Functional Reassembly of ATP-Dependent Xenobiotic Transport by the N- and C-Terminal Domains of RLIP76 and Identification of ATP Binding Sequences†

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Functional Reassembly of ATP-Dependent Xenobiotic Transport by the N- and C-Terminal Domains of RLIP76 and Identification of ATP Binding Sequences†

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ABSTRACT: We have recently shown that RLIP76, a Ral-binding, GTPase-activating protein, is an ATP-dependent transporter of doxorubicin (DOX) as well as glutathione conjugates [Awasthi, S., et al. (2000) *Biochemistry 39*, 9327−9334]. RLIP76 overexpressed in human cells or transformed *E. coli* undergoes proteolysis to yield several fragments, including two prominent peptides, N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$, from the N- and C-terminal domains, respectively. To investigate whether the fragmentation of RLIP76 has any relevance to its transport function, we have studied the characteristics of these two peptide fragments. Recombinant N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ were purified from overexpressing transformed *E. coli*. While N-RLIP76$^{1-367}$ readily underwent proteolysis, showing SDS-gel patterns similar to those of RLIP76, C-RLIP76$^{410-655}$ was resistant to such degradation. Both N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ had ATPase activity (K_m for ATP, 2.5 and 2.0 mM, respectively) which was stimulated by DNP-SG, DOX, and colchicine (COL). ATP binding to both peptides was confirmed by photoaffinity labeling with 8-azido-ATP that was increased in the presence of compounds that stimulated their ATPase activity. Photoaffinity labeling was also increased in the presence of vanadate, indicating trapping of a reaction intermediate in the ATP binding site. The ATP binding sites in N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ were identified to be ^{69}GKKKGK^{74} and ^{418}GGIKDLSK^{425}, respectively. Mutation of K^{74} and K^{425} to M residues, in N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$, respectively, abrogated their ATPase activity as well as azido-ATP labeling. Proteoliposomes reconstituted with either N-RLIP76$^{1-367}$ or C-RLIP76$^{410-655}$ alone did not catalyze ATP-dependent transport of DOX or COL. However, proteoliposomes reconstituted with a mixture of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ mediated such transport. Proteoliposomes reconstituted with the mixture of mutant peptides lacking ATPase activity did not exhibit transport activity. Present studies have identified the ATP binding sites in RLIP76, and show that DOX and COL transport can be reconstituted by two fragments of RLIP76.

We have recently shown (*1*) that RLIP76, a previously characterized Ral-binding, GTPase-activating protein involved in the Ras-Ral-mediated signal transduction cascade (*2*), can also mediate the ATP-dependent transport of chemotherapeutic agents such as doxorubicin (DOX)[1] as well as of glutathione conjugates (GS−E). Furthermore, we have demonstrated (*1*) that RLIP76 is structurally, immunologically, and functionally similar to *S*-(dinitrophenyl)glutathione (DNP-SG) ATPase, a transport protein with wide substrate specificity characterized by us previously from various human (*3*−*7*) and rat (*8*, *9*) tissues. Recombinant RLIP76 expressed in prokaryotic (*E. coli*) or eukaryotic (human cell lines K562 and H69) cells could be resolved into multiple bands on SDS−PAGE, and all these bands originated from the parent RLIP76 (*1*). The total rec-RLIP76 purified from transformed *E. coli*, showing multiple bands at approximate kDa values of 28, 38, 49, and 95 (all containing internal sequences of RLIP76), catalyzed ATP hydrolysis which was stimulated by GS−E as well as DOX. When reconstituted

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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); COL, colchicine; Pgp, P-glycoprotein; MDR, multi-drug resistance; MRP, multi-drug resistance-associated protein; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; BHT, butylated hydroxytoluene; 4-HNE, 4-hydroxynonenal.

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in proteoliposomes, RLIP76 mediated the ATP-dependent transport of DNP-SG and DOX (*1*). The observed fragmentation of rec-RLIP76, even in the presence of PMSF in the purification buffers, suggested that RLIP76 easily undergoes proteolytic cleavage. We have therefore hypothesized that RLIP76 may be a precursor that undergoes proteolytic cleavage yielding mature proteins/peptides, and that this processing may be required for its transport function. If this contention is correct, the various fragments of RLIP76 must play specific roles in the 'transporter complex' reassembled from these fragments. Thus, the ATP hydrolysis by RLIP76, its association with the membrane, and the translocation of the substrates across the membrane against a concentration gradient by RLIP76 (*1*) may be attributed to specific peptides generated during the processing of RLIP76, and the characteristics of these fragments of RLIP76 must be investigated.

As pointed out above, several peptide fragments of RLIP76 were observed during its purification from transformed *E. coli* (*1*). The apparent molecular mass values of these peptides in SDS−PAGE were consistently observed to be higher than their actual molecular masses estimated from their sequences. For example, full-length RLIP76, with an estimated molecular mass of about 76 kDa, appeared as a band at 95 kDa. Likewise, a C-terminal peptide (residues 410−655) with an estimated molecular mass of about 28 kDa was observed at 38 kDa in SDS-gels. A prominent peptide band originating from the N-terminal was also seen at 49 kDa (Figure 1 in ref *1*), and its electrophoretic mobility in SDS-gels was identical to that of the recombinant N-terminal peptide of RLIP76 containing residues 1−367. These results suggested that during the observed fragmentation of RLIP76, two prominent peptides were generated, which originated from the N-terminal (residues 1 to about 367) and the C-terminal (residues 410−655) domains of RLIP76. Present studies were designed to investigate the possible roles of these two peptides (designated as N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$) in the transport function of RLIP76. We have prepared recombinant N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ and investigated their ATPase activity, ATP binding, and transport characteristics. Furthermore, through site-directed mutagenesis studies, we have identified the ATP binding sequences in these fragments. The results of these studies show that both N- and C-terminal peptides of RLIP76 catalyzed ATP hydrolysis, and that the ATP binding residues of these fragments were distinct from each other and also from the Walker A and B motifs (*10*) characteristic, among others, of the family of ABC transporter proteins (*11*). Neither N-RLIP76$^{1-367}$ nor C-RLIP76$^{410-655}$, when separately reconstituted into proteoliposomes, catalyzed the transport of DOX or GS−E, even though their ATPase activity was stimulated by these compounds. However, when equimolar mixtures of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ were incorporated into proteoliposomes, ATP-dependent transport of DOX as well as COL was observed. Mutations in the ATP binding sites of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ abrogated their ATPase activity as well as transport function. These results provide further evidence that RLIP76 indeed represents a novel transporter of xenobiotics which is distinct from the family of ABC transporters, and suggests that the proteolytic fragmentation of RLIP76 observed by us previously (*1*) is compatible and may perhaps be essential for its functionality as a transporter.

EXPERIMENTAL PROCEDURES

Reagents. Sources of all chemicals used in these studies have been described in our recent publications (*1, 3, 4*).

