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Review

Transport functions and physiological significance of 76 kDa Ral-binding GTPase activating protein (RLIP76)✪

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We have recently demonstrated that a previously known Ral-binding GTPase activating protein, RLIP76, can also catalyze ATP-dependent transport of various structurally unrelated xeno- and endobiotics irrespective of their net charge (Awasthi *et al.*, 2000, *Biochemistry,* 39: 9327). RLIP76 is a non-ATP binding cassette (ABC) protein but it has two ATP-binding sites and shows basal ATPase activity which is stimulated

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Abbreviations: ABC, ATP-binding cassette; CYP450, cytochrome P450; DNP-SG, *S*-(2,4-dinitrophenyl) glutathione; DOX, doxorubicin; GAP, GTPase activating protein; GSH, glutathione; GST, glutathione *S*-transferase; 4-HNE, 4-hydroxynonenal; GS-HNE, glutathione conjugate of 4-HNE; GS-X, GSH-conjugate; IOV, inside-out oriented vesicle; JNK, c-Jun N terminal kinase; LPO, lipid peroxidation; LTC4, leukotriene C4; Met-ATP, methylene-adenosine triphosphate; MRP, multidrug resistance associated protein; NSCLC, non-small cell lung cancer; Pgp, P-glycoprotein; RLIP76, 76 kDa Ral-binding GTPase activating protein (RalBP1); SCLC, small cell lung cancer.

in the presence of its transport substrates (allocrites) such as doxorubicin (DOX) and *S*-(2,4-dinitrophenyl) glutathione (DNP-SG). Proteoliposomes reconstituted with purified RLIP76 catalyze ATP-dependent, saturable transport of DOX, as well as of glutathione-conjugates including leukotrienes (LTC4) and the GSH-conjugate of 4-hydroxynonenal (GS-HNE). In erythrocytes the majority of transport activity for DOX, GS-HNE, and LTC4 is accounted for by RLIP76. Cells exposed to mild oxidative stress show a rapid and transient induction of RLIP76 resulting in an increased efflux of GS-HNE and acquire resistance to oxidative stress mediated toxicity and apoptosis. Cells transfected with RLIP76 acquire resistance to DOX through increased efflux of the drug suggesting its possible role in the mechanisms of drug-resistance. In this article, we discuss the significance of transport functions of RLIP76 highlighting its role in the defense mechanisms against oxidative injury, and modulation of signaling mechanisms.

The biotransformation of xenobiotics is carried out in two phases (Williams, 1959). In Phase I, enzymes such as cytochromes P450 (CYP450s), epoxide hydrolases, esterases, and amidases bioactivate xenobiotics by introducing or exposing reactive groups. Bioactivated metabolites of xenobiotics are conjugated to hydrophilic compounds, such as glutathione (GSH), glucuronate, sulfate, etc., by reactions catalysed by the Phase II enzymes. These conjugates must be excluded from the cells because their accumulation can cause inhibition of biotransformation enzymes (Awasthi *et al.*, 1993) and eventual toxicity. The transport mechanisms involved in the efflux of these metabolites have been designated as Phase III of the detoxification process (Ischikawa, 1992). It is established that similar to Phase I (e.g. CYP450s) and Phase II enzymes (e.g. glutathione *S*-transferases — GSTs) these transporters also belong to several superfamilies (Saier & Paulsen, 2001) which have attracted a great deal of interest in recent years, particularly because of their roles in multidrug resistance of bacteria, parasites, and human cancer cells (Saier & Paulsen, 2001; Bambeke *et al.*, 2000; Gottesman & Pastan, 1993; Johnstone *et al.*, 2000; Leslie *et al.*, 2001; Renes *et al.*, 2000a).

Among these transporters, the members of the ABC family (Higgins, 1992; Holland & Blight, 1999) have been studied most extensively. We have recently demonstrated (Awasthi *et al.*, 2000) that a previously described GTPase activating protein, RLIP76 (Jullien-Flores *et al.*, 1995; Cantor *et al.*, 1995; Park & Weinberg, 1995), that does not belong to the ABC superfamily of proteins, can also catalyze the ATP-dependent transport of xenobiotics and their metabolites and is a component of Phase III detoxification system. RLIP76 may be one of the major transporters involved in the extrusion of xenobiotics/their metabolites, as well as endogenously generated toxins. In this mini-review we summarize these studies highlighting the transport function and physiological significance of this novel transporter.

