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Review

Transport of glutathione-conjugates in human erythrocytes★✪

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The last step of detoxification of both endogenous and environmental toxicants is typically a conjugation that produces a bulky hydrophilic molecule. The excretion of such conjugates out of cells is of sufficient biological importance to have led to the evolution of ATP-driven export pumps for this purpose. The substrate specificity of such transporters is broad, and in some cases it has been shown to include not only anionic conjugates but also neutral or weakly cationic drugs. In the present article, we review the molecular identity, functional and structural characteristics of these pumps, mainly on the example of human erythrocytes, and discuss their physiological role in detoxification and in the multidrug resistance phenotype of cancer cells.

Living cells defend themselves from toxicants/xenobiotics present in the environment through biotransformation of these compounds to relatively non-toxic metabolites and their subsequent elimination through transport mechanisms. Most cells are equipped with a multitude of Phase I and Phase II biotransforming enzymes [1]. Reactive groups such as –OH, $-NH_2$, or =O are introduced/exposed on relatively hydrophobic

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Abbreviations: ABC, ATP-binding cassette; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, *S*-(2,4-dinitrophenyl)-glutathione; DOX, doxorubicin (adriamycin); GAP, GTPase-activating protein; GEFs, guanine nucleotide exchanges factors; GS-E, glutathione-conjugate; GSH, glutatione; GSSG, oxidized glutathione; GSTs, glutathione *S*-transferases; IOVs, inside-out-oriented vesicles; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; RLIP76, Ral-binding protein.

xenobiotics by Phase I enzymes which include cytochrome P450, monooxygenases, epoxide hydrolases, esterases and amidases. The resultant products of these transformations can then be conjugated to hydrophilic compounds such as glutathione (GSH), glucuronate, sulfate, etc., by the Phase II enzymes such as glutathione *S*-transferases (GSTs) and other transferases. These conjugates (usually but not always less toxic, more hydrophilic) are then excreted through transport processes across the cellular membranes.

GSH and GSTs play a central role in the detoxification of exogenous as well as the endogenous electrophiles [2–6] which can alkylate cellular DNA and proteins leading to cellular dysfunction and toxicity. GSH can conjugate these electrophiles nonenzymatically as well as through reactions catalyzed by GSTs. A multitude of GST isozymes belonging to several distinct gene families are present in mammalian tissues [4–6]. These enzymes have wide range of substrate affinities towards the electrophilic compounds and are expressed in a tissue specific manner, perhaps, to meet the detoxification needs of specific organs. The GSH-conjugates (GS-E) are either transported as such or they are converted to mercapturic acid for their eventual excretion. The transport of the GS-E is crucial for normal function of erythrocytes and other cells, because their intracellular accumulation can cause toxicity, inhibition of some glycolytic pathway enzymes and key detoxification enzymes such as GSTs and glutathione reductase [7–11]. Therefore, mechanisms must exist for their transport from cells. In recent years a great interest has arisen in the transport mechanisms for GS-E because of their role in the mechanism of multidrug resistance (MDR) of cancer cells. Accumulation of GS-E in the cell can affect the intracellular concentration of chemotherapeutic drugs either by inhibiting GSTs or by interfering with the transport of GSH-conjugates of chemotherapeutic alkylating agents. Moreover, in recent years it has been shown that GS-E and chemotherapeutic agents such as doxorubicin (DOX), vinblastin and vincristin share common transport mechanisms, further emphasizing the significance of GS-E transport in MDR [11–16]. While it is clear that GS-E are transported from human erythrocytes and other cells through an ATP-dependent active transport process [17–20], the molecular characterization of GS-E transporter(s) has been hampered because of the difficulty in their purification.

Erythrocytes provide a valuable model for studies on GS-E transporters chiefly because a homogenous population of these cells is easily attainable and purified membrane preparation can be easily obtained from these cells for transport studies. Even though the active transport of oxidized glutatione (GSSG) and GS-E from erythrocytes has been known for a long time [17, 18, 21], the molecular mechanism for the transporters was poorly understood until recently. In recent years there have been significant advances in our understanding of the transport mechanism for GS-E in erythrocytes. In the present article, we have briefly reviewed the earlier studies on transport of GS-E from erythrocytes and have provided an updated account of recent findings, which link GS-E transport mechanism in erythrocytes and other cells with signal transduction pathways [22]. The mechanisms of GS-E transport have also been widely studied in other tissues besides erythrocytes but for the sake of brevity we have focused the present review primarily on the studies dealing with erythrocyte GS-E transporters giving the historical perspective, current status, and the physiological, clinical, and toxicological significance of these proteins.

