteoliposomes. Further purification followed the protocol used by us to obtain highly active preparations of DNP-SG ATPase from human erythrocyte ghosts (*12*, *13*), and yielded RLIP76 preparations with ATPase activity comparable to that reported by us for purified DNP-SG ATPase (*7*, *8*). For comparison, we have also purified RLIP76 from detergent extracts without prior reconstitution into proteoliposomes. However, this resulted in RLIP76 preparations with significantly lower ATPase activity as compared to the enzyme purified from reconstituted vesicles (data not presented). Based on these results, we adopted the protocol for purification of RLIP76 from proteoliposomes for all further kinetic and transport studies. While somewhat unconventional, this method takes advantage of a protection of the enzyme by the membrane, and closely parallels the successful purification of DNP-SG ATPase from erythrocyte membranes.

The results of a representative purification protocol are presented in Table 1. Since a crude *E. coli* extract, as expected, contained substantial amounts of other ATPases besides RLIP76, the purification of RLIP76 was monitored using the DOX- and DNP-SG-stimulated ATPase activity as an index, which is a characteristic feature of DNP-SG ATPase (*7*, *8*, *12*, *13*). DOX-stimulated ATPase activity accounted for about 1% of the total ATPase activity present in the crude detergent extract of *E. coli*. The DOX-dependent ATPase was isolated in about 50% yield, and an approximately 50-fold purification was achieved. Taken together, these results indicate that recombinant RLIP76 is immunologically similar to DNP-SG ATPase (*12*), that it can be purified by a protocol identical to that used to purify DNP-SG ATPase, and that during this process it undergoes degradation, perhaps proteolytic, resulting in multiple bands in SDS gels that are similar to those observed with DNP-SG ATPase.

RLIP76 protein expressed in *E. coli* migrated on SDS−PAGE as a 95 kDa major band (Figure 1A) which was recognized by anti-DNP-SG ATPase antibodies (Figure 1B). These results were consistent with those of previous studies in which bands at 95−110 kDa have been shown in SDS gels for the purified preparations of rat and mouse orthologs of RLIP76 (91−92% identity with RLIP76) which have calculated molecular masses of about 75 kDa (*17*, *18*).

Purified recombinant RLIP76 showed a characteristic pattern in SDS gels (Figure 1C). In addition to the band at 95 kDa, clearly visible bands at 49, 41, 38, and 35 kDa were also observed along with variable appearance of other faintly stained bands, all of which were recognized by the antibodies against DNP-SG ATPase (Figure 1D). Previously reported Western blot analysis of rat brain tissue using antibodies against RalBP1, the rat ortholog of RLIP76, also showed multiple bands including a prominent band at 38 kDa (*18*). The relative amounts of the additional bands on SDS gels were dependent on the detergent concentrations used for the extraction of RLIP76 as observed in a shift from larger to smaller fragments when membranes were extracted with a 2% rather than a 1% nonionic detergent, $C_{12}E_9$ (Figure 1C,D, lanes 3 and 2, respectively).

N-Terminal Sequences and Amino Acid Analysis. The N-terminal sequences of the peptides with apparent molecular masses in SDS−PAGE of 95, 41, 38, and 35 kDa presented in Table 2 were found to be identical to sequences in RLIP76 starting at residues 2, 410, 424, and 472, respectively (numbering the initiator methionine as 1). Similar to parent RLIP76 protein, these peptides also had larger apparent molecular masses in SDS−PAGE than expected from their sequences. For example, the estimated molecular masses of the fragments starting with residues 410, 424, and 472 are 28.5, 27.2, and 21.4 kDa, respectively, while their apparent size on gels corresponds to 41, 38, and 35 kDa, respectively. Quantitative amino acid analysis of the purified total RLIP76 fraction showed residue yields within 95% of the expected yields computed on the basis of the derived sequence of RLIP76. Together, these data confirmed the purity of recombinant RLIP76 and strongly indicated that the major and minor peptides observed in SDS−PAGE originated from