TRANSPORTERS OF ABC FAMILY

A brief description of the ABC transporters, which have been extensively reviewed previously (Leslie *et al.*, 2001; Renes *et al.*, 2000a; Higgins, 1992; Holland & Blight, 1999), is pertinent to this article. ABC transporters catalyze ATP-dependent translocation of substrates (or allocrites) across the membranes. These transporters have ATP-binding sites (Walker motifs) and several transmembrane segments or helices in their sequences (Holland & Blight, 1999), as exemplified by the structures of P-glycoprotein (Pgp) and multidrug resistance associated protein (MRP1). Overexpression of ABC transporters has been associated with drug resistance of certain bacteria, parasites and human cancer cells (Saier & Paulsen, 2001; Bambeke *et al.*, 2000; Gottesman & Pastan, 1993; Johnstone

et al., 2000; Leslie *et al.*, 2001; Ruetz *et al.*, 1996). Overexpression of Pgp, MRP1, or both, has been demonstrated in a variety of cancer cells exhibiting the multidrug resistance phenotype (Gottesman & Pastan, 1993; Johnstone *et al.*, 2000; Leslie *et al.*, 2001). Cells overexpressing Pgp, when exposed to chemotherapeutic agents such as adriamycin, vinblastine, and colchicine, show decreased accumulation of these drugs (Gottesman & Pastan, 1993; Ambudkar *et al.*, 1999). Purified Pgp reconstituted in proteoliposomes catalyzes ATP-dependent (Shapiro & Ling, 1994) transport of doxorubicin (DOX) and related amphiphilic cationic drugs, such as colchicine, vinblastine, and daunomycin (Ambudkar, 1995; Ambudkar *et al.*, 1999; Shapiro & Ling, 1994; Sharom *et al.*, 1993). Excellent review articles on the role of Pgp in drug resistance, its structure and the proposed mechanisms of primary active transport are available (Ambudkar *et al.*, 1999; van Veen *et al.*, 2000; Sauna *et al.*, 2001).

MRP (MRP1 or ABCC1), originally cloned (Cole *et al.*, 1992) from a drug resistant lung cancer cell line, H69, selected for resistance to DOX, has been shown to mediate ATP-dependent transport of the conjugates of GSH, glucuronate, and sulfate (Mao *et al.*, 2000; Leier *et al.*, 1994; 1996; Loe *et al.*, 1998; Jedlitschky *et al.*, 1996; Muller *et al.*, 1994). MRP1 has also been shown to catalyze the transport of physiological GSH-conjugates, such as leukotriene (LTC4) and GS-HNE, the GSH conjugate of 4-hydroxynonenal (4-HNE), which suggests that it plays a physiological role in normal tissues (Mao *et al.*, 2000; Renes *et al.*, 2000b). While it has been reported that MRP1 is ubiquitously expressed in human tissues (Leslie *et al.*, 2001), studies in our laboratory indicate that MRP1 expression in humans may not be ubiquitous because in contrast to the reported presence of MRP1 in human erythrocytes (Pulaski *et al.*, 1996) we could not find any detectable expression of MRP1 in this tissue (Sharma *et al.*, 2001). The reasons for these conflicting findings are not known and must be resolved through further studies. The transport of GSH-conjugates is crucial for the survival of erythrocytes because of the inability of these cells to metabolize these conjugates to mercapturic acids. Our studies have shown that the majority of the transport activity of erythrocyte membranes for GSH-conjugates including LTC4 and GS-HNE is accounted for by RLIP76 (Sharma *et al.*, 2001). Thus, it appears that MRP1 is not the only GSH-conjugate (GS-X) efflux pump in all human tissues and additional transporters for GSH-conjugates are likely to be present. This idea is consistent with earlier studies on the transport of xenobiotics in erythrocyte membranes, which suggest heterogeneity among anion conjugate transporters (Zimniak & Awasthi, 1993).