Cloning and Expression of RLIP76 N-Terminal and C-Terminal Fragments in E. coli. The RLIP76 λgt11 clone (*1*) was used as the template for PCR amplification of the RLIP76 coding sequence. The upstream (5′-GGCGGATCCATGACTGAGTGCTTCCT-3′) and downstream (5′-GCACTCGAGTCAGATGGACGTCTCCTTCCT-3′) PCR primers were designed to introduce a *Bam*HI restriction site (underlined) immediately upstream of the initiator codon, and a *Xho*I site (underlined) immediately downstream of the stop codon (TGA) of the RLIP76 open reading frame. The PCR product was cloned into vector pGEM-T (Promega) and confirmed by sequencing. For expression of RLIP76 protein carrying a 6×His tag at its N-terminus which was used in initial experiments, the *Bam*HI−*Xho*I fragment was purified from pGEM-RLIP76 and inserted between the same sites of pET-30a(+) (Novagen), creating the pET30-His-RLIP76 plasmid. However, all work on full-length RLIP76 reported in the present paper was performed with protein free of extraneous sequences such as a His tag. For this purpose, the QuickChange Site-Directed Mutagenesis Kit (Stratagene Inc.) was used to introduce in the pET30-His-RLIP76 plasmid an *Nde*I site spanning nucleotides −3 to +3 relative to the initiator ATG. The *Nde*I−*Nde*I fragment was then excised, and the large fragment of the plasmid was self-ligated. To characterize the functional domains of RLIP76, two truncated fragments (N-terminal, amino acids 1−367; and C-terminal, amino acids 410−655) carrying His tags at their N-termini were expressed. DNA encoding the N-terminal fragment was amplified by PCR using the upstream primer 5′-GGCCTCGAGATGACTGAGTGCTTC-3′ (containing a *Xho*I site, underlined) and the downstream primer 5′-AGAGCGGCCGCCTAGAGTTCTT GCACATGTGT-3′ (containing a *Not*I site, underlined), respectively. The PCR fragment was cloned into pGEM-T, the plasmid was cut with *Xho*I, the cohesive ends were blunted with Klenow DNA polymerase and dNTP, and the construct was then cut with *Not*I. The resulting fragment was purified and subcloned into pET-30c(+) cut with *Eco*RV and *Not*I. DNA encoding the C-terminal fragment was amplified with primers 5′-AGCCATATGCACCACCACCACCACCACAATTG-TTTACATCG AGAT-3′ (upstream) and 5′-GCACTCGAGT-CAGATGGACGTCTCCTTCCT-3′ (downstream). The upstream primer contained a *Nde*I site (underlined) followed by six histidine codons and a stretch of RLIP76-coding sequence starting with codon 410, and the downstream primer contained a *Xho*I restriction site (underlined). The PCR product was subcloned into pGEM-T, liberated with *Nde*I and *Xho*I, purified, and subcloned into pET-30b(+) previously cut with the same enzymes.

Site-Directed Mutagenesis. The QuikChange Site-Directed Mutagenesis Kit (Stratagene Inc.) was used. To replace lysine residues 74 and 425 with methionine residues, the mutagenic primers 5′-AAGAAAAAAGGGATGTTTAAGAAAAAG-3′ (with its reverse-complement) and 5′-AAGGATTTGTC-TATGGAAGAAAGATTAT-3′ (with its reverse-complement), respectively, were used. The mutation sites are underlined. Clones of full-length RLIP76 or its N- and

C-terminal fragments in pET-30 expression vectors served as templates. Mutations were confirmed by DNA sequencing.

Expression of RLIP76 and Purification by GS−E Affinity Chromatography. E. coli strain BL21(DE3) transformed with the pET-30a(+) plasmid containing the full-length RLIP76 cDNA clone was grown at 37 °C until A_{600} reached 0.6, and was induced with 0.4 mM IPTG at 37 °C overnight. Bacteria were lysed in 1% (w/v) $C_{12}E_9$ (polyoxyethylene-9 lauryl ether or polidocanol, Sigma), 10 mM Tris-HCl, pH 7.4, 1.4 mM β-mercaptoethanol, 100 μM EDTA, 50 μM butylated hydroxytoluene (BHT), and 100 μM PMSF. After incubation in the above buffer for 16 h at 4 °C with gentle shaking followed by sonication and centrifugation at 20000*g* for 30 min, RLIP76 was purified from the supernatant by DNP-SG affinity chromatography using a protocol identical to that described previously (*1*).

Purification of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$. Purification of these recombinant truncated RLIP76 peptides was achieved by metal affinity chromatography (*12*) over Ni−NTA Superflow resin (QIAGEN) used for histidine-tagged proteins with slight modifications as described below. *E. coli* BL21(DE3) expressing N-RLIP76$^{1-367}$ were suspended in 1% $C_{12}E_9$−TBS (pH 7.4) containing 100 μM PMSF, sonicated, and shaken for 4 h at 4 °C, followed by centrifugation at 13 000 rpm for 20 min. The supernatant was mixed with Ni−NTA Superflow resin preequilibrated with the same buffer. The resin was incubated at 4 °C for 2 h and washed with TBS containing 1% $C_{12}E_9$. Protein was eluted with 8 mL of elution buffer (400 mM imidazole, 500 mM NaCl, 100 μM PMSF, 20 mM Tris-HCl, pH 7.9). While the extraction of N-RLIP76$^{1-367}$ required detergent, the C-terminal peptide, C-RLIP76$^{410-655}$, could be extracted without detergent and was purified as described below: Bacteria containing C-RLIP76$^{410-655}$ were suspended in binding buffer (5 mM imidazole, 500 mM NaCl, 100 μM PMSF, 20 mM Tris-HCl, pH 7.9), sonicated, and centrifuged. Ni−NTA Superflow resin (Qiagen) was used for binding the resultant supernatant. The resin was washed with binding buffer and eluted with elution buffer. The eluates containing purified N-RLIP76$^{1-367}$ or C-RLIP76$^{410-655}$ were concentrated using Amicon Centriprep concentrators, followed by sequential dialysis against lysis buffer (10 mM Tris-HCl, pH 7.4, 1.4 mM β-mercaptoethanol, 50 μM BHT, 100 μM EDTA, 100 μM PMSF) containing 0.025% $C_{12}E_9$. The purity of these peptides was confirmed by their N-terminal sequencing.

ATPase Activity. ATPase activity was measured by organic extraction of hydrolyzed γ-phosphate of ATP as phosphomolybdate according to the method of Knowles and Leng (*13*) with slight modifications as described by us previously (*1*). Blanks obtained in the absence of protein were subtracted from all values. ATPase activity was measured in the absence and presence of activators. The difference of these two activities is taken to represent activator-dependent ATPase. Protein was determined by the dye binding assay described by Minamide and Bamburg (*14*).

Functional Reconstitution in Proteoliposomes. To determine whether purified RLIP76 and its C- and N-terminal fragments could function as ATP-dependent transporters of GS−E, as well as DOX and COL as previously demonstrated for DNP-SG ATPase (*3*) and RLIP76 (*1*), we reconstituted the purified protein fractions into artificial liposomes as described by us recently (*1, 4*) with minor modifications. Briefly, purified protein was first dialyzed overnight against a buffer containing 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA, 100 mM KCl, 40 mM sucrose, 2.8 mM β-mercaptoethanol, and 0.025% (v/v) polidocanol (reconstitution buffer). The reconstitution mixture contained 50 μg of protein, 4 mg of asolectin (soybean phospholipids), and 1 mg of cholesterol/mL. The reaction mixture was sonicated for 15 s at 50 W, after which 200 mg of Biobeads SM-2 (Bio-Rad) preequilibrated in the reconstitution buffer (without polidocanol) was added to initiate vesiculation by removal of detergent. After incubation for 4 h at 4 °C, Biobeads were removed by centrifugation at 3600*g*, and the proteoliposomes were used for transport studies. Control proteoliposomes were prepared using an equal amount of crude protein from *E. coli* transformed with insert-free plasmid.

