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Functional reconstitution of Ral-binding GTPase activating protein, RLIP76, in proteoliposomes catalyzing ATP-dependent transport of glutathione conjugate of 4-hydroxynonenal✪

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Earlier studies from our laboratories have shown that RLIP76, a previously described Ral-binding GTPase activating protein (Jullien-Flores *et al.*, 1995, *J. Biol. Chem*. 270: 22473), is identical with the xenobiotic transporter DNP-SG ATPase, and can catalyze ATP-dependent transport of glutathione-conjugates as well as doxorubin (Awasthi *et al.*, 2000, *Biochemistry*, 39: 9327). We have now reconstituted purified bacterially expressed RLIP76 in proteoliposomes, and have studied ATP-dependent uptake of the glutathione conjugate of 4-hydroxynonenal (GS-HNE) by these vesicles. Results of these studies show that RLIP76 reconstituted in proteoliposomes catalyzes ATP-dependent transport of GS-HNE against a concentration gradient. The transport of GS-HNE is saturable with respect to ATP as well as GS-HNE with K_m values of 1.4 mM and 2.5 μM, respectively. These studies demonstrate that RLIP76 mediates

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Abbreviations: DOX, doxorubicin; DNP-SG, *S*-(2,4-dinitrophenyl) glutathione; GAP, GTPase activating protein; GSH, glutathione; GS-HNE, glutathione conjugate of 4-HNE; GST, glutathione *S*-transferase; 4-HNE, 4-hydroxynonenal; HRT, horse radish peroxidase; IOV, inside-our oriented vesicle; JNK, c-Jun N terminal kinase; LTC4, leukotriene C_4; MRP, multi-drug resistance associated protein; Pgp, P-glycoprotein; RLIP76, 76 kDa Ral-binding GTPase activating protein (RalBP1).

active transport of GS-HNE, and are consistent with our previous work showing that RLIP76-mediated efflux of GS-HNE regulates the intracellular concentration of 4-HNE and thereby affects 4-HNE mediated signaling.

4-Hydroxynonenal (4-HNE), a relatively stable and toxic end product of lipid peroxidation, has been implicated in cell cycle signaling mechanisms. Cells in culture exposed to 4-HNE show activation of c-Jun N terminal kinase (JNK) and caspase 3 and eventually undergo apoptosis (Uchida *et al.*, 1999; Cheng *et al.*, 2001a; 2001b; Yang *et al.*, 2001). Exposure to 4-HNE has also been shown to induce erythroid differentiation in human erythroleukemia cells (Cheng *et al.*, 1999). On the other hand, at low concentrations, 4-HNE is reported to cause cell proliferation in at least some cell types (Ruef *et al.*, 1998; Cheng *et al.*, 1999). These studies suggest that 4-HNE influences signaling pathways in a concentration dependent manner and that the regulation of its intracellular concentrations may be crucial for some of the signaling pathways. 4-HNE is generated during lipid peroxidation, the extent of which depends on uncontrollable extracellular factors such as exposure to radiation, xenobiotics, oxidative stress, and heat. Thus, the intracellular concentrations of 4-HNE must be regulated by its metabolism and/or exclusion from the cell (Cheng *et al.*, 2001b).