MRP1-mediated transport of GSH-conjugates of lipid peroxidation (LPO) products has also been suggested to constitute one of the mechanisms of resistance to drugs in cancer cells (Renes *et al.*, 2000a). Exposure to chemotherapeutic drugs, such as DOX, results in LPO leading to the formation of toxic products including 4-HNE. It has been suggested that MRP1 may contribute to drug resistance by transporting GS-HNE. While this may be true for some cell types it appears that other transporters, including RLIP76, may play a major role in these mechanisms (Awasthi *et al.*, 2000; 2001a; 2001b; Sharma *et al.*, 2001).

Despite identification of a large number of putative transporters in human genome, the information about their functions is limited. Our laboratories have focused on the functional characterization of transporters involved in the primary active transport of xenobiotics, xenobiotic conjugates, endogenously generated toxins, and the agents used in cancer chemotherapy. These efforts have led to the finding that a Ral-binding GTPase activating protein, RLIP76, first reported by Jullien-Flores and co-workers (Jullien-Flores *et al.*, 1995), can also catalyze ATP-dependent transport of organic cations and anions (Awasthi *et al.*, 2000; 2001a; 2001b; 2001c;

Sharma *et al.*, 2001; 2002). Surprisingly, RLIP76 turned out to be identical to *S*-(2,4-dinitrophenyl) glutathione ATPase (DNP-SG ATPase), a versatile transporter reported previously that is capable of transporting organic anions, as well as cations (LaBelle *et al.*, 1988; Awasthi *et al.*, 1994; 1998a; 1998b).

DNP-SG ATPase

GSH-conjugates of electrophilic xenobiotics formed within erythrocytes are transported through an ATP-dependent primary active transport process (LaBelle *et al.*, 1986). We identified a protein in membranes of human erythrocytes which catalyzed ATP hydrolysis in the presence of GSH-conjugates (LaBelle *et al.*, 1988). This ouabain and EGTA insensitive ATPase was designated as DNP-SG ATPase because its ATPase activity was stimulated by DNP-SG. The presence of DNP-SG ATPase was subsequently demonstrated in most of the human tissues, including liver, heart, lungs, muscles, kidneys, erythrocytes, leukocytes, and various human cell lines derived from diverse tissue origins (LaBelle *et al.*, 1988; Awasthi *et al.*, 1994; 1998a; 1998b; Sharma *et al.*, 1990; Singhal *et al.*, 1991; Saxena *et al.*, 1992). The basal ATPase activity of DNP-SG ATPase was stimulated in the presence of a variety of organic anions, such as DNP-SG, glucuronides, sulfates, and cations, such as DOX, and its metabolites (LaBelle *et al.*, 1988; Awasthi *et al.*, 1994; 1998a; 1998b; Sharma *et al.*, 1990; Singhal *et al.*, 1991). These findings were complemented by the results of transport studies with membrane vesicles and with proteoliposomes reconstituted with purified DNP-SG ATPase which showed that DNP-SG ATPase catalyzed the transport of the anionic GSH-conjugates, as well as of cationic drugs, such as DOX and colchicine (Awasthi *et al.*, 1994; 1998a; 1998b; 1999). The transport of both organic cations and anions was saturable, temperature-dependent, and sensitive to the osmolarity of the assay medium. ATP hydrolysis was required for transport because the transport activity was not observed in the presence of methylene-adenosine triphosphate (Met-ATP), a non-hydrolyzable analogue of ATP. Polyclonal antibodies raised against DNP-SG ATPase inhibited the ATP-dependent uptake of DOX and DNP-SG by inside-out oriented vesicles (IOVs), prepared from erythrocyte membranes, indicating that the transport was specifically catalyzed by DNP-SG ATPase (Awasthi *et al.*, 1994). Immunological studies also indicated that DNP-SG ATPase was distinct from MRP1 or Pgp (Awasthi *et al.*, 1998a; 1998b). A transport protein immunologically similar to DNP-SG ATPase was also identified in rodent liver (Zimniak *et al.*, 1992; Pikula *et al.*, 1994a; 1994b). This protein, when purified and reconstituted in proteoliposomes, also catalyzed ATP-dependent uphill transport of DNP-SG with kinetic parameters similar to those of human DNP-SG ATPase (Pikula *et al.*, 1994b). These results suggest that transporters similar to DNP-SG ATPase are present in rodent tissues.