Transport Studies. ATP-dependent transport of DOX and COL in proteoliposomes was measured as described by us recently (*1*). Briefly, reconstituted proteoliposomes were diluted 2−20-fold in 90 μL of transport buffer, which had a composition identical to that of the reconstitution buffer except for omission of polidocanol, containing either [^{14}C]-DOX (5.8×10^4 cpm/nmol) or [^{3}H]COL (6.9×10^4 cpm/nmol). DOX concentrations from 0.2 to 10 μM and COL concentrations from 0.2 to 20 μM were used. Measurement of ATP-dependent transport was initiated by addition of 10 μL of ATP prepared in transport buffer to the experimental group and 10 μL of transport buffer alone to the control group. The final concentration of ATP in the experiments was 4 mM. After incubation with ATP for 5 min, aliquots of the reaction mixtures containing reconstituted protein were filtered using a Millipore Multiscreen 96-well plate vacuum filtration system as described by us previously (*1, 4, 6*). The filtration membranes were individually cut out and solubilized in scintillation cocktail overnight before radioactivity counting. As reported previously (*1*), the 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 effects of RLIP76 and its mutants on substrate uptake by vesicles were studied by comparing uptake with or without ATP by liposomes reconstituted with or without the protein. Possible nonspecific effects of nucleotides on substrate uptake by the vesicles were studied by substituting equal concentrations of ADP as described previously (*3*).

Photoaffinity Labeling of RLIP76 with Azido[α-^{32}P]ATP and Vanadate Trapping. For the photoaffinity labeling of the RLIP76, N-RLIP76$^{1-367}$, C-RLIP76$^{410-655}$, or mutant protein, 2 μg of purified protein was incubated in a final volume of 50 μL in reaction buffer (50 mM Tris-HCl, 2 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, pH 7.0) supplemented with 5−20 μM 8-azido[α-^{32}P]ATP (ALT, INC., 11.7 Ci/mmol). Where specified, unlabeled ATP or ADP was also added to the reaction mixtures. The reaction mixtures were kept for 5 min at room temperature, then kept on ice for 3 min, and irradiated for 5 min with a UV lamp (UVSL25, Ultra-Violet Products Inc., San Gabriel, CA) at a distance of 3 cm. Thereafter, the protein was precipitated with 1 mL of ice-cold 6% trichloroacetic acid solution containing 15 mM Tris-HCl, pH 7.4, centrifuged at 3000*g* for 20 min at 4 °C, and washed 3 times with 3 mL of the same trichloroacetic acid solution with similar centrifugations. The final pellet

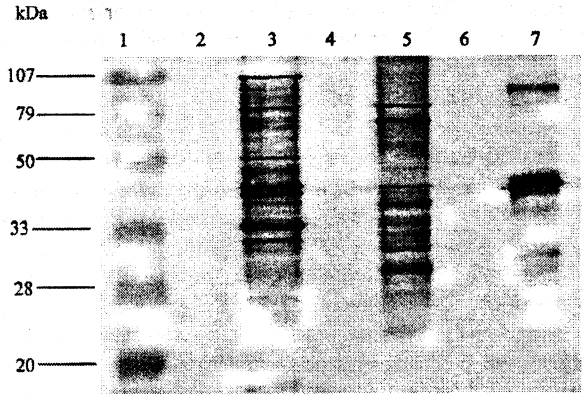

FIGURE 1: Expression and Western blot analysis of rec-RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$. Protein (2 μg) from the lysates of *E. coli* transformed with pET-RLIP76, pET-N-RLIP76$^{1-367}$, and pET-C-RLIP76$^{410-655}$ was subjected to Western blot analyses using anti-RLIP76 antibodies. Lane 1, molecular mass markers; lanes 2 and 3, protein from the total lysates of noninduced and IPTG-induced *E. coli* transformed with pET-RLIP76, respectively; lanes 4 and 5, protein from the total lysates of noninduced and IPTG-induced *E. coli* transformed with pET-N-RLIP76$^{1-367}$, respectively; lanes 6 and 7, protein from the total lysates of noninduced and IPTG-induced *E. coli* transformed with pET-C-RLIP76$^{410-655}$, respectively.

was dissolved in 15 μL of SDS–PAGE sample buffer and electrophoresed in 12% SDS–polyacrylamide gels. The gels were dried and subjected to autoradiography and evaluation by the AlphaImager 2000 Documentation & Analysis System (Alpha Innotech Co.). Vanadate-induced nucleotide trapping was analyzed according to the method of Urbatsch et al. (*15*). Orthovanadate solutions (100 mM) were prepared from Na_3VO_4 and boiled for 2 min before each use to break down polymeric species. The purified protein was incubated for 5 min with 20 μM 8-azido[α-^{32}P]ATP (about 2 μCi in each reaction), 3 mM $MgCl_2$, 200 μM vanadate, 20 mM Tris-HCl, pH 8.0, 0.1 mM EGTA, in a total volume of 50 μL at 37 °C. Incubations were started by addition of protein and stopped by transfer to ice. Samples were kept on ice and irradiated with UV light for 5 min. 'Dark controls' where samples were not irradiated with UV light were included in all experiments.

RESULTS

Expression in E. coli and Purification of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$. We have recently shown that the purification of rec-RLIP76 either from eukaryotic cells or from *E. coli* overexpressing this protein results in preparations showing several bands in SDS–PAGE, which arise through fragmentation of RLIP76 (*1*). The preparations of rec-RLIP76 containing these bands catalyzed the hydrolysis of ATP stimulated by DOX or DNP-SG, and when reconstituted in proteoliposomes, mediated the ATP-dependent transport of DOX and DNP-SG (*1*). To test the hypothesis that RLIP76 may be a precursor protein which undergoes proteolytic cleavage to give peptides which reassemble to form a 'transporter complex', we prepared rec-N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ by separately expressing these peptides in *E. coli*. The expression was examined by Western blot analyses of crude bacterial lysates using polyclonal antibodies raised against RLIP76 (*1*). The results presented in Figure 1 show that rec-RLIP76, rec-N-RLIP76$^{1-367}$, and rec-C-RLIP76$^{410-655}$ were expressed in transformed *E. coli* in the presence (lanes 3, 5, and 7, respectively, in Figure 1) but not in the absence of IPTG (lanes 2, 4, and 6, respectively). Interestingly, N-RLIP76$^{1-367}$ showed multiple bands (lane 5, Figure 1) recognized by antibodies against RLIP76, indicating that this peptide undergoes fragmentation similar to that of full-length RLIP76 (lane 3, Figure 1). Furthermore, the appearance of bands in N-RLIP76$^{1-367}$ at molecular masses greater than the expected value of 49 kDa suggests that some of the peptides generated from N-RLIP76$^{1-367}$ undergo aggregation. As opposed to N-RLIP76$^{1-367}$, the C-terminal peptide, C-RLIP76$^{410-655}$, was relatively resistant to degradation, and a major band at its expected apparent molecular mass value of about 38 kDa was observed along with some minor bands (lane 7, Figure 1). Collectively these results show that RLIP76 readily undergoes degradation, perhaps by proteolysis, and that its N-terminal domain is much more susceptible to degradation as compared to the C-terminal domain. This contention is supported by the results of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ purification presented below.