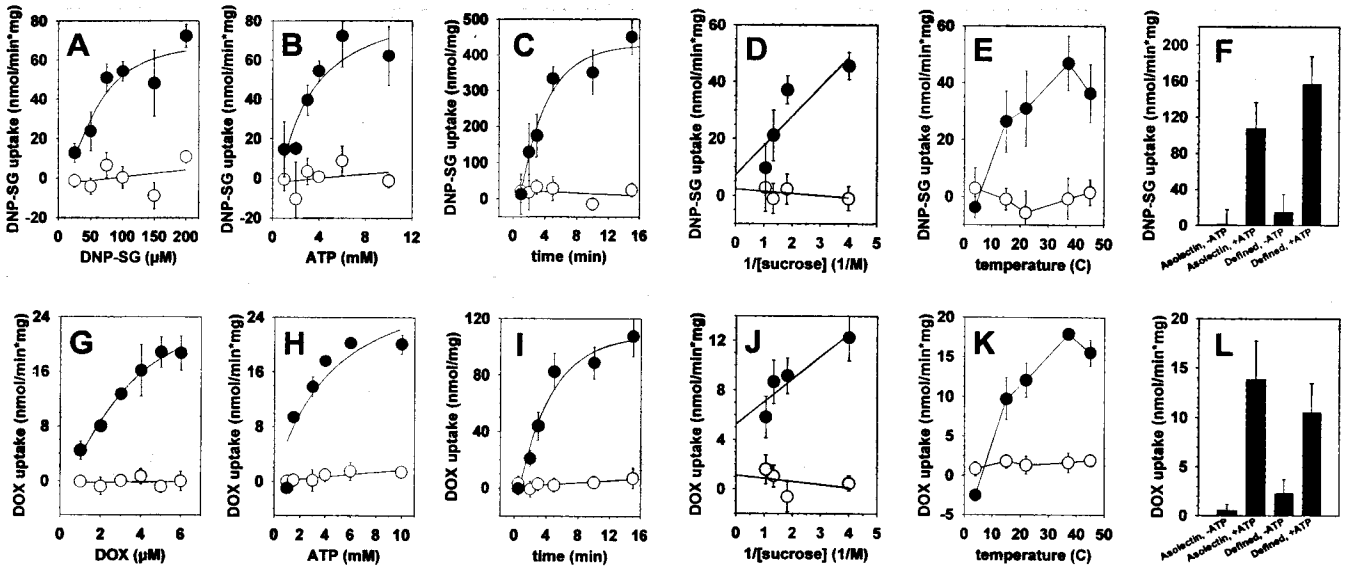

FIGURE 2: DNP-SG and DOX transport by reconstituted RLIP76. Purified recombinant RLIP76 (●) or crude protein from untransformed *E. coli* (○) was reconstituted into asolectin−cholesterol proteoliposomes. Transport of [^{3}H]DNP-SG (panels A−F) and [^{14}C]DOX (panels G−L) was measured in the presence and absence of ATP as previously described (*8, 13*). Vesicles equivalent to 250 ng of purified RLIP76 were used per 30 μL filtered reaction mixture. Assays were carried out at 37 °C for studies shown in all panels except E and K. Osmolarity of intra- and extravesicular fluid was held constant at 250 mOsm for studies shown in all panels except D and J. Uptake time was held constant at 5 min for studies shown in all panels except C and I. ATP was held constant at 4 mM for studies shown in all panels except B and H. DNP-SG was held constant at 100 μM for studies shown in all panels except A, and DOX was held constant at 3.6 μM except for panel G. A comparison of DNP-SG and DOX amounts associated with proteoliposomes in the absence of ATP is shown in panels F and L. The ATP-dependent transport shown in the figure was obtained by subtracting the uptake by the corresponding proteoliposomes in the absence of ATP. Means ± SE (n = 9) are shown. Panels A and G: dependence of transport rate on substrate concentration; panels B and H: saturable by ATP; panels C and I: time dependence of transport; panels D and J: inhibition of substrate accumulation by osmotic shrinking of vesicles; panels E and K: temperature dependence of transport; panels F and L: comparison of the effect of substituting purified defined lipids (phosphatidylcholine/phosphatidylethanolamine/phosphatidylinositol/cardiolipin, 40:33:14:4, by weight) for asolectin during reconstitution. Under present conditions, as previously shown (*13*), precision of the present assay allowed detection of a 2% increase in counts over filter background caused by the proteoliposomes or liposomes in the absence of ATP, $p < 0.001$ by a 2-tailed, homoscedastic t-test. Under varying transport conditions, addition of ATP resulted in a 5−50% increase in counts as compared with background or liposome/proteoliposomes in the absence of ATP, $p < 0.0001$. Calculated kinetic parameters for DNP-SG transport are as follows: K_M for ATP, 2.5 mM; K_M for DNP-SG, 60 μM; k_{cat} for DNP-SG (assuming half of the protein was reconstituted in transport-competent orientation), 3.4 s^{-1}. The calculated kinetic parameters for DOX transport are as follows: K_M for ATP, 4.6 mM; K_M for DOX, 3.1 μM; k_{cat} for DOX (assuming half of the protein was reconstituted in transport-competent orientation), 1.9 s^{-1}.