GSSG AND GS-E TRANSPORT IN HUMAN ERYTHROCYTES: EARLIER STUDIES

GSH is present in high concentration in erythrocytes (about 2 mM), where its major role is believed to be the protection of haemo-

globin from oxidation. In this process, GSSG is generated. A significant portion of GSSG thus formed is cleared out from erythrocytes through an active, energy-dependent transport [21]. This ATP-dependent transport of GSSG has been shown *in situ*, in resealed erythrocyte ghosts and in inside-out-oriented vesicles (IOVs) prepared from erythrocyte membranes [21, 23–25].

The formation of the GSH-conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) in erythrocytes through a reaction catalyzed by GST was demonstrated by Awasthi *et al.* [26] and the resultant conjugate, *S*-(2,4-dinitrophenyl)-glutathione (DNP-SG), has served as a valuable model substrate for the studies on GS-E transport in human erythrocytes. Active transport of DNP-SG from erythrocytes *in situ* has been demonstrated [17, 18, 27]. Studies with IOVs prepared from erythrocyte membranes demonstrated that this transport was ATP-dependent [19]. Subsequent studies [20] revealed that DNP-SG transport in erythrocyte membranes was an ATP-dependent primary active process independent of inorganic ion pumps and membrane potential. Because of the similarities in the structures of GSSG and GS-E, the obvious question arose whether or not the mechanisms for the transport of GSSG [21] and GS-E [18] were similar. This question still remains unresolved despite numerous studies [25, 28–32].

Kondo *et al.* [19] through kinetic studies of ATP-dependent uptake of GSSG in erythrocyte IOVs demonstrated it to be a biphasic transport with high and low affinity components (K_m for GSSG, 0.1 mM and 7.3 mM, respectively). Since the low affinity component of this transport process was inhibited by DNP-SG, it was suggested that DNP-SG and GSSG were transported by shared mechanism(s). In contrast, the studies on transport of DNP-SG by erythrocytes *in situ* or in IOVs have shown that DNP-SG transport was unaffected by GSSG [20, 27], indicating that these compounds were transported by distinct mechanisms. Similar to GSSG transport, DNP-SG transport in human erythrocyte IOVs exhibits biphasic kinetics and shows different pH profiles and inhibition patterns by organic anions [30, 32, 33]. Increased transport rate of DNP- SG with increasing extracellular pH suggested that the anionic GS-E may be co-transported with a proton [32]. These studies also revealed heterogeneity in the transport system with at least two components, one having high affinity and other having low affinity for DNP-SG (K_m 3.9 μM and 1.6 mM, respectively). Both these components were inhibited by various polyvalent anions including the conjugates of steroid hormones, bile salts, and bilirubin [30], suggesting wide substrate specificity for this/these transporter(s). It has been suggested that the low K_m systems may have evolved for the transport of GS-E and polyvalent anions, whereas the high K_m component may be responsible for the transport of monovalent anions [30, 31]. However, in the absence of transport studies with purified transporter(s) it is difficult to assign well-defined substrate specificities to these components. Nonetheless, these and other numerous studies on the transport of GS-E in erythrocytes and other tissues strongly suggested that the transport system(s) for the efflux of GSSG and GS-E, could utilize a wide variety of substrates and was heterogenous in nature [30–42].