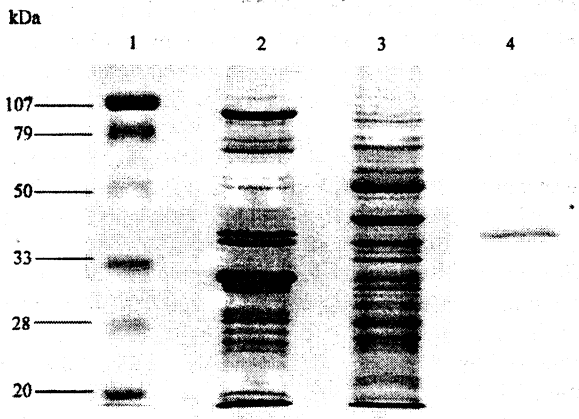

FIGURE 2: SDS–PAGE of purified rec-RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$. Purified peptides were subjected to SDS–PAGE and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular mass markers; lane 2, RLIP76 (10 μg); lane 3, N-RLIP76$^{1-367}$(10 μg); lane 4, C-RLIP76$^{410-655}$(2 μg).

Purification of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$. To evaluate the partitioning of rec-N-RLIP76$^{1-367}$ and rec-C-RLIP76$^{410-655}$ in the membrane and soluble fractions, crude lysates of transformed *E. coli* prepared in the absence of detergent were centrifuged at 28000*g*. The results of Western blot analyses of the supernatants and pellets (after solubilization with detergent) revealed that the majority of N-RLIP76$^{1-367}$ was present in the membrane fraction, while C-RLIP76$^{410-655}$ was predominantly present in the soluble fraction (data not presented). Therefore, for the purification of N-RLIP76$^{1-367}$, the bacterial lysates were prepared in the lysis buffer containing 1% polidocanol, and the purification of C-RLIP76$^{410-655}$ was performed from lysates prepared in the absence of detergent. As a control, purification of full-length RLIP76 was also performed in parallel from detergent extracts of *E. coli* expressing RLIP76. Coomassie-stained SDS–PAGE of purified RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$ are presented in Figure 2. Consistent with the results of Western blot analysis of crude lysates (Figure 1), purified N-RLIP76$^{1-367}$ yielded multiple bands (Figure 2, lane 3), all of which were recognized by the antibodies

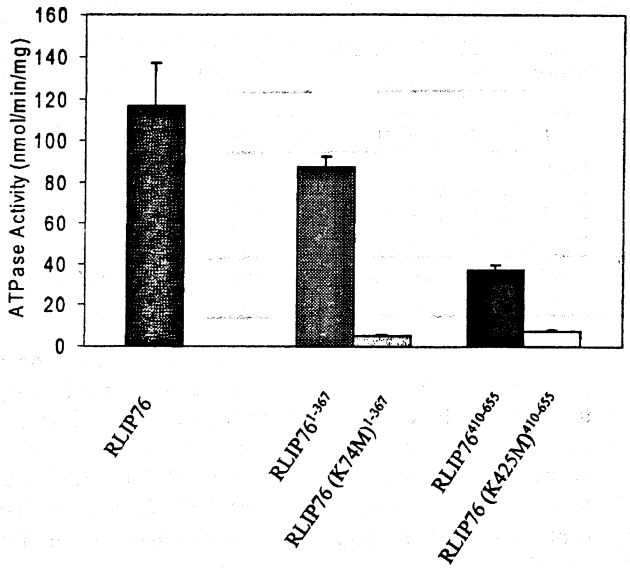

FIGURE 3: ATPase activity of wild-type and mutant N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$. ATPase activity was measured as previously described by us (*3*). Purified protein (2.5 μg) was preincubated at 37 °C for 5 min in 0.5 mL of reaction mixture (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 0.8 mM sodium phosphate, 2.8 mM β-mercaptoethanol, and 1 mM ouabain). The reaction was started by addition of [γ-^{32}P]ATP (final concentration 1.6 mM). After incubation for 60 min at 37 °C, the reaction was terminated by addition of 2.5 mL of an ice-cold mixture of 1 M perchloric acid and 5% ammonium molybdate (4:1). The reaction mixture was extracted with 2.5 mL 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. Means ± SD ($n = 3$) are shown.

against RLIP76 in Western blots (data not presented). In addition to the band at its expected position of 49 kDa, multiple bands higher as well as lower than 49 kDa were observed, and the pattern of these bands closely resembled that of full-length RLIP76 (Figure 2, lane 2). The presence of bands at molecular mass values higher than 49 kDa in the preparations of purified N-RLIP76$^{1-367}$ suggests aggregation of N-RLIP76$^{1-367}$ and/or its fragments and supports our hypothesis of the assembly of transport complex. As opposed to N-RLIP76$^{1-367}$, the C-terminal peptide, C-RLIP76$^{410-655}$, showed predominantly a single band corresponding to its expected apparent molecular mass value of about 38 kDa. This was also consistent with the results of Western blot analyses of crude lysates presented in Figure 1. Taken together, these results confirmed that the fragmentation and aggregation patterns of N-RLIP76$^{1-367}$ observed by Western blotting in crude bacterial lysates (Figure 1) are reflected in the purified protein and that C-RLIP76$^{410-655}$ is relatively resistant to such fragmentation.

ATPase Activity of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$. As shown in Figure 3 and Table 1, both peptides had ATPase activity. This activity was Mg^{2+}-dependent, linear with respect to protein concentration and time of incubation (data not presented), and saturable with respect to ATP. The specific activity of N-RLIP76$^{1-367}$ was significantly higher than that of C-RLIP76$^{410-655}$, but the activities of both peptides were noticeably lower than that of full-length RLIP76 (Table 1 and Figure 3). The K_m values for ATP of both peptides (Table 2) were in the same range as that

Table 1: Stimulation of ATPase Activity of RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$ by DNP-SG, DOX, and COL[a]

additions	specific activity [nmol min^{-1} (mg of protein)$^{-1}$]		
	RLIP76	N-RLIP76$^{1-367}$	C-RLIP76$^{410-655}$
none	116.5 ± 9.8	87.3 ± 5.2	37.2 ± 4.0
DNP-SG (120 μM)	200.7 ± 18.1	157.5 ± 15.9	80.0 ± 10.0
DOX (10 μM)	242.2 ± 28.2	186.8 ± 10.2	88.4 ± 6.2
COL (5 μM)	229.0 ± 34.8	194.2 ± 11.5	90.6 ± 6.9

[a] The proteins were purified from the transformed *E. coli* cultures (100 mL) as described in the text. The ATPase activity was determined as described by us previously in the presence of the specified concentrations of the stimulating compounds. Values represent means ± SD of $n = 3$.

reported for rec-RLIP76 (*1*) and tissue-purified DNP-SG ATPase (*3*). We have previously shown that various anionic as well cationic compounds, that are substrates for tissue-purified DNP-SG ATPase (*3*) and rec-RLIP76-mediated ATP-dependent transport (*1*), stimulate the ATPase activity of these proteins. The results presented in Table 1 demonstrated that the ATPase activity of both N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ was stimulated by weakly cationic compounds such as DOX and COL, as well as by the anionic DNP-SG. For each of these substrates, the K_m values for stimulation of the ATPase activity were noticeably similar for RLIP76 and its N- and C-terminal peptides (Table 2). Moreover, the K_m values for DNP-SG-, DOX-, and COL-stimulated ATPase activity were in the same range as the K_m values for ATP-dependent transport of these compounds reported for tissue-purified DNP-SG ATPase (*4*) or rec-RLIP76 (*1*) in reconstituted proteoliposomes. The ATPase activities of RLIP76 and its N- and C-terminal peptides were also stimulated in the presence of vinblastine, 17-β-estradiol-17(β-D-glucuronide), and lithocholic acid 3-*O*-sulfate (data not presented).