IDENTITY OF DNP-SG ATPase WITH RLIP76

Attempts to purify DNP-SG ATPase consistently showed that this protein was prone to degradation, and depending on the conditions of purification, peptides of varying chain lengths were observed in denaturing SDS gels. Thus, for over a decade the molecular identity of DNP-SG ATPase could not be established due to lack of purified protein. Purified preparations of DNP-SG ATPase consistently included a 38 kDa peptide fragment which displayed ouabain, EGTA insensitive ATPase activity that was stimulated by DNP-SG, leading us earlier to an erroneous conclusion that this peptide was the intact DNP-SG ATPase (Awasthi *et al.*, 1998a; 1998b). The polyclonal antibodies against

DNP-SG ATPase were eventually used to clone DNP-SG ATPase. Immunoscreening of a human bone marrow cDNA library using these antibodies yielded RLIP76 (Awasthi *et al.*, 2000), a previously known Ral binding, GTPase activating protein (GAP), which is believed to bridge the Ral, Rac, and CDC42 pathways (Jullien-Flores *et al.*, 1995).

Similar to DNP-SG ATPase, recombinant RLIP76 could be purified by DNP-SG affinity chromatography, and during the purification it underwent proteolytic degradation yielding peptide patterns in SDS gels similar to those observed during purification of DNP-SG ATPase (Awasthi *et al.*, 2000). These observations, and the fact that RLIP76 was cloned using antibodies against DNP-SG ATPase, strongly suggested that these two proteins were closely similar if not identical. The authors who initially described RLIP76 and its rat and mouse orthologs, RalBP1 and RIP, respectively (Jullien-Flores *et al.*, 1995; Cantor *et al.*, 1995; Park & Weinberg, 1995) had also noted the aberrant behavior of these proteins in SDS gels. The proteins migrated in SDS gels as a major band in the range of molecular mass values of 95–110 kDa, which was higher than their molecular mass predicted from the sequences. In addition, several bands corresponding to lower molecular mass also appeared in these gels, suggesting proteolytic degradation of the parent protein. Our preparations of recombinant RLIP76 also showed the 95 kDa band along with several smaller molecular mass peptides; among these, a 38 kDa fragment was consistently prominent. All these peptides were recognized by antibodies raised against DNP-SG ATPase. Also, N-terminal amino-acid sequences of these peptides matched internal sequences of RLIP76, indicating that all these fragments arose from RLIP76, due to its proteolytic processing (Awasthi *et al.*, 2000). The major among these fragments were the C-RLIP76$^{410\text{-}654}$ and N-RLIP76$^{1\text{-}367}$ derived from the C- and N-terminus of RLIP76, respectively (Awasthi *et al.*, 2001c). Both these peptides had inherent ATPase activity similar to DNP-SG ATPase, which could be stimulated by DNP-SG and DOX. The similarities in peptide patterns, immunological crossreactivity, and retention on DNP-SG affinity columns indicated that DNP-SG ATPase and RLIP76 were identical.