GSSG AND GS-E TRANSPORT IN ERYTHROCYTES IS LINKED TO SPECIFIC MEMBRANE ATPases

GSSG-stimulated Mg^{2+}-ATPase

Kondo *et al.* [35, 41] showed the presence of a Mg^{2+}-dependent ATPase in erythrocyte membranes which was stimulated in the presence of GSSG. This ATPase activity, designated as GSSG, Mg^{2+}-ATPase, was shown to have biphasic kinetics similar to that reported for GSSG transport in erythrocyte IOVs, and

found to be and independent of other known ATPases. This ATPase was purified from erythrocyte membranes using S-hexyl glutathione affinity chromatography [35] and shown to be composed of two non-identical subunits with molecular mass of 62 kDa and 82 kDa. The purified protein was reconstituted in proteoliposomes and shown to have both GSSG-dependent ATPase activity and ATP-dependent transport of GSSG, linking transport of GSSG to this ATPase. In the absence of transport studies with GS-E it was, however, not established whether GSSG, Mg^{2+}-ATPase catalyzed the transport of GS-E or other anions as observed in erythrocyte IOVs [19].

DNP-SG ATPase

An ATPase activity stimulated by DNP-SG was first characterized by LaBelle *et al.* [28] in erythrocyte membranes. This ATPase was distinct from the known ATPases present in erythrocyte membrane and had K_m values for ATP and DNP-SG similar to those reported for the ATP-dependent transport of DNP-SG in erythrocyte IOVs [20]. It was, therefore, suggested that this ATPase, designated as DNP-SG ATPase, catalyzes the ATP-dependent transport of DNP-SG in erythrocytes. Purification of DNP-SG ATPase from erythrocyte membranes using DNP-SG affinity chromatography yielded enzyme preparations exhibiting DNP-SG-stimulated ATPase activity, and showing a band at 38 kDa in SDS/PAGE [36]. However, minor bands with molecular mass values higher and lower than 38 kDa were always discernible in these preparations. Polyclonal antibodies against the 38 kDa peptide of DNP-SG ATPase were raised, and expression of this transporter in other tissues besides erythrocytes was demonstrated [12, 36, 38]. ATP hydrolysis by DNP-SG ATPase was not stimulated by GSSG [12, 13, 28], suggesting that it was different from GSSG, Mg^{2+}-ATPase characterized by Kondo *et al.* [35, 41]. DNP-SG ATPase was, however, stimulated by various physiologic organic anions such as conjugates of bile acids, estradiol, bilirubin as well as physiological GS-E such as leukotrienes [34, 38, 42, 43], suggesting that it was involved in the transport of these organic anions. DNP-SG ATPase has been purified from liver [42], lung [12] and muscle [38], and its presence in other human tissue has been demonstrated [36]. These studies suggested that DNP-SG ATPase used various organic anions as substrates, that it was distinct from GSSG, Mg^{2+}-ATPase, and that it was widely expressed in human tissues.

A transport protein immunologically related to DNP-SG ATPase has also been identified in rat liver [44–46]. This protein had ATPase activity stimulated by DNP-SG, could be photoaffinity labeled by 8-azido-ATP, and mediated ATP-dependent transport of DNP-SG when reconstituted in proteoliposomes [45, 46]. The overlapping substrate affinities and the immunological similarities between DNP-SG ATPase and the above-mentioned rat liver protein indicated a close similarity between these two transporters and suggested that transporter(s) similar to the erythrocyte DNP-SG ATPase were present not only in other humans tissues but also in other mammalian species.

Erythrocyte GS-E transporters and drug resistance

The transport mechanisms for xenobiotics and their metabolites have enormous clinical implications in cancer chemotherapy because these transporters are believed to be the major determinants of the intracellular concentration of chemotherapeutic agents and their metabolites. Two widely studied transporters, the P-glycoprotein (Pgp) [47, 48] and the multidrug resistance-associated protein or MRP [49], and members of their families have been associated with multidrug resistance of cancer cells, and the overexpression of these ATP-binding cassette (ABC) proteins has been reported in cancer cells exhibiting the MDR

phenotype. The Pgp and MRP families of proteins have been extensively reviewed [47, 48, 50, 51] and hence they are not covered in this article. The erythrocyte GS-E transporter, DNP-SG ATPase, is distinct from MRP and Pgp families of transporters as suggested by immunological and functional studies. However, it mediates ATP-dependent transport of compounds covering the substrate profiles of both MRP and Pgp [11–15, 22]. The recent studies summarized below underscore the relevance of DNP-SG ATPase to the mechanisms of MDR and show that transport mechanisms besides Pgp and MRP are involved in MDR.