Photoaffinity Labeling with 8-Azido[α-^{32}P]ATP. The ATPase activity of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ indicates that ATP binding sites must be present in these peptides. The existence of such sites was confirmed by their labeling with 8-azido[α-^{32}P]ATP and by trapping of the label in the presence of vanadate. The full-length RLIP76 (Figure 4A) as well as the truncated peptides, N-RLIP76$^{1-367}$ (Figure 4B) and C-RLIP76$^{410-655}$ (Figure 4C), were photoaffinity labeled with 8-azido[α-^{32}P]ATP proportionally to the amount of protein (lanes 1, 2, and 3 in panels A, B, and C of Figure 4). No labeling was observed in controls in which the reaction mixtures were not irradiated with UV light (data not presented). The characteristic fragmentation patterns for RLIP76 and N-RLIP76$^{1-367}$ were evident (Figure 4, panels A and B, respectively). In contrast, photoaffinity labeling of C-RLIP76$^{410-655}$ resulted in a single labeled band (Figure 4, panel C), consistent with the lack of fragmentation of this peptide (Figure 2). The labeling of all three proteins was Mg^{2+}-dependent (lane 6 of Figure 4 in all panels) and was inhibited by ATP (lane 7 of Figure 4 in all panels) and ADP (lane 8 of Figure 4 in all panels).

Vanadate is a strong inhibitor of multiple ATPases. Among others, vanadate has been shown to inhibit ATPase activities and transport functions of P-glycoprotein (*15, 16*) and DNP-SG ATPase (*17*). Vanadate is known to increase the apparent affinity of many ATP binding proteins for Mg^{2+}ADP, resulting in its trapping in the catalytic site (*18, 19*). As

Table 2: Kinetic Parameters of RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$ for ATPase Activity Stimulated by Different Compounds[a]

	RLIP76			N-RLIP76$^{1-367}$			C-RLIP76$^{410-655}$		
substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)
DNP-SG	67.9	142.3	2.1	89.3	110.4	1.24	61	34.6	0.57
DOX	2.5	211.1	84.4	2.3	123.7	53.8	2.34	50.7	21.7
COL	2.1	160.3	76.3	2.6	107.5	41.3	1.96	28.5	14.5
ATP	2100	–	–	2500	–	–	2000	–	–

[a] ATPase activity was determined using a fixed concentration of ATP (1.6 mM) in the presence of various concentrations of DNP-SG (5–200 μM), DOX (0.2–10 μM), and COL (0.2–20 μM).

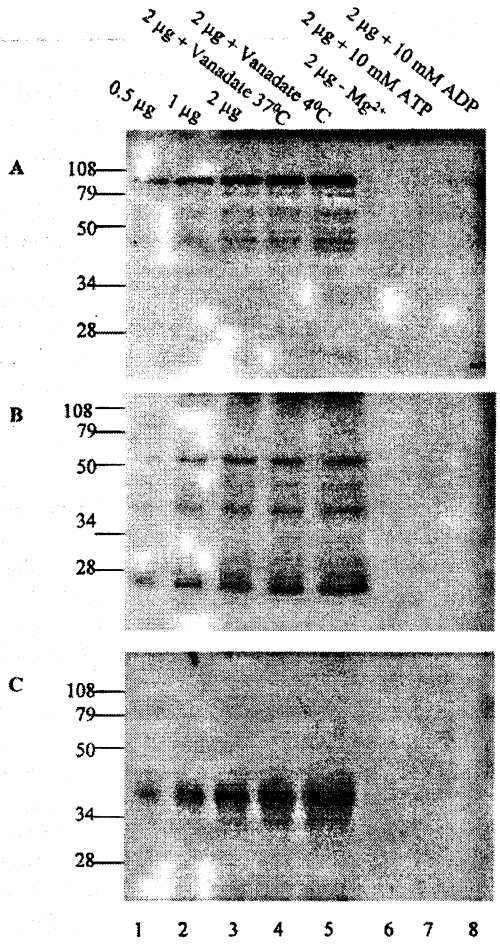

FIGURE 4: Photoaffinity labeling of rec-RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$ with [α-^{32}P]-8-azido ATP. The specified amounts of proteins were subjected to photoaffinity labeling as detailed in the text. Panel A, RLIP76; panel B, N-RLIP76$^{1-367}$; panel C, C-RLIP76$^{410-655}$. In all panels, lanes 1–3, labeling of increasing amounts (0.5, 1, and 2 μg) of proteins. Lanes 4 and 5, labeling in the presence of 200 μM vanadate at 37 and 4 °C, respectively. Lane 6, labeling in the absence of Mg^{2+}. Lanes 7 and 8, labeling in the presence of 10 mM ATP and ADP, respectively.

shown in lanes 4 and 5 of all three panels in Figure 4, vanadate caused increased labeling of intact RLIP76 and of its N- and C-terminal fragments. Vanadate-mediated trapping of the label was higher at 4 °C (lane 5 in Figure 4) as compared to that at 37 °C (lane 4 in Figure 4).

Effect of Stimulation of ATPase Activity on 8-Azido[α-^{32}P]ATP Photoaffinity Labeling. Compounds which stimulate the ATPase activity of RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$ may affect the affinity, and thus labeling, of these proteins by 8-azido[α-^{32}P]ATP. Therefore, we examined the effect of DNP-SG, DOX, and COL on the photoaffinity labeling. Results presented in Figure 5 showed that each of these compounds enhanced the labeling of all three proteins. In these experiments, the characteristic degradation pattern was also observed for RLIP76 and N-RLIP76$^{1-367}$, while C-RLIP76$^{410-655}$ showed predominantly a single band suggesting minimal degradation of this peptide.