RLIP76 and that RLIP76 undergoes degradation during purification even in the presence of protease inhibitors in the purification buffers.

ATPase Activity of RLIP76. RLIP76 purified by DNP-SG affinity chromatography had a baseline ATPase activity of 224 nmol/(min·mg), similar to DNP-SG ATPase (*12*), which was stimulated in the presence of DNP-SG or DOX to 381 and 430 nmol/(min·mg), respectively. Corresponding protein fractions purified from control *E. coli* did not show detectable ATPase activity. The ATPase activity of RLIP76 was confirmed by demonstrating the formation of ADP in the reaction mixture by the ion-pair HPLC method for determination of nucleotides (data not presented). The K_m values for ATP, DOX, and DNP-SG were found to be 2.0 mM, 1.5 μM, and 65 μM, respectively, which were in the same range as those reported for DNP-SG ATPase (*12*).

Functional Reconstitution of RLIP76. After establishing that RLIP76 catalyzes a partial reaction expected of a primary active transporter, i.e., substrate-stimulated ATPase, we proceeded to show transport itself by reconstituting purified RLIP76 into artificial proteoliposomes using asolectin. These proteoliposomes were stringently characterized for their suitability for transport studies as described by us for the functional reconstitution of DNP-SG ATPase (*13*). Reconstituted RLIP76 catalyzed ATP-dependent uptake of [^{3}H]-DNP-SG and [^{14}C]DOX into vesicles (Figure 2). Transport by RLIP76-containing (but not by control) vesicles was ATP-dependent, saturable with respect to the transported substrate (Figure 2, panels A and G) and ATP (Figure 2, panels B and H), and time-dependent (Figure 2, panels C and I). Substrate accumulation was predominantly within the lumen of vesicles, as demonstrated by decreased accumulation when vesicles were osmotically shrunk (Figure 2, panels D and J). Extrapolation to zero intravesicular volume indicated that approximately one-third of DOX uptake was due to binding to the lipid bilayer, while essentially all DNP-SG entered the lumen of proteoliposomes (Figure 2, panels D and J). On the basis of the intravesicular volume measured by inulin entrapment and analysis of data from Figure 2, panels C, D, I, and J, it can be calculated that RLIP76 sustained steady-state concentration gradients of 11- and 51-fold for DNP-SG and DOX, respectively. The activation energies for transport of the above two substrates were 61 and 96 kJ/mol, and the temperature optimum was at 37 °C with inactivation at 45 °C, consistent with an enzyme-catalyzed reaction (Figure 2, panels E and K). Inclusion of 1 mM GSH in the transport buffer had no effect on the ATP-dependent transport of DOX or DNP-SG by the proteoliposomes. As in the case of DNP-SG ATPase isolated from erythrocytes, ATP-dependent transport of DOX by reconstituted RLIP76 was inhibited by DNP-SG in a concentration-dependent manner; the converse was true as well. Taken together, these

results indicate a close similarity between the biochemical characteristics of transport catalyzed by recombinant RLIP76 and by DNP-SG ATPase purified from erythrocytes.