Cells metabolize 4-HNE through its conjugation to glutathione (GSH) which is catalyzed by glutathione *S*-transferases (GSTs) (Alin *et al.*, 1985). 4-HNE and its GSH-conjugate (GS-HNE) are also substrates for aldose reductase, which catalyzes their NADPH dependent reduction to corresponding alcohols (Srivastava *et al.*, 1995; 1998). GS-HNE is inhibitory to GSTs, and to sustain the GST-mediated cellular mechanisms of detoxification, it must be removed from cells through ATP-dependent transport (Ishikawa, 1989; Awasthi *et al.*, 2000; Sharma *et al.*, 2001). We have recently demonstrated that RLIP76 (Ral interacting protein or Ral-BP1), is the major transporter of GS-HNE in erythrocytes and plays a role in the regulation of intracellular concentration of 4-HNE (Awasthi *et al.*, 2000; Cheng *et al.*, 2001b; Sharma *et al.*, 2001). RLIP76 is a GTPase activating protein (GAP) first described by Jullien-Flores and co-workers (Jullien-Flores *et al.*, 1995). Our studies have shown that DNP-SG ATPase, a previously described transport protein (LaBelle *et al.*, 1988; Awasthi *et al.*, 1994; 1998a; 1998b) which catalyzed ATP-dependent primary active transport of various GSH-conjugates as well as cationic amphiphilic drugs such as doxorubicin (DOX), is identical with RLIP76 (Awasthi *et al.*, 2000). Transfection of K562 cells with RLIP76 accelerates the efflux of GS-HNE (Awasthi *et al.*, 2000; Cheng *et al.*, 2001b). Likewise, induction of RLIP76 in cells subjected to oxidative stress is accompanied by increased transport of GS-HNE (Cheng *et al.*, 2001b). RLIP76 and hGST5.8, a GST isoenzyme with substrate preference for 4-HNE, are induced in a variety of cells upon exposure to conditions causing enhanced lipid peroxidation and 4-HNE formation (Cheng *et al.*, 2001b). Cells with induced RLIP76 show accelerated efflux of GS-HNE and acquire resistance to 4-HNE- or H_2O_2- induced apoptosis through this mechanism. The transport of GS-HNE and resistance to 4-HNE-mediated apoptosis can be abrogated by coating the cells with anti-RLIP76 IgG (Cheng *et al.*, 2001b). These studies suggest that a coordinated action of GSTs and RLIP76 plays a major role in regulating the intracellular concentrations of 4-HNE. Studies with inside-out oriented vesicles (IOVs) prepared from human erythrocyte membranes also indicate that the majority of GS-HNE transport from erythrocytes is catalyzed by RLIP76, and that this transport can be inhibited by antibodies against RLIP76 (Sharma *et al.*, 2001; Cheng *et al.*, 2001b). It has been shown that about 70% of the transport of GS-HNE from erythrocytes is mediated by this transporter (Sharma *et al.*,

2001). Together, these studies strongly suggest that RLIP76-mediated transport of GS-HNE is a major determinant of the intracellular concentrations of 4-HNE.

RLIP76-mediated, ATP-dependent uptake of GS-HNE has been studied primarily in the IOVs prepared from membrane vesicles (Ishikawa, 1989; Awasthi *et al.*, 2000; Sharma *et al.*, 2001). Since this transport is inhibited by anti-RLIP76 IgG (Cheng *et al.*, 2001b; Sharma *et al.*, 2001), it has been concluded that it is specifically mediated by RLIP76 but direct evidence for the ATP-dependent transport of GS-HNE by RLIP76 was lacking. To conclude unequivocally that RLIP76 indeed catalyzes transport of GS-HNE, a functional reconstitution of RLIP76 in proteoliposomes capable of catalyzing ATP-dependent transport of GS-HNE must be achieved. In the present studies, we demonstrate that proteoliposomes reconstituted with highly purified, bacterially expressed RLIP76 can catalyze ATP-dependent primary active transport of GS-HNE. In this communication we describe the kinetic characteristics of RLIP76-mediated transport of GS-HNE, and discuss the physiologic significance of RLIP76 in defense mechanisms against oxidative stress.

MATERIALS AND METHODS

Chemicals. [Glycine-2-^{3}H]GSH (specific activity 44 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). 4-HNE was purchased from the Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All other chemicals and reagents were obtained from the Sigma Chemical Company (St. Louis, MO, U.S.A.).

Synthesis of 3-(4-hydroxynonanyl) glutathione (GS-HNE). 4-Hydroxy-2-*trans*-[^{3}H]nonenal was synthesized by the method of Srivastava *et al.* (1999). Purified 4-hydroxy-2-*trans*-[^{3}H]nonenal (10 μmol, specific activity 1 μCi/mmol) was enzymatically conjugated to GSH (1 μmol) using our previously described method (Sharma *et al.*, 2001), and GS-HNE thus formed was purified by preparative thin-layer chromatography on silica gel G plates using butanol/acetic acid/water (4:1:1, by vol.) as the mobile phase. For the synthesis of unlabeled GS-HNE, a similar procedure was used except that 4-hydroxy-2-*trans*-[^{3}H]nonenal was replaced by unlabeled 4-HNE.