Identification of ATP Binding Sites of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ by Site-Directed Mutagenesis. Analysis of the sequence of the two RLIP76-derived proteins for known ATP binding motifs failed to identify any such sites in either N-RLIP76$^{1-367}$ or C-RLIP76$^{410-655}$. However, N-RLIP76$^{1-367}$ contained the sequence ^{69}GKKKGK^{74} which was markedly similar to that of the Walker A motif GXXXXGK present in the ABC-family of transporters (*10, 11*). Likewise in C-RLIP76$^{410-655}$, the sequence ^{418}GGIKDLSK^{425} was similar to the ATP binding motif GGXKVXXK, characterized in phosphoglycerate kinases (*20*). Reasoning that these may be the putative ATP binding sequences in N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$, respectively, we targeted these sequences for site-directed mutagenesis, and examined the resultant mutants for ATPase activity and 8-azido-ATP labeling. As shown in Figure 3, when the K^{74} residue in the sequence ^{69}GKKKGK^{74} of N-RLIP76$^{1-367}$ was mutated to M, the mutant peptide containing the sequence ^{69}GKKKGM^{74} lost most of its ATPase activity. Likewise, when the K^{425} residue in the sequence ^{418}GGIKDLSK^{425} in C-RLIP76$^{410-655}$ was mutated to M, the resultant mutant containing the sequence ^{418}GGIKDLSM^{425} also lost most of its ATPase activity. Compounds such as DOX, DNP-SG, and COL which stimulated ATPase activity of the wild-type RLIP76, N-RLIP76$^{1-367}$, and C-RLIP76$^{410-655}$ failed to stimulate ATPase activity in the mutant peptides (data not presented). This loss of ATPase activity was consistent with our finding that both mutations abrogated 8-azido[α-^{32}P]ATP photoaffinity labeling of the peptides. The purified N-RLIP76$^{1-367}$-(K74M) and C-RLIP76$^{410-655}$(K425M) mutant peptides showed in Coomassie blue-stained SDS-gels fragmentation patterns similar to those of their wild-type counterparts (Figure 6, panels A and C, respectively). However, none of the fragments derived from the mutant peptides were labeled with azido-ATP (Figure 6, panels B and D, respectively). These results strongly suggest that the sequences ^{69}GKKKGK^{74} and ^{418}GGIKDLSK^{425} in N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$, respectively, are required for ATP binding and ATPase activity of these peptides. This conclusion is consistent with the observation that many but not all fragments of the wild-type N-RLIP76$^{1-367}$ were labeled by azido-ATP (Figure 6A,B). The unlabeled fragments were

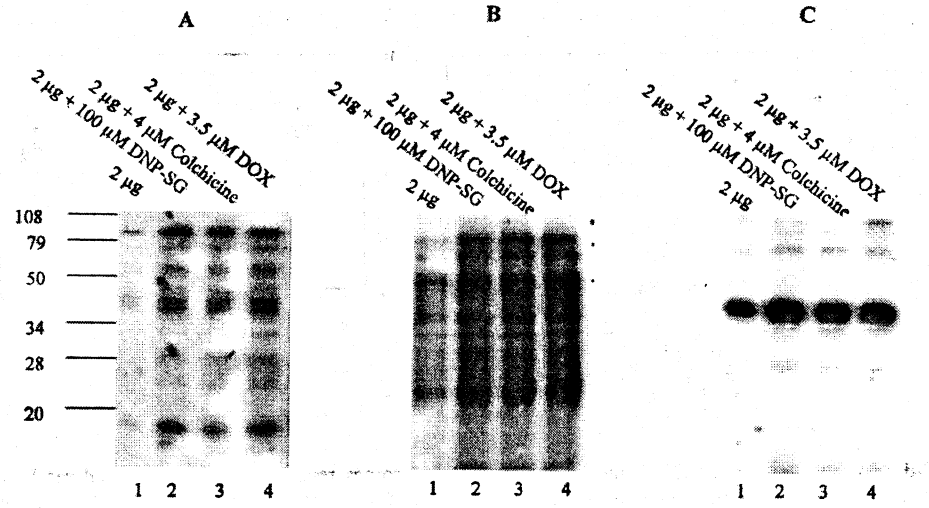

FIGURE 5: Effect of stimulation of ATPase activity by DNP-SG, COL, and DOX on photoaffinity labeling. Panel A, RLIP76; panel B, N-RLIP76$^{1-367}$; and panel C, C-RLIP76$^{410-655}$. In all panels, lane 1, labeling of 2 μg of protein; lanes 2–4, labeling of 2 μg of protein in the presence of 100 μM DNP-SG, 4 μM COL, and 3.5 μM DOX, respectively, added to the reaction mixture prior to the addition of 8-azido[α-^{32}P]ATP. Details of photoaffinity labeling are described in the text.

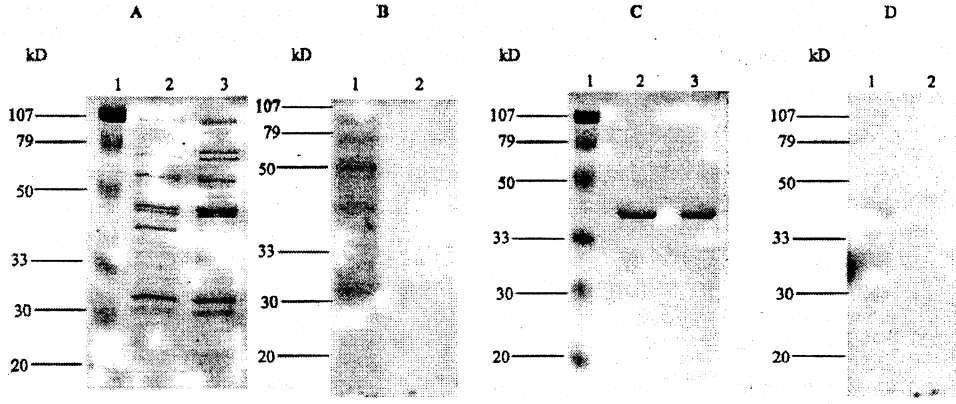

FIGURE 6: Photoaffinity labeling of wild-type and mutant N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$. Two micrograms of purified protein was loaded in each lane. (Panel A) Coomassie blue-stained SDS–PAGE: lane 1, molecular mass markers; lane 2, N-RLIP76$^{1-367}$; lane 3, N-RLIP76$^{1-367}$(K74M). (Panel B) Autoradiogram of proteins subjected to photoaffinity labeling with 8-azido[α-^{32}P]ATP followed by SDS–PAGE: lane 1, N-RLIP76$^{1-367}$; lane 2, N-RLIP76$^{1-367}$(K74M). (Panel C) Coomassie blue-stained SDS–PAGE: lane 1, molecular mass markers; lane 2, C-RLIP76$^{410-655}$; lane 3, C-RLIP76$^{410-655}$(K425M). (Panel D) Autoradiogram of proteins subjected to photoaffinity labeling with 8-azido[α-^{32}P]ATP followed by SDS–PAGE: lane 1, C-RLIP76$^{410-655}$; lane 2, C-RLIP76$^{410-655}$(K425M).

presumably derived from parts of the sequence that does not include the ^{69}GKKKGK^{74} target motif.

Reconstitution of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ in Proteoliposomes. Recombinant total RLIP76, when reconstituted in proteoliposomes prepared from either asolectin or phospholipids of defined composition, mediates ATP-dependent transport of DOX (*1*), DNP-SG (*1*), and COL (unpublished observations in our laboratory). Since both N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ had ATPase activity that was stimulated by the above substrates (Table 1), the peptides were reconstituted into proteoliposomes to determine whether they were able to catalyze transport. The results (Figure 7) demonstrated that neither N-RLIP76$^{1-367}$ nor C-RLIP76$^{410-655}$, when separately incorporated in proteoliposomes, catalyzed ATP-dependent transport of DOX or COL. As expected, proteoliposomes containing the full-length RLIP76 used as a positive control catalyzed ATP-dependent transport of DOX and COL, and the kinetic characteristics of this transport (data not presented) were essentially similar to those described by us recently (*1*).