Transport Studies with Proteoliposomes of Defined Phospholipid Composition. Soybean phospholipids (asolectin) were used for the above reconstitution studies. Asolectin may contain proteins, especially proteolipids, some of which have ionophoric properties. Thus, it could be argued that transport is not catalyzed by RLIP76 itself, but by its complex with an asolectin-derived protein. To examine this possibility, we simulated the phospholipid composition of asolectin (*25*) using highly purified, defined phospholipids. This phospholipid mixture, when combined with cholesterol in a 4:1 ratio, supported RLIP76-catalyzed transport of DNP-SG and DOX (Figure 2, panels F and L). Thus, we conclude that the preparation of recombinant RLIP76 alone is sufficient for the transport function.

K562 Cells Transfected with RLIP76 Acquire Resistance to DOX and 4-Hydroxynonenal. We have also considered the question whether transport-related functions of RLIP76 are physiologically relevant in mammalian cells. If so, cells overexpressing RLIP76 would be expected to acquire resistance to the cytotoxic effects of DOX. In agreement with our prediction, K562 human promyelocytic leukemia cells expressing RLIP76 acquired an approximately 2-fold resistance to DOX as compared to control or vector-transfected cells (Figure 3, panel A). A cell biological assay for GS-E cytotoxicity was less straightforward since most GS-E added externally to cells would remain inaccessible to an export pump. We utilized the fact that 4-hydroxynonenal (4-HNE), an endogenous toxicant generated during lipid peroxidation, is hydrophobic enough to cross membranes, and that is it metabolized primarily to GS-E by glutathione *S*-transferases (GSTs) (*26*). GS-E can inhibit glutathione *S*-transferases (*4*) and thus become toxic unless metabolized or transported out of the cell. K562 cells transfected with RLIP76 were resistant to 4-HNE toxicity (Figure 3, panel B), consistent with the transport activity of RLIP76 for GS-E.

Transport Studies with Inside-Out Vesicles Prepared from K562 Cell Membranes. The transport function was verified by demonstrating ATP-dependent uptake of DNP-SG and DOX by inside-out plasma membrane vesicles prepared from RLIP76-transfected cells which showed severalfold higher ATP-dependent uptake as compared to those prepared from control or vector-transfected cells (Figure 3, panels C−F). This indicates that the acquired resistance of transfected cells was due to enhanced RLIP76-mediated efflux of DOX or GS-E. Furthermore, the results demonstrate that RLIP76 expressed in mammalian cells is correctly processed, targeted, and inserted into the membrane in a transport-competent orientation. Our previous studies have shown that DNP-SG ATPase can mediate the transport of various GS-E, *Vinca* alkaloids (*8*), colchicine (*14*), and metabolites of DOX (*8*). In view of the close similarity between tissue-purified DNP-SG ATPase and recombinant RLIP76 demonstrated here, it is reasonable to assume that RLIP76 also mediates transport of these substrates. Thus, RLIP76 up-regulation may constitute a novel mechanism of the multidrug resistance (MDR) phenotype attributable to a non-ABC transporter.

Effect of Intracellular Concentrations of RLIP76 on DOX Cytotoxicity. Because transfection of mammalian cells and the resulting overexpression of heterologous proteins can result in positional effects and compensatory responses which may complicate the interpretation of results, we compared the transfection of cells with RLIP76 cDNA with delivering RLIP76 protein directly to adherent cells using RLIP76 liposomes. We chose the well-characterized H358 human bronchioalveolar non-small-cell lung cancer (NSCLC) line for these studies. Increasing amounts of RLIP76 were reconstituted in a fixed amount of liposomes. Incubation of these proteoliposomes with the adherent H358 cells resulted in a proportional increase in RLIP76 in the membrane fraction as shown by Western blot analysis (Figure 4A). The observed increase was not due to nonspecific adherence of RLIP76 liposomes to the culture dish since the supernatant fraction obtained prior to detergent extraction of cell membranes contained no detectable RLIP76. These studies suggested that RLIP76 could be successfully delivered to cells using liposomes. The effects of increased RLIP76 on cellular sensitivity to DOX were determined by growing H358 cells in 96 well plates and treating with RLIP76 or control liposomes for 24 h prior to measuring the IC_{50} for DOX. Although the liposomes alone offered some protection from DOX, the effect of RLIP76 liposomes was significantly greater and increased with increasing amount of RLIP76 (Figure 4B,C). Taken together, results of these studies