Bacterial expression and purification by metal affinity chromatography of RLIP76 carrying an N-terminal histidine tag. *Escherichia coli* strain BL21(DE3) was transformed with a pET-30a vector containing the full length *RLIP76* cDNA ligated in frame to the plasmid-supplied upstream sequence encoding a 6x histidine tag. The bacteria were grown at 37°C until A_{600} reached 0.6, and were induced with 0.4 mM IPTG at 37°C overnight (Awasthi *et al.*, 2001). The bacterial culture was centrifuged at 1400 × ***g***, and the pellet was lysed in 5 ml buffer containing 50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0, 100 μM phenylmethylsulfonyl fluoride (PMSF), and 0.5% polidocanol by sonication on ice (3 × 15 s at 50 W). The lysate was incubated for 2 h at 4°C with gentle shaking followed by centrifugation at 13 000 r.p.m. for 20 min. The supernatant was mixed with Ni-NTA superflow resin (Qiagen) which was preequilibrated with the above lysis buffer. The resin was incubated for 2 h at 4°C with gentle shaking, and washed with buffer containing 50 mM sodium phosphate, 300 mM NaCl, 0.5% polidocanol and 10 mM imidazole, pH 8.0, until A_{280} returned to zero. The protein bound to the resin was eluted with buffer containing 50 mM sodium phosphate, 300 mM NaCl, 0.025% polidocanol, 100 μM PMSF, 50 μM butylated hydroxytoluene (BHT) and 250 mM imidazole, pH 8.0. The eluted protein was concentrated using Amicon Centriprep concentrators followed by sequential dialysis against buffer containing 10 mM Tris/HCl, pH 7.4, 1.4 mM β-mercaptoethanol, 50 μM BHT, 100 μM EDTA, 100 μM PMSF and 0.025% polidocanol.

SDS/PAGE and Western blotting. Purified RLIP76 was subjected to SDS/PAGE analysis on 12.5% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. Western blot analysis of the purified protein was performed by blotting the polyacrylamide gels to nitrocellulose using the tank blotting method of Towbin *et al.* (1979). The membranes were then incubated with polyclonal antibodies raised in rabbit against bacterially expressed RLIP76 (Sharma *et al.*, 2001) followed by goat anti-rabbit secondary antibodies conjugated with horse radish peroxidase (HRP). The blots were developed using a HRP color developing reagent (Bio-Rad). The protein concentration was determined by the Bradford assay (Bradford, 1976).

Reconstitution of RLIP76 into proteoliposomes. Reconstitution of purified RLIP76 into proteoliposomes was performed by the method described by us previously (Awasthi *et al.*, 2000). Briefly, the purified RLIP76 was dialyzed against reconstitution buffer (10 mM Tris/HCl, pH 7.4, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 40 mM sucrose, 2.8 mM β-mercaptoethanol, and 0.025% (by vol.) polidocanol. A mixture of soybean lipids (asolectin; 40 mg/ml) and cholesterol (10 mg/ml) was prepared in the reconstitution buffer by sonication, and a 100 μl aliquot of this mixture was added to 0.9 ml of dialyzed purified RLIP76 containing 10 μg of protein. The mixture was sonicated for 15 s at 50 W after which 200 mg of SM-2 biobeads preequilibrated with reconstitution buffer (without polidocanol) were added to initiate vesiculation by removal of detergent. After incubation for 4 h at 4°C, SM-2 beads were removed by centrifugation at 3620 × ***g***.