Reassembly of Functional Transporter. To test our hypothesis that the processed peptide fragments of RLIP76 may reassemble to form a functional transport complex, we prepared proteoliposomes in which a mixture containing equal amounts of N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ was incorporated. Results of transport measurements in these proteoliposomes (Figure 7) clearly demonstrated that ATP-dependent transport of DOX and COL was mediated by these proteoliposomes. This transport was linear with protein concentrations and time, sensitive to osmotic shrinking of the vesicles and to temperature (optimal temperature 37 °C), could be abolished by heat inactivation, and was saturable with respect to ATP as well as DOX or COL with kinetic constants for these substrates (data not presented) similar to those reported for RLIP76 (*1*). Since the N-RLIP76$^{1-367}$ undergoes facile fragmentation and the preparations used for transport studies contain a mixture of these fragments, it is at present not possible to ascertain which fragment(s) is (are) crucial for the transport activity of the complex. Proteoliposomes were also made from the mutant proteins

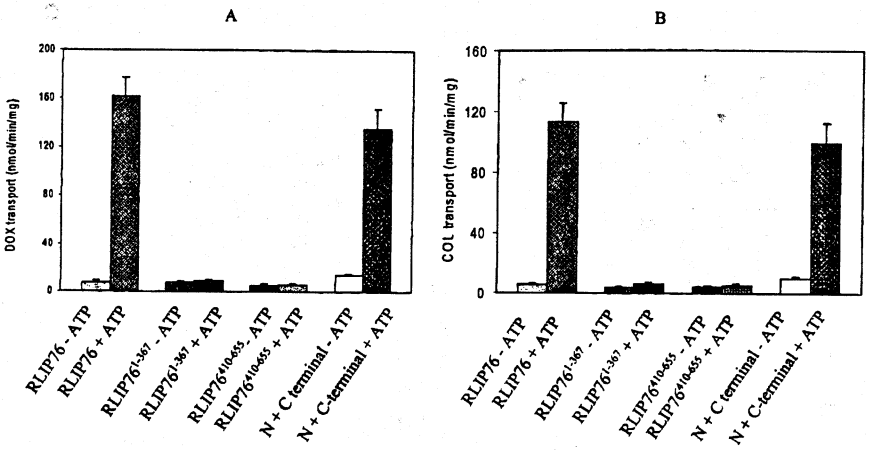

FIGURE 7: ATP-dependent transport of DOX and COL by proteoliposomes reconstituted with purified rec-RLIP76, N-RLIP$^{1-367}$, and C-RLIP76$^{410-655}$. The proteins were expressed, purified, and reconstituted as described under Experimental Procedures. Control liposomes were prepared using equal amounts of protein from the extract of noninduced *E. coli*. Transport assays were performed according to method of Awasthi, S., et al. using a 96-well plate filtration device (*4*). Reaction mixtures (108 μL) containing reconstituted liposomes or proteoliposomes (0.125 μg of protein) were incubated with [^{14}C]-DOX (left panel) or [^{3}H]-COL (right panel) for 15 min at 37 °C. Transport reaction was started by addition of 12 μL of 40 mM ATP. The final concentration of ATP, DOX, or COL was 4 mM, 3.6 μM, or 5 μM, respectively. Equi-osmolar amounts of NaCl were added to the controls measuring transport in the absence of ATP. Transport was measured for 5 min at 37 °C, after which the reaction mixture was filtered through a 0.45 μm membrane in the Millipore 96-well plate manifold. Background binding of [^{14}C]-DOX or [^{3}H]-COL to the filter membrane was estimated by omitting liposomes from the transport reaction mixtures. The values for ATP-dependent and ATP-independent transport presented in bar graphs represent mean ± SD (n = 3).

N-RLIP76$^{1-367(K74M)}$ and C-RLIP76$^{410-655(K425M)}$, which, as described above, are devoid of ATPase activity (Figure 3). Proteoliposomes reconstituted with a mixture containing equal amounts of these mutant peptides did not show any detectable ATP-dependent transport activity for any of the substrates used in this study (data not presented).

DISCUSSION

Our recent discovery that RLIP76 is an ATP-dependent transporter of medium-size organic compounds and that it is similar to DNP-SG ATPase (*1*), a transport protein widely expressed in human tissues (*3*−*7*), has evoked exciting possibilities of additional physiological roles of this protein besides Ral-mediated signaling mechanisms (*2*). We have previously suggested its role in transport-mediated mechanisms of multidrug resistance (MDR) of cancer cells and shown (*1*) that cancer cells transfected with RLIP76 acquire partial resistance to DOX and 4-hydroxynonenal (4-HNE). Present studies add COL, a classical substrate of P-glycoprotein (*21*), to the list of substrates or allocrites (a term coined by Holland and Blight, ref *22*, to accurately define the substrates of transporters) of RLIP76. In addition, the present studies show that the ATPase activity of RLIP76 and its two peptides is stimulated by a wide variety of compounds including 17-β-estradiol-17(β-D-glucuronide), lithocholic acid 3-*O*-sulfate, and vinblastine, suggesting these compounds to be allocrites of RLIP76. Thus, RLIP76 appears to have a wide allocrite spectrum, a characteristic RLIP76 shares with the two widely studied transporters Pgp (*23*) and MRP (*24*) involved in the mechanisms of MDR. Transport of GS−E by RLIP76 may be important not only for the detoxification of electrophilic xenobiotics but also in leukotriene (LTC)-mediated signaling in the mechanisms of chemotaxis. LTC, an important group of physiologic GS−E, serves as a intercellular signal for chemotaxis, a process suggested to be regulated by the Rho pathway (*25*). RLIP76 has been postulated to be a link between Ral and Rho because of its GTPase activating (GAP) activity toward CDC42 (*2*). DNP-SG ATPase and/or RLIP76 have remarkably higher affinity for LTC_4 as compared to other GS−E allocrites (*26, 27*), and its transport by RLIP76 may be a major determinant of LTC_4 concentration. The transport of the GSH conjugate of 4-HNE by RLIP76 may also be highly relevant for the regulation of intracellular concentrations of 4-HNE, which is implicated in signaling for apoptosis and differentiation (*28, 29*). This idea is consistent with our unpublished observations that human leukemia K562 cells overexpressing RLIP76 acquire 2−5-fold resistance to 4-HNE-induced apoptosis. Studies to explore these potential roles of RLIP76 with major clinical implications must be conducted. Also it may be important to elucidate the interrelationship between previously characterized transporters of GS−E and oxidized glutathione (*30, 31*).

The functionality of RLIP76 as a versatile ATP-dependent transporter raises interesting mechanistic questions. Foremost is the problem of how to reconcile the primary active transport function of RLIP76, which requires an integral membrane protein, with its previously assigned role in Ral signaling (*2*) which presumably predicts a cytosolic (or peripheral membrane) localization. The present studies may provide some clues to this puzzling question. A primary active transporter must satisfy at least two criteria. First, it must catalyze substrate-stimulated ATP hydrolysis, and second, it must span the membrane to create a path for the transported compound. The results of the present studies confirm our previous observations that RLIP76 fulfills the first criterion of substrate-stimulated ATP hydrolysis. Our results not only show that the ATPase activity of RLIP76 and its peptide fragments is stimulated by different allocrites, but also demonstrate photoaffinity labeling of RLIP76 and its peptides which is increased in the presence of allocrites. This is consistent with the transport function of RLIP76 since a conformational coupling of ATP binding and/or hydrolysis with substrate binding is a hallmark of transporting ATPases.