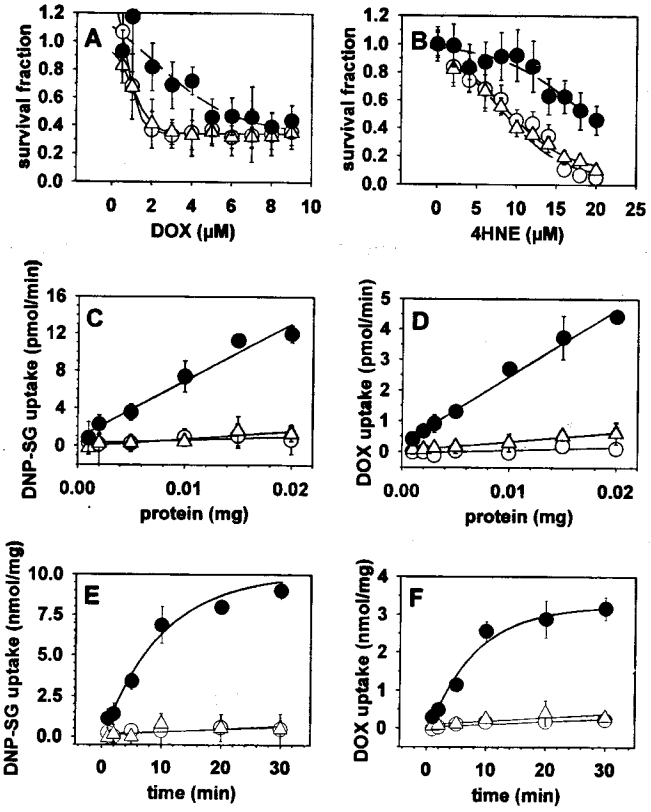

FIGURE 3: Effect of RLIP76 transfection on cytotoxicity and transport of DOX and 4HNE. DOX (panel A) and 4-HNE (panel B) cytotoxicity in wild-type K562 cells (○), cells transfected with insert-free pcDNA3 plasmid (△), and cells transfected with pcDNA3/RLIP76 (●) was determined using the MTT assay. Means ± SE presented are from three separate determinations (each with 8 replicates, $n = 24$). ATP-dependent uptake of [^{3}H]DNP-SG (panels C and E) and [^{14}C]DOX (panels D and F) by inside-out plasma membrane vesicles from control (○), pcDNA3-transfected (△), and pcDNA3/RLIP76-transfected (●) K562 cells. For panels C and D, uptake time was 10 min, and for panels E and F, protein was 15 μg per 30 μL of filtered reaction mixture. Means ± SE ($n = 9$) are shown.