Transport studies. ATP-dependent transport of [^{3}H]GS-HNE by RLIP76 proteoliposomes were measured by the rapid filtration technique described by Awasthi *et al.* (1994). Proteoliposomes were equilibrated at 37°C for 5 min in 120 μl of reconstitution buffer containing 10 μM [^{3}H]GS-HNE (100 c.p.m./pmol). The transport reaction was started by addition of ATP (final concentration 4 mM), and the reaction mixture was incubated for the indicated time at 37°C with gentle shaking. Control proteoliposomes were incubated with 4 mM 5′AMP in place of ATP. The reactions were stopped by placing the tubes on ice. Aliquots (30 μl) of the reaction mixture were filtered through nitrocellulose membrane (0.45 μm) in a 96 well filtration plate under uniform suction. The plate was then air-dried, and the filters were cut by Millipore punch hole assembly. The filters were individually placed in glass scintillation vials containing 10 ml of scintillation fluid, and radioactivity was determined in a scintillation counter (Beckman LS6800). Measurements were performed in triplicate, and the mean ±S.D. is reported. Net ATP-dependent transport of GS-HNE was calculated by subtracting vesicle-associated radioactivity in the presence of AMP from that determined in the presence of ATP. The dependence of transport on RLIP76 protein was demonstrated by comparing protein-containing with protein-free liposomes with or without ATP. Kinetic constants for the ATP-dependent uptake of GS-HNE were determined by varying the concentration of [^{3}H]GS-HNE from 0.5 μM to 20 μM, and that of ATP from 0.5 to 8 mM.

RESULTS AND DISCUSSION

Purification of RLIP76

Bacterially expressed RLIP76 was purified from transformed *E. coli* by chromatography on Ni-affinity columns as described by us previously (Awasthi *et al.*, 2001). Purified preparations showed several bands on SDS polyacrylamide gel electrophoresis (Fig. 1A), and these bands were recognized by antibodies against RL1P76 (Fig. 1B). We and others have previously shown that even though the molecular mass of RLIP76 calculated from its amino-acid composition is 76 kDa, the protein shows several bands at higher as well lower

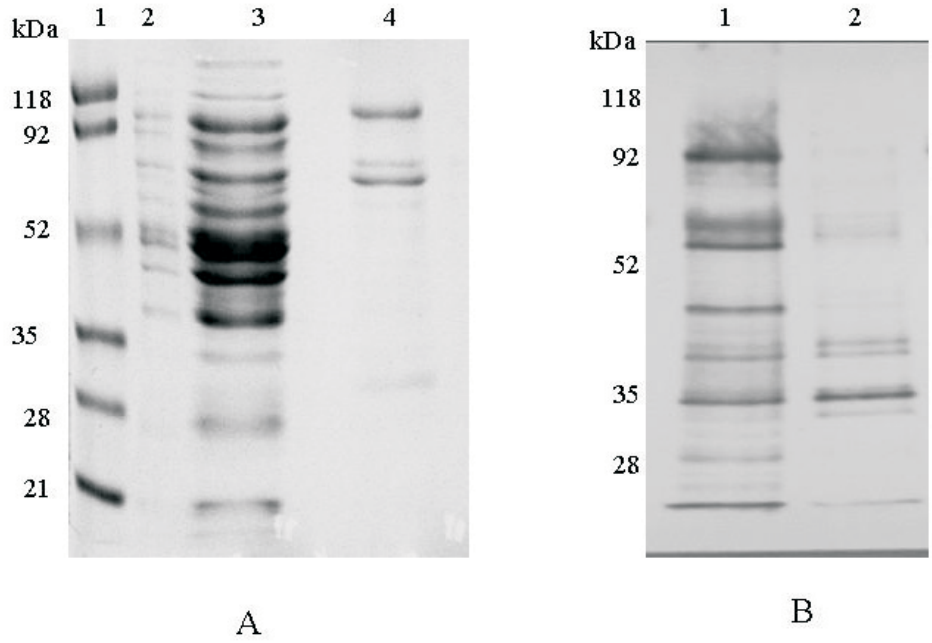

Figure1. SDS/PAGE and Western blot analyses of bacterially expressed RLIP76.