Inspection of the RLIP76 amino acid sequence revealed the presence of two motifs with considerable similarity (although not identity) with the consensus for a known ATP binding site, the P-loop (Walker motif) (*10, 32*). One of these two sequences was located in the N-terminal part, and the other in the C-terminal part of RLIP76. Both are functional, as shown by (i) photoaffinity labeling of both N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$, (ii) ATPase activity of both of the above peptides, and (iii) loss of photoaffinity labeling and ATPase activity upon mutation of the critical conserved lysine (*32*) in either of the above peptides. The N-terminal site resembled those found in a variety of ATPases including ABC proteins, while the C-terminal motif was more closely related to the site found in phosphoglycerate kinases (*20*). By virtue of their similarity to the Walker A motif, these sites are likely to interact with the phosphates of ATP (*32*). Future studies will determine the possible roles and interactions of these two sites.

The question whether RLIP76 fulfills the criterion of spanning the membrane deserves a closer scrutiny. There is compelling evidence for the localization of RLIP76, at least partly, in membranes. DNP-SG ATPase, a transport protein (*3*−*7*) whose identity has been established with RLIP76 (*1*), was first characterized (*5*) and isolated (*3, 6*) from erythrocyte membrane vesicles free of cytosol. Purification of rec-RLIP76 from transformed *E. coli* (ref *1* and present studies) requires detergent extraction. Likewise, a closer examination of results on purification of RalBP1 (*33*) and RIP (*34*), the rat and mouse orthologs of RLIP76, respectively, suggests that these are also associated, at least in part, with membranes. Present studies are consistent with these findings and reveal that the N-terminal domain of RLIP76 is more tightly associated with membrane. However, the nature of this association is not clear.

Scanning of the RLIP76 amino acid sequence does not reveal any obvious transmembrane helices. However, the algorithms that identify transmembrane alpha helices may be fairly accurate but are not infallible, and structures other than alpha helices may enter into favorable interactions with the hydrophobic core of the membrane (*35*). Nevertheless, the apparent lack of transmembrane helices in RLIP76 indicates that it may undergo a substantial rearrangement to become an integral membrane protein. Several mechanisms leading to such rearrangement can be considered. One, for which there is an experimental precedent, is a drastic conformational change which probably moves structural elements previously buried in the hydrophobic interior of the protein to its surface, and which may make use of polar domains found originally on the surface to form a hydrophilic channel useful for transport. A conformational change of this type has been observed for certain annexins (*36*−*38*).

While the mechanism discussed above may apply to RLIP76, a different possibility was suggested by the facile fragmentation of the protein. We have now confirmed (ref *1* and present work) that the fragmentation of RLIP76 occurs not only upon purification from tissues as observed in earlier studies with DNP-SG ATPase (*3*), but also when the protein is expressed in *E. coli*. The pattern of fragments is reproducible, and not only is present in purified preparations but also is discernible by Western blotting in total lysates of transformed bacteria, suggesting that it is not an artifact of purification. The purification of RLIP76 in the presence of unusually high concentrations of PMSF (500 μM vs against 100 μM used in ref *1* and in the present studies) resulted in a noticeable increase in the yield of intact RLIP76, but the fragmentation patterns were similar for the preparations of RLIP76 purified in the absence or presence of 500 μM PMSF, and their transport as well as ATPase activities were comparable (data not presented). The fragmentation of RLIP76 is strikingly persistent since it occurs in eukaryotic as well as in prokaryotic cells. Interestingly, the resulting fragments show a propensity for aggregation leading to structures that are stable even under denaturing conditions since they can be observed on SDS−PAGE (Figure 2). These results suggest that the proteolytic fragmentation of RLIP76 and subsequent reassembly of at least some of these fragments into stable complexes may be part of normal RLIP76 physiology required for its transport function. This is consistent with our working hypothesis that the full-length RLIP76 is a precursor subject to processing into mature peptides which then reassemble to form the transport-competent complex. The stoichiometry and geometry of reassembly could provide the flexibility needed to convert an apparent soluble protein into an integral membrane protein. Processing of precursor proteins to mature, functional forms is a widespread phenomenon in biology. It spans a range of complexity from relatively simple cases of proenzyme conversion into a catalytically active form, to complex situations such as proteolytic liberation of multiple distinct proteins from a viral polyprotein (*39*). The classical processing of insulin is conceptually analogous to the proposed processing of RLIP76, since both chains of mature insulin are derived from a single precursor. While our results suggest that the processing of RLIP76 may be required for its transport function, it is not possible to definitively conclude that such processing is an essential prerequisite for transport. This could be ascertained only by comparing the transport activity of intact RLIP76 with that of preparations containing fragmented RLIP76. At present, this question is unresolved because of our inability to obtain intact RLIP76 even when purified in the presence of a protease inhibitor.

Since no obvious transmembrane helices and thus channel structures could be identified in the sequence of RLIP76, it is possible that such a channel, which is probably a structural prerequisite for transport, is formed by the cooperative action of more than one fragment. On the other hand, the modular nature of proteins (see ref *40* for a recent review) and the strict geometrical requirements for a nucleotide binding site may indicate that ATP binding and hydrolysis is probably contained within a domain, rather than being attributable to structures formed by subunit interactions. Thus, it may be predicted that at least some of the fragments derived from RLIP76 may retain ATPase activity, but that its transport function should require the complete, assembled complex. This prediction is borne out by the results presented in this paper. We have demonstrated that the two major fragments of RLIP76 obtained during its purification have ATPase activity, but neither can mediate the ATP-dependent transport process on its own. However, upon reconstitution, these fragments can reassemble to form a complex capable of ATP-dependent transport of various xenobiotics. As expected for a primary, ATP-dependent pump, the ATPase activity of the constituent peptides of this complex is essential for transport, as shown by the lack of transport activity of the complex

containing mutant peptides devoid of ATPase activity. While it is premature to predict the exact structural organization of this transport complex, a working model could be proposed in which the predominantly cytosolic C-terminal domain may act as a gating protein while the hydrophobic peptide components of the N-terminal domain provide the channel structures. Although speculative at this stage, this model provides a testable working hypothesis for the mechanisms for RLIP76-mediated transport of structurally diverse xenobiotics.

It should be noted that our results are consistent with the model of proteolytic processing of RLIP76 into smaller peptides which would then reassemble into a transport-competent complex, but do not yet prove such a model. The alternative explanation, not ruled out by the present results, is that the N- and C-terminal fragments of RLIP76 associate into a structure closely resembling native RLIP76. Such structure, despite a gap in its polypeptide backbone, could behave similarly to the intact RLIP76. The gain of transport function could then be due to a conformational change of the initially soluble protein followed by its insertion into a membrane. Thus, the fragmentation of RLIP76 would be compatible with, but not necessary for, transport. Numerous precedents exist for proteins retaining their function despite a nick in the polypeptide backbone. In the field of membrane transport, examples are furnished, among others, by the sarcoplasmic reticulum Ca^{2+}-ATPase, whose tryptic fragments regain function when co-reconstituted (*41*), or the MRP1 protein, whose function can be restored by coexpression of its N- and C-terminal halves (*42*). However, it should be noted that a major difference between the above examples and RLIP76 is that the generation of fragments from RLIP76 seems to be physiologic while fragmentation in the above examples is a purely experimental intervention. Thus, fragmentation may indeed be an obligatory step in RLIP76 processing.

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