DNP-SG ATPase: A MULTIDRUG TRANSPORTER

It was shown [52] that DNP-SG transport in erythrocytes *in situ* was inhibited by DOX which is a classical substrate of Pgp. Therefore, the possibility of DOX and other Pgp substrates being also substrates for DNP-SG ATPase was explored. These studies [12] revealed that the ATP hydrolysis catalyzed by DNP-SG ATPase purified from erythrocytes is stimulated in the presence of DOX and its metabolites. DOX-stimulated ATP hydrolysis by DNP-SG ATPase was saturable with respect to ATP, as well as DOX concentrations (K_m 2.0 mM and 1.2 μM, respectively). Erythrocyte IOVs showed ATP-dependent transport of DOX, daunomycin and vinblastin, and the transport of these substrates by IOVs was diminished when the vesicles were exposed to antibodies against the 38 kDa peptide of DNP-SG ATPase [12]. ATP-dependent transport of DOX in IOVs was competitively inhibited by DNP-SG, and *vice-versa*. Transport of DOX as well as DNP-SG by DNP-SG ATPase was confirmed by studies showing that proteoliposomes reconstituted with purified DNP-SG ATPase catalyzed ATP-dependent transport of both compounds [13, 14]. ATP-dependent transport of DOX and DNP-SG in these proteoliposomes was found to be linear with respect to time, was sensitive to osmolarity of the assay medium and to temperature, and was saturable with respect to DNP-SG, as well as DOX. The energy of activation of transport was 12 and 15 kcal/mol for DNP-SG and DOX, respectively. DOX was found to be a competitive inhibitor of DNP-SG transport, and *vice-versa*, suggesting that the DOX and DNP-SG share a common transport mechanism [13, 14, 16].

These studies for the first time demonstrated that the erythrocyte GS-E transporter, DNP-SG ATPase, could mediate ATP-dependent transport of weakly cationic antitumor agents as well as the organic anions such as DNP-SG. The stimulation of erythrocyte membrane ATPase activity by the two classical uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and carbonylcyanide *p*-fluoromethoxyphenylhydrazone, has been reported [53, 54]. Both these compounds competed with DNP-SG for the activation of GS-E ATPase activity suggesting that small anionic, lipophilic compounds may also be transported by this system. That a single transporter can mediate the ATP-dependent transport of compounds having diverse charge has since been further substantiated by studies demonstrating transport of organic anions as well as DOX by MRP (for review see [50]). The transport of DOX and other antitumor agents by DNP-SG ATPase strongly suggested that the MDR phenotype of cancer cells is associated with additional transporter(s) besides Pgp and MRP.

IDENTITY OF DNP-SG ATPase WITH RLIP76, A Ral-BINDING, GTPase-ACTIVATING PROTEIN

Attempts of a molecular characterization of DNP-SG ATPase through its purification from erythrocytes and other human tissues were only partly successful because of its aberrant behavior in SDS/PAGE and low yields during the purification. Polyclonal antibodies raised against the 38 kDa peptide of DNP-SG ATPase

were, therefore, used to screen a human bone marrow expression library. This resulted in the isolation of a cDNA clone of human RLIP76, a known Ral-binding protein cloned previously by Jullien-Flores *et al.* [55]. RLIP76 has been suggested to be involved in Ral–Ras–Rho signaling pathways but no clearly defined functions was assigned to it previously.