RLIP76 was expressed in *E. coli* BL21(DE3) transfected with full length RLIP76 cDNA, and purified by Ni-NTA metal affinity chromatography as described in the Materials and Methods. Panel A: Coomassie Brilliant Blue stain of SDS/PAGE, lane1, protein molecular mass markers; lane 2, 28 000 × ***g*** supernatant (5 μl) of bacterial extract prior to addition of 0.4 mM IPTG, lane 3, 28 000 × ***g*** supernatant (5 μl) of bacterial extract, 12 h after addition of 0.4 mM IPTG; lane 4, 10 μg of purified RLIP76. Panel B: Western blot probed with anti-RLIP76 IgG and visualized by HRP color developing reagent (Bio-Rad), lane 1, 28 000 × ***g*** supernatant (5 μl) of bacterial lysate (induced); lane 2, purified RLIP76 (10 μg).

molecular mass in SDS gels (Jullien-Flores *et al.*, 1995; Awasthi *et al.*, 2000; Cheng *et al.*, 2001b; Sharma *et al.*, 2001; Awasthi *et al.*, 2001; Sighal *et al.*, 2001). The N-terminal sequences of all these bands represent internal sequences of RLIP76, indicating that the peptides arose from proteolytic processing of RLIP76. The amino acid composition of purified RLIP76 also indicates that the preparations are more than 96% pure (Awasthi *et al.*, 2000), and thus suitable for transport studies.

ATP-dependent uptake of GS-HNE by proteoliposomes reconstituted with RLIP76

Purified RLIP76 was reconstituted into proteoliposomes as described previously (Awasthi *et al.*, 2000; 2001) and detailed in the Materials and Methods Section. Electron microscopic evaluation indicated that the proteoliposomes prepared by this method were closed and mostly unilamellar. We have previously determined by inulin entrapment experiments that the internal volume of vesicles prepared under these conditions as is approximately 19 μl/ml of proteoliposomes (Awasthi *et al.*, 1998b). In preliminary experiment it was shown that the ATP-dependent uptake of [^{3}H]GS-HNE by proteoliposomes was linear for up to 10 min. Thus, for transport experiments the reaction mixtures were incubated for 10 min.

As shown in Fig. 2, a significant amount of [^{3}H]GS-HNE was associated with liposomes reconstituted without RLIP76. However, this association was not dependent on the presence of ATP (Fig. 2) or the presence of equiosmolar NaCl, ADP, or Met-ATP, the non-hydrolyzable analog of ATP (data not presented). These results are consistent with our previous studies on transport of DOX and DNP-SG which show a significant amount of

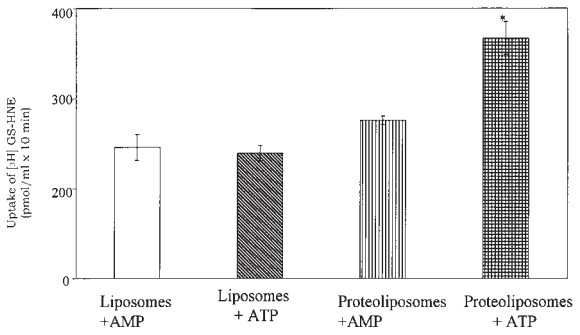

Figure 2. Uptake of GS-HNE in the presence of 5′AMP and ATP by liposomes and proteoliposomes.

Uptake by liposomes and proteoliposomes of [^{3}H]GS-HNE (10 μM, specific radioactivity 100 c.p.m./pmol) was compared in presence of AMP and ATP. Proteoliposomes used for these studies were reconstituted with 10 μg of purified RLIP76 protein and 5 mg of lipid/ml reconstitution buffer. Protein was omitted for reconstitution of liposomes. Liposomes and proteoliposomes were diluted 6-fold in transport buffer containing 10 μM [^{3}H]GS-HNE. After equilibrating at 37°C for 5 min, transport was initiated by addition of either 5′AMP (4 mM) or ATP (4 mM). Aliquots of reaction mixture (30 μl) were filtered in triplicate after 10 min of incubation, and radioactivity remaining on the nitrocellulose filter of each well in the 96 well plate was quantified. Legends for different bars are shown on the figure. *Indicates a statistically significant difference between uptake of GS-HNE by proteoliposomes in presence of ATP as compared to AMP.

non-specific association of DOX and DNP-SG with liposomes (Awasthi *et al.*, 2000), perhaps due to the amphiphilic nature of these compounds.