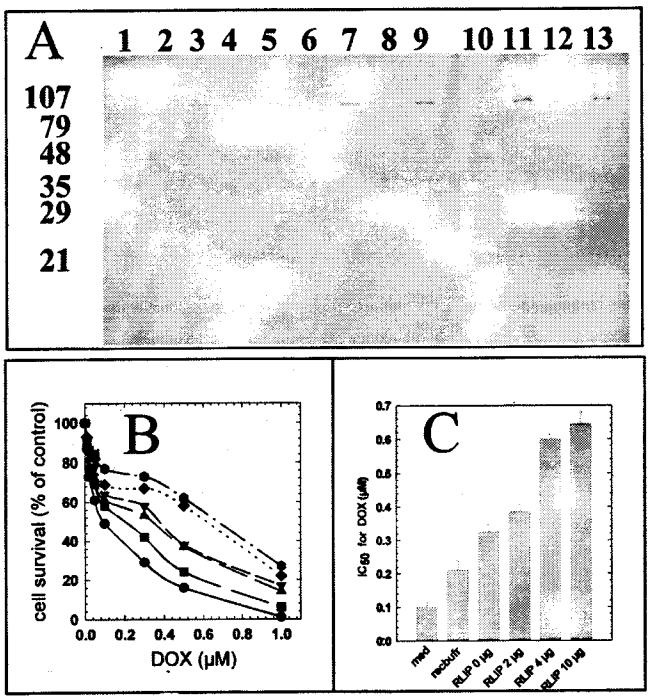

FIGURE 4: Delivery of RLIP76 to membranes of H358 human bronchioalveolar NSCLC cell line using RLIP76 liposomes and effect on DOX sensitivity. H358 cells were grown in 2 cm diameter plates for 24 h after inoculation of 2×10^6 cells, followed by addition of equal amounts of liposomal lipid reconstituted with increasing amounts of purified recombinant RLIP76 protein. After 24 h incubation, the medium was removed, and cells were washed 4 times with 2 mL of PBS. The last wash was saved and concentrated by lyophilizing. The adherent cells were harvested by addition of buffer containing detergent ($C_{12}E_9$ 0.025% v/v), and equal volumes of supernatant were subjected to SDS–PAGE and subjected to Western blot analysis using the polyclonal antibodies raised in rabbit against recombinant total RLIP76 purified by DNP-SG affinity chromatography probed with antibodies. A composite figure from two separate gels is shown. Lane 1 contains prestained marker. Odd-numbered lanes correspond to membrane fraction of cells treated with liposomes containing 0, 2.5, 5, 10, 20, and 50 μg of recombinant RLIP76, and even-numbered lanes are corresponding PBS washes (panel A). The effect of RLIP76 proteoliposomes on drug sensitivity was determined by measuring the IC_{50} value of DOX (0−1 μM) 24 h after the addition of 40 μL of medium alone (●), reconstitution buffer (■), reconstituted liposomes without RLIP76 (▲), or liposomes reconstituted with 2 (▼), 4 (◆), or 10 μg (hexagon) of RLIP76. After growing for 96 h, cells were stained with MTT and solubilized with DMSO. Plates were read at 570 nm. OD_{570} values normalized to control for one of three experiments are presented (panel B). Mean and SE of IC_{50} values from three separate experiments are presented (panel C).

complemented the results of studies with K562 cells, offering additional evidence for the protective effect of RLIP76 toward DOX.

DISCUSSION

Molecular cloning of DNP-SG ATPase yielded cDNA clones of RLIP76, a previously characterized Ral-GTP-binding protein which has the ability to activate downstream GTPases and is thus involved in signal transduction. The cloning result shows that DNP-SG ATPase, a protein previously defined by its functional and biochemical properties, is in fact identical or at least closely related to RLIP76. The conclusion that DNP-SG ATPase functionality can be attributed to the RLIP76 protein was verified by the results of our present studies which demonstrated that RLIP76 can mediate ATP-dependent transmembrane movement of anionic GS-E (DNP-SG) as well as a weakly cationic chemotherapeutic drug (DOX) in several independent systems. The above statement is based on the following experimental evidence: (1) transport studies on proteoliposomes (reconstituted with either asolectin or defined phospholipids) containing purified recombinant RLIP76; (2) acquired resistance to DOX and 4HNE of K562 cells transfected with cDNA encoding RLIP76; (3) acquired resistance to DOX of H358 cells upon direct incorporation of RLIP76 by fusion of the cells with proteoliposomes. Corollary evidence consistent with the identity of DNP-SG ATPase and RLIP76 includes the finding that RLIP76 binds to a DNP-SG affinity column from which it can be eluted with ATP, indicating the presence of binding sites for both substrates. Furthermore, the kinetic parameters of RLIP76-mediated ATPase activity and ATP-dependent transport of DNP-SG and DOX are closely similar to those of tissue-isolated DNP-SG ATPase reported by us previously (*13*).