TRANSPORT ALLOCRITES OF RLIP76

Bacterially expressed RLIP76 showed catalytic properties similar to those of DNP-SG ATPase purified from human tissues. RLIP76 had constitutive ATPase activity that was stimulated by anionic (e.g., DNP-SG), as well as cationic (e.g., DOX) ligands (Awasthi *et al.*, 2000; 2001c; Singhal *et al.*, 2001). The K_m for the ATPase activity of RLIP76 for ATP, DNP-SG, GS-HNE, DOX, and colchicine were similar to those of DNP-SG-ATPase. When reconstituted in proteoliposomes, RLIP76 catalyzed ATP dependent, uphill transport of DNP-SG, DOX, colchicines, and daunomycin (Awasthi *et al.*, 2000; 2001c; Singhal *et al.*, 2001). These results indicated that the substrate profile of RLIP76 was clearly distinct from either Pgp or MRP1. While the transport of DOX and other amphiphilic cationic drugs has been demonstrated in proteoliposomes reconstituted with Pgp (Ambudkar *et al.*, 1999; Shapiro & Ling, 1995), Pgp does not seem to catalyze the transport of anionic conjugates. On the other hand, MRP1 mediates the transport of organic anions, such as DNP-SG, leukotrienes, GS-HNE, and glucuronides (Leslie *et al.*, 2001). MRP1 also catalyzes transport of vincristine and daunomycin but it requires GSH co-transport (Renes *et al.*, 2000a; Loe *et al.*, 1998). The direct evidence for MRP1 mediated transport of DOX is lacking even though it was originally cloned from cells selected to resistance against this drug. Our studies clearly demonstrate that the substrate spectrum of RLIP76 is wider than that of either Pgp or MRP1, because RLIP76 can

transport organic anions, as well as cations, without the requirement of GSH co-transport.

The physiological significance of the ATP-dependent transport of DOX and GSH-conjugates by RLIP76 was demonstrated by experiments in which RLIP76-overexpressing cells acquired resistance to both DOX and 4-HNE induced cytotoxicity by accelerating the efflux of DOX and GS-HNE (Awasthi *et al.*, 2000). These results, together with the ability of RLIP76 to transport leukotrienes (Sharma *et al.*, 2001), suggest that transport catalyzed by RLIP76 has toxicological, as well as physiological, significance, and could be a modulator of drug resistance.

STRUCTURAL STUDIES WITH RLIP76

As indicated earlier in this article, RLIP76 expressed in cultured cells or in *Escherichia coli* undergoes proteolytic processing (Awasthi *et al.*, 2000). The functional consequences of these post-translational modifications are presently unclear. We have investigated properties of two of the most prominent peptides, N-RLIP76$^{1\text{-}367}$ and C-RLIP76$^{410\text{-}654}$, generated during its proteolytic processing (Awasthi *et al.*, 2001c). These two fragments are cleaved off from the N- and C-termini of RLIP76, respectively, and show bands in SDS gels close to 38 kDa. Both peptides have constitutive ATPase activity that can be stimulated by the anionic and cationic ligands, such as DNP-SG and DOX that are transport allocrites of RLIP76. Both peptides bind ATP, as indicated by photo-affinity labeling, which is increased in the presence of vanadate (Awasthi *et al.*, 2001c). The sequence of the putative ATP binding sites was similar but not identical with the consensus for the P-loop (Walker motif). The N-terminal ATP-binding site, ^{69}GKKKGK^{74}, resembled that of ABC proteins, while the C-terminal site, ^{418}GGIKDLSK^{425}, had similarity with the motif found in phosphoglycerate kinases (Saraste *et al.*, 1990).

When reconstituted in proteoliposomes, neither of these peptides is capable of transport function. However, proteoliposomes reconstituted with a mixture of both fragments catalyze ATP-dependent transport of DNP-SG as well as DOX with kinetic parameters similar to those of RLIP76 (Awasthi *et al.*, 2001c) or tissue purified DNP-SG ATPase (Awasthi *et al.*, 1998a; 1998b). Thus, it appears that both ATP-binding sites are required for the transport function of RLIP76. This idea is consistent with the results of site directed mutagenesis experiments, which showed that mutations of K^{74} and K^{425} to methionine residues in the N- and C-terminal peptides, respectively, abrogated their ATPase activity, ATP-binding capacity and transport function (Awasthi *et al.*, 2001c).