The association of [^{3}H]GS-HNE with RLIP-76-containing proteoliposomes in the absence of ATP was similar to that observed with protein-free liposomes, with or without ATP. However, in the presence of ATP, a significant increase was observed in the uptake of [^{3}H]GS-HNE by RLIP76 proteoliposomes (Fig. 2). Such an increase was not observed in the presence of Met-ATP or equiosmolar NaCl or ADP used as controls (not shown). These results indicated that [^{3}H]GS-HNE uptake was ATP-dependent, and that the hydrolysis of ATP was required for the uptake. The uptake was temperature-dependent, and maximal uptake was observed at 37°C (data not presented). Based on the specific radioactivity of [^{3}H]GS-HNE (100 c.p.m./pmol), the internal vesicle volume (19 μl/ml of reaction mixture), and the assumption that [^{3}H]GS-HNE is in the lumen of the vesicles, the intravesicular concentration of GS-HNE after 10 min incubation in absence of ATP was estimated to be about 13 μM which was similar to the extravesicular concentration of GS-HNE in the reaction mixture. In the presence of ATP, the estimated concentration of GS-HNE in RLIP76 reconstituted proteoliposomes was found to be about 20 μM, indicating that the transport of GS-HNE into the vesicles was against a concentration gradient.

Uptake of [^{3}H]GS-HNE by RLIP76 proteoliposomes was found to be saturable with respect to GS-HNE and ATP (Fig. 3A and 3B, respectively). The K_m of RLIP76-mediated transport for ATP was found to be 1.4 mM, while the K_m for GS-HNE was 2.5 μM, values similar to those observed for GS-HNE transport in crude membrane vesicles prepared from human erythrocytes. These results also demonstrate that RLIP76 has a significantly higher affinity for GS-HNE as compared to that of the non-physiological glutathione conjugate DNP-SG (K_m of approx. 60 μM) (Sharma *et al.*, 2001).

We have previously demonstrated that the efflux of GS-HNE from K562 cells can be blocked by anti-RLIP76 IgG (Cheng *et al.*, 2001b; Awasthi *et al.*, 2002). Likewise, the ATP-dependent uptake of GS-HNE by erythrocyte membrane IOVs can be blocked by anti-RLIP76 IgG (Sharma *et al.*, 2001). Even though these studies strongly suggested that the transport of GS-HNE was mediated by RLIP76, direct evidence for its involvement in GS-HNE transport was lacking. The present studies clearly demonstrate that RLIP76 can be functionally reconstituted in proteoliposomes, and that it is capable of catalyzing