Cloning of RLIP76 by DNP-SG ATPase antibodies was a surprising finding, and the possibility of it being a cloning artifact was considered. However, results showing that the recombinant (rec) RLIP76 could be purified to apparent homogeneity from the transformed *E. coli* [22] by an identical protocol of DNP-SG affinity chromatography used to purify DNP-SG ATPase from human erythrocytes [13] suggested a similarity between these proteins. Furthermore, rec-RLIP76 thus obtained catalyzed ATP hydrolysis which was stimulated by DOX, DNP-SG, and other compounds known to stimulate DNP-SG ATPase activity. The kinetic parameters for ATP hydrolysis stimulated by these compounds were similar for RLIP76 and DNP-SG ATPase, further suggesting a close similarity between these two proteins. The behavior of purified rec-RLIP76 in SDS/PAGE was also similar to that observed for DNP-SG ATPase. It has been consistently observed in our laboratories that during purification, DNP-SG ATPase undergoes degradation/aggregation, giving rise to multiple bands in SDS/PAGE [36, 38, 44]. Similar behavior was observed for the purified preparation of rec-RLIP76. The molecular mass of RLIP76 calculated from its sequence is 76 kDa. Purified rec-RLIP76, however, showed no recognizable band at 76 kDa. Instead, a band at 95 kDa was observed which was consistent with the report of Jullien-Flores *et al.* [55] who were the first to clone RLIP76. In addition to the 95 kDa band, rec-RLIP76 showed additional bands. The electrophoretic mobility of one of these bands was identical to the 38 kDa band observed for DNP-SG ATPase [12–14, 36, 38]. Other prominent bands corresponding to molecular mass of 41, 49 and 28 kDa were also observed in rec-RLIP76 preparations, along with several minor bands. That all the peptides originated from RLIP76 was confirmed by their N-terminal sequence analyses, which yielded internal sequences of RLIP76. All these peptides were recognized by antibodies against the 38 kDa peptide of DNP-SG ATPase. Conversely, the antibodies raised against rec-RLIP76 recognized the 38 kDa peptide in erythrocyte membranes along with bands of higher and lower molecular mass. Together, these studies indicated structural functional and immunological similarities between RLIP76 and DNP-SG ATPase, and showed that during purification, DNP-SG ATPase as well as rec-RLIP76 undergo degradation/aggregation leading to similar profiles in SDS/PAGE. This may explain why peptides of different molecular masses have been observed by us and other investigators during purification of the GS-E or GSSG transporters [12, 35, 36, 56].

Functional similarity between RLIP76 and DNP-SG ATPase has been demonstrated by studies showing that proteoliposomes reconstituted with DNP-SG ATPase or RLIP76 mediate ATP-dependent transport of DNP-SG as well as DOX. The kinetic parameters of the transport for DOX and DNP-SG by these proteoliposomes were observed to be similar to those reported for the transport of these compounds by DNP-SG ATPase purified from human erythrocytes [14]. As yet unpublished studies from our laboratory show that rec-RLIP76 mediates transport of not only DOX but also of colchicine, daunorubicin and other substrates of Pgp. Furthermore, we have shown (manuscript in preparation) that RalBP1 [55], the rat ortholog of RLIP76, also exhibits functional properties and transport characteristics similar to that observed for RLIP76/DNP-SG ATPase. RalBP1 thus appears to be identical with the rat hepatic transporter isolated and characterized by us previ-

ously [45, 46] which was immunologically and functionally similar to DNP-SG ATPase.

The role of RLIP76/DNP-SG ATPase in MDR is suggested not only by the biochemical studies summarized above, but also more directly by our studies showing that transfection of cancer cells (K562) with RLIP76 confers on these cells a resistance to DOX that is several fold higher than that of mock-transfected cells [22]. These studies suggest that these GS-E transporters are linked not only with the mechanisms of drug resistance but also with Ral–Ras–Rho-mediated signaling pathways.

The identity of DNP-SG ATPase with RLIP76 implies that the protein has diverse functionality, i.e. GTPase activation as well as a membrane xenobiotic transport, that is mechanistically difficult to explain. It has been observed that RLIP76 as well as its rat and mouse orthologs (RalBP1 and RIP, respectively [57, 58]) have the ability to bind to membranes [22]. Since it is known that some soluble proteins such as annexins or bacterial toxins can insert into the membrane and turn into transporters, it may be envisaged that RLIP76 and its orthologs function as transporters through similar mechanisms. In this regard it is possible that the observed proteolytic fragmentation (or processing) of RLIP76/DNP-SG ATPase and the subsequent oligomerisation of the resultant peptides may be essential for the formation of the complex responsible for the transport function of RLIP76/DNP-SG ATPase. The exact mechanisms through which RLIP76/DNP-SG ATPase mediates the transport of structurally diverse organic anions as well as cations are not clear and must be investigated by studying the mechanisms leading to various peptides from RLIP76 and the functional properties of these peptides.

RLIP76 was