Immunohistochemical studies using antibodies specific to RLIP76 indicate that RLIP76 is associated with membranes. The fact that RLIP76 requires detergent for extraction also suggests that it is an integral membrane protein (Awasthi *et al.*, 2000; 2001c; Singhal *et al.*, 2001). Recent studies demonstrating its role in endocytosis of EGFR, TGFβ and insulin receptors (Matsuzaki *et al.*, 2002; Yamaguchi *et al.*, 1997; Ikeda *et al.*, 1998), exocytosis, and membrane ruffling (Moskalenko *et al.*, 2002) are also consistent with its membrane association. Surprisingly, unlike the ABC transporters, no transmembrane alpha helices are evident in the RLIP76 sequence. The mechanisms through which RLIP76 associates with membranes are not clear and must be investigated. Differences in the ATP-binding sites and transmembrane domains between RLIP76 and ABC proteins suggest that the transporters may be more diverse, in terms of structural elements defining ATP-binding and modes of membrane insertion. RLIP76 undergoes facile proteolytic cleavage, and some of the resulting peptides can be reconstituted into an active transport complex (Awasthi *et al.*, 2001c). This could be viewed as post-translational processing, and may be relevant to

the functions of RLIP76. Further studies are needed to investigate functional consequences of the post translational modifications of RLIP76. It is likely that it may also be relevant to other functions of RLIP76 including its activity as a GAP protein (Jullien-Flores *et al.*, 1995), its role in pathways regulating endocytosis (Matsuzaki *et al.*, 2002; Yamaguchi *et al.*, 1997; Ikeda *et al.*, 1998), exocytosis and membrane ruffling (Moskalenko *et al.*, 2002). The presence of various motifs in the primary structure of RLIP76 (Table 1) may also suggest that the proteolytic fragments resulting from its processing could perform distinct functions. It is possible that these fragments individually, or in association with other proteins, may be involved in the known and as yet unknown functions of RLIP76. It has been shown RLIP76 contains a Ral- binding domain (residues 403–499) while the C-terminal domain (residues 500–647) is involved in binding of Reps1 and POB1, linking RLIP76 to insulin receptor and TGF-β receptor (Jullien-Flores *et al.*, 1995; Yamaguchi *et al.*, 1997). It is possible that fragments containing leucine zipper (residues 547–578), potential N-myristoylation sites if unmasked by cleavage (residues 21–26, 40–45, 191–196), tyrosine kinase phosphorylation site (residues 308–315), and several protein kinase C phosphorylation sites, may be involved in its as yet unknown functions.

Table 1. Various motifs in the primary structure of RLIP76

Motif	Residues	References
ATP-binding sites	N-terminal residues 69–74	Awasthi *et al.*, 2001c
	C-terminal residues 418–425	
Ral-binding region	403–499	Jullien-Flores *et al.*, 1995
Protein kinase C phosphorylation sites*	118–120, 297–299, 353–355, 509–511	
Tyrosine kinase phosphorylation site*	308–315	
N-myristoylation sites*	21–26, 40–45, 191–196	
Leucine zipper pattern*	547–578	
cAMP-dependent protein kinase phosphorylation site*	113–116*	
cGMP-dependent protein kinase phosphorylation site*	650–653	

http://www.ncbi.nml.nih. gov/genome/sts/accession NM_006788.2

RLIP76-MEDIATED TRANSPORT OF GS-HNE AND ITS PHYSIOLOGICAL SIGNIFICANCE

RLIP76 mediates ATP-dependent transport of GS-HNE (Awasthi *et al.*, 2000; 2001b; Sharma *et al.*, 2001) and other electrophilic products of lipid peroxidation. Thus, the transport function of RLIP76 in coordination with GSTs may be important in defense mechanisms against LPO caused by oxidative stress. Furthermore, by regulating the intracellular concentration of 4-HNE, a relatively stable end product of LPO, RLIP76 may affect cell cycle signalling mechanisms. 4-HNE has been shown to trigger apoptosis (Cheng *et al.*, 1999; 2001a; 2001b; Uchida *et al.*, 1999; Yang *et al.*, 2001), cell differentiation (Cheng *et al.*, 1999; Dianzani *et al.*, 1999) and, at low concentration, cell proliferation (Cheng *et al.*, 1999; Ruef *et al.*, 1998). A role of RLIP76 in the regulation of the intracellular concentrations of 4-HNE is indicated by our