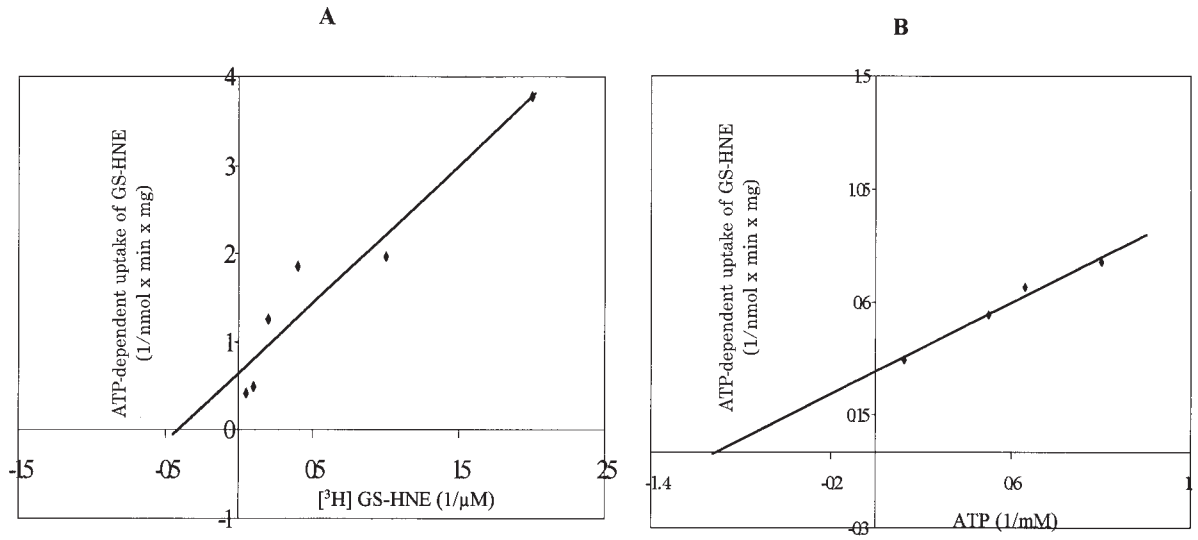

Figure 3. Double reciprocal plots of the kinetics of GS-HNE uptake by proteoliposomes with respect to varying GS-HNE and ATP concentrations.

Proteoliposomes with 10 μg of RLIP76 protein were diluted 6-fold in transport buffer containing 0.5–20 μM [^{3}H]GS-HNE (Panel A). After equilibration at 37°C for 5 min, transport was initiated by the adding 4 mM of either AMP or ATP. Aliquots of the reaction mixture (30 μl) were filtered in triplicate after 10 min. Radioactivity remaining on the nitrocellulose filter of each well in 96 well plate was quantified. ATP-dependent uptake of GS-HNE was calculated by subtracting uptake in presence of AMP from that observed in presence of ATP. Points represent averages of triplicate determinations. Panel B, the uptake of GS-HNE by proteoliposomes measured at varying concentrations (0.5–8 mM) of ATP at invariant 10 μM of [^{3}H]GS-HNE.

ATP-dependent, saturable transport of GS-HNE against a concentration gradient.

ATP-dependent transport of 4-HNE conjugate by membrane vesicles from cells overexpressing MRP1 has been previously reported (Renes *et al.*, 2000). Results of our studies show that the K_m of RLIP76 for GS-HNE is in a similar range to that of MRP1 (Renes *et al.*, 2000). The V_{max} of RLIP76-mediated transport of GS-HNE found in present studies is about 1.4 ± 0.1 nmol/min per mg protein. Considering that a substantial expression of RLIP76 is observed in all human tissues and cell lines examined so far, the present studies suggest that RLIP76 may be a major contributor to transport of GS-HNE in human tissues. Particularly in tissues such as erythrocytes where, in our hands, the expression of MRP1 is undetectable, RLIP76 contributes to a major portion of the transport of GS-HNE or other physiologic GSH-conjugates such as leukotriene C4 (LTC4) (Sharma *et al.*, 2001). It is possible that RLIP76-mediated transport of GS-HNE and LTC4 is predominant in normal tissues, and MRP1-mediated transport may take over in cells overexpressing MRP1. This postulate must, however, be substantiated by further experimental evidence.

In summary, the present studies clearly demonstrate that RLIP76 catalyzes ATP-dependent transport of GS-HNE. These results are consistent with our previous studies suggesting that RLIP76-mediated transport of GS-HNE is an important component of defense mechanisms against oxidative stress and lipid peroxidation, and that RLIP76 can modulate cellular signaling mechanisms by regulating intracellular concentrations of 4-HNE (Cheng *et al.*, 2001b). The latter compound has been implicated (Ruef *et al.*, 1998; Cheng *et al.*, 1999; Uchida *et al.*, 1999; Cheng *et al.*, 2001a; Yang *et al.*, 2001; Cheng *et al.*, 2001b) in signaling for apoptosis, differentiation, and the cell cycle.

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