The aberrant behavior of DNP-SG ATPase and other GS-E transporters in SDS gels has been noted previously (*12, 23, 27*−*29*). In the present studies, we observed a similar pattern for recombinant RLIP76. Moreover, SDS gel electrophoresis of RLIP76 gave rise to multiple bands, including a prominent fragment migrating at 38 kDa. All of the fragments originated from the parent protein as confirmed by immunological cross-reactivity and the results of N-terminal sequence analysis. Interestingly, the ratios of these bands could be altered by changing the detergent concentration during solubilization, in analogy to our previous observations on DNP-SG ATPase. The propensity of RLIP76 to degrade into a set of fragments whose relative amounts depend on isolation conditions may explain the seemingly contradictory results obtained in various laboratories purifying erythrocyte GS-E transporters. Proteins with apparent molecular masses of 28 (*27*), 38 (*12*), and 62 and 82 kDa (*28*) have been reported. Likewise, in our earlier studies (*23, 29*), the purification of an organic anion transporter from rat liver has also yielded preparations showing bands at 90, 60, and 30 kDa which were recognized by anti-DNP-SG ATPase antibodies. Significantly, the ratios of these three bands were variable between experiments, and the 90 kDa band could be converted to 60 kDa, and 60 kDa to 30 kDa, by mild proteolysis (*23, 29*). Because of susceptibility to proteolysis and the unusual behavior of DNP-SG ATPase (and/or related transporters) in gels, its molecular characterization has eluded investigators in the past.

Several questions arise from the results presented in this report. In view of the role of RLIP76 in signal transduction, it could be proposed that RLIP76 is not itself a transporter, but activates or induces another transport protein. We believe that this hypothesis is incorrect for the several reasons. The first argument is based on our as yet unpublished preliminary findings that the C-terminal part of RLIP76 (amino acids 424−655), which we expressed in *E. coli* and purified by DNP-SG affinity chromatography, has DNP-SG- and DOX-stimulated ATPase activity and can be photoaffinity labeled with azido-ATP, but does not mediate transport upon reconstitution. Uncoupling of transport from ATPase activity cannot be reasonably explained if RLIP76 is assumed to induce another transporter. Second, RLIP76 that is pure by several criteria has ATPase and transport activity in well-

defined experimental systems. Third, both transformation of bacteria and transfection of mammalian cells with RLIP76 cDNA result in a gain of transport function, a very unlikely outcome for a protein with only regulatory function. Finally, direct uptake of purified RLIP76 protein into mammalian cells reconstituted into liposomes confers DOX resistance.

Mechanistically, it may be difficult to conceptualize RLIP76, a protein thought to be cytosolic and involved in signal transduction, as a membrane transporter. In this context, it is worth pointing out that RLIP76 has the ability to bind to membranes. In rat brain, more RalBP1 was found in the particulate than in the soluble fraction (Figure 4 in ref *18*), and in our experiments, 80% of RLIP76 expressed in *E. coli* was in the particulate fraction in the absence of detergent, but could be extracted with $C_{12}E_9$. RLIP76 has no obvious transmembrane helices. However, some soluble proteins, e.g., bacterial toxins and certain annexins, can insert into membranes and turn into transporters. Potential mechanisms (*30, 31*) include oligomerization, conformational changes of the protein which unmask or create transmembrane structures, and protein-induced destablization of the lipid bilayer. It is tempting to speculate that the fragmentation of RLIP76 may rearrange the protein to enable transport functionality. From this perspective, the fragmentation would not be an experimental artifact, but could be viewed as processing of a precursor into mature protein(s). The already mentioned C-terminal fragment of RLIP76 (residues 424−655) could contribute the energy transducing subunit to the transport complex, with other fragments providing the channel element. So far, we could not address the latter possibility experimentally because our attempt to purify the recombinant N-terminal part of RLIP76 (amino acids 1−376) was unsuccessful due to its apparently tight association with the membrane.

It is interesting to speculate whether the signal transduction and transport functions of RLIP76 are independent or functionally linked. For example, the binding of RalBP1, the rat homologue of RLIP76, to Ral appears to recruit RalBP1 to membranes, which could affect transport activity. The ability of RLIP76 to mediate GS-E transport could be relevant to leukotriene-mediated signaling of chemotaxis. As already mentioned, transport of drugs could contribute to multi-drug resistance of human cancers to chemotherapy. This is particularly relevant because the MRP and Pgp are insufficient to explain all forms of transport mediated resistance. Although further work will be required to elucidate the mechanistic and regulatory aspects of RLIP76 function and its physiological role, our present results demonstrate that RLIP76 is closely related or identical with DNP-SG ATPase, and that it can catalyze ATP-dependent transport of GS-E and DOX across biological membranes.

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