studies (Cheng *et al.*, 2001a) showing that a mild, transient heat shock or oxidative stress induces RLIP76 prior to inducing heat shock proteins or the antioxidant enzymes. In these studies, when cells were exposed to mild oxidative stress (50 μM H_2O_2, 20 min) or heat shock (42°C, 30 min) and allowed to recover for 2 h, increased levels of 4-HNE were observed. In the stressed cells, there was an approximately 3-fold induction of hGSTA 5.8, a GST isoenzyme which catalyzes the conjugation of 4-HNE to GSH, leading to the formation of GS-HNE. A concomitant induction of RLIP76 (about 3-fold) was also observed in these cells (Cheng *et al.*, 2001a). Consistent with the induction of RLIP76, cells preconditioned with stress shock transported GS-HNE at a three-fold higher rate. The transport of GS-HNE in stressed cells was specifically due to RLIP76 induction because the efflux of GS-HNE by these cells could be blocked by coating them with anti-RLIP76 IgG (Cheng *et al.*, 2001a). Interestingly, the stress-preconditioned cells, with induced hGSTA 5.8 and RLIP76, were more resistant to H_2O_2 or 4-HNE-mediated cytotoxicity and apoptosis, as compared to the controls (Cheng *et al.*, 2001a). In the stress-preconditioned cells, activation of JNK (c-Jun N terminal kinase) was suppressed, which was consistent with their resistance to apoptosis, because JNK activation is implicated in stress-mediated apoptosis (Uchida *et al.*, 1999; Cheng *et al.*, 2001b; Yang *et al.*, 2001). This resistance of stress preconditioned cells to H_2O_2 or 4-HNE induced apoptosis could be abrogated by coating the cells with anti-RLIP76 IgG, which inhibited the transport of GS-HNE from cells. Thus, it appears that the RLIP76-mediated transport of GS-HNE in cells plays an important role in the regulation of the intracellular levels of 4-HNE, which has been implicated in apoptosis, signalling and differentiation (Cheng *et al.*, 1999; 2001a; 2001b; Yang *et al.*, 2001; Dianzani *et al.*, 1999; Ruef *et al.*, 1998). A protective role of RLIP76 against stress mediated LPO and subsequent toxicity is also suggested by results of studies showing that cells enriched with RLIP76 by exposure to RLIP76-containing proteoliposomes become more resistant to radiation evoked toxicity (Awasthi *et al.*, 2002a). The proposed physiological significance of the transport functions of RLIP76, including its role in defense against LPO, modulation of stress mediated signaling, and drug resistance and the transport of physiologic ligands such as LTC4, is summarized in Fig. 1.

RELEVANCE OF RLIP76-MEDIATED TRANSPORT TO MULTI-DRUG RESISTANCE

Since RLIP76 can catalyze ATP-dependent primary active transport of not only anionic conjugates, but also of cationic chemotherapeutic drugs, such as DOX, daunomycin, and colchicine, the protein may play a role in the acquired multidrug resistance of cancer cells. RLIP76 overexpression can confer resistance to DOX, as well as to alkylating toxins, by increasing the efflux of these agents or their conjugates from cells (Awasthi *et al.*, 2000). Antibodies against RLIP76 have been shown to enhance cytotoxicity of DOX to cancer cells, by blocking the transport of drugs (Cheng *et al.*, 2001a; Awasthi *et al.*, 2002b). It has been suggested that higher resistance of non-small cell lung cancer (NSCLC) cells to DOX, as compared to the small cell lung cancer (SCLC) cells, correlates with a higher RLIP76-mediated efflux of DOX in NSCLC cells (Awasthi *et al.*, 2001a; 2002b). Furthermore, coating of NSCLC with anti-RLIP76 antibodies sensitizes these cells to DOX by blocking its efflux. These studies strongly suggest that RLIP76 modulates drug sensitivity of cancer cells. The relevance of RLIP76 to the mechanisms of multidrug resistance may be two fold; its transport function can protect cells through drug efflux and it can affect signaling mechanisms through the modulation of the intracellular concentration of 4-HNE,

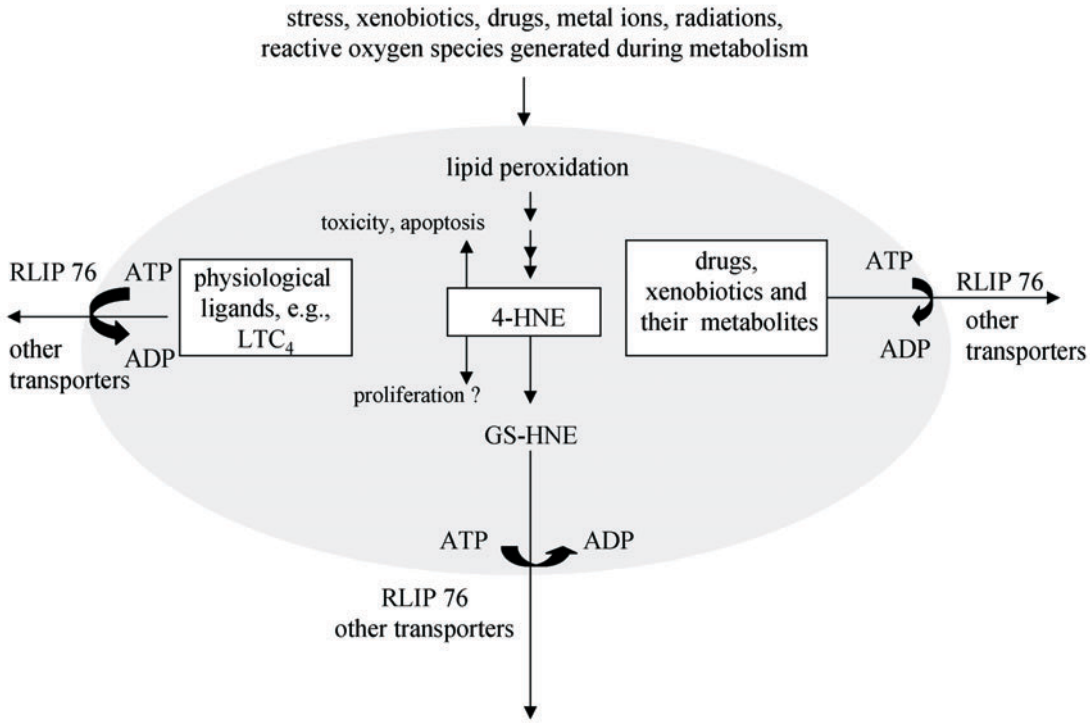

Figure 1. Physiological significance of RLIP76 transport functions.

RLIP76 catalyzes ATP-dependent efflux of GS-HNE formed mostly through enzymatic conjugation of 4-HNE to GSH. Removal of GS-HNE is crucial for effective functioning of GST because it inhibits GST. Thus, RLIP76, in coordination with GSTs, is a major regulator of intracellular concentration of 4-HNE. RLIP76-mediated efflux of xenobiotics and their metabolites which can cause oxidative stress and toxicity. RLIP76 also catalyzes transport of LTC4.

which is known to be involved in cell cycle arrest, cell differentiation, and apoptosis (Uchida *et al.*, 1999; Cheng *et al.*, 1999; 2001b; Yang *et al.*, 2001; Dianzani *et al.*, 1999; Ruef *et al.*, 1998). It has been suggested that the effects of 4-HNE on cell cycle signalling is concentration dependent. Higher concentrations of 4-HNE cause differentiation and apoptosis (Uchida *et al.*, 1999; Cheng *et al.*, 1999; 2001b; Yang *et al.*, 2001; Dianzani *et al.*, 1999). On the other hand, in the presence of low 4-HNE levels cell proliferation has been reported (Cheng *et al.*, 1999; Ruef *et al.*, 1998). Induction of RLIP76, perhaps by oxidative or chemical stress due to anticancer drugs, would deplete 4-HNE and thus promote the proliferation of cancer cells. RLIP76 could, therefore, play an important role not only by catalyzing drug efflux but also by influencing the signaling pathway in favor of cell proliferation. Thus, it may be worthwhile to explore the putative role(s) of RLIP76 in the mechanisms of multidrug resistance, not only because of its distinct transport properties, but also because of its potential role in signaling pathways, which may affect cell proliferation and cell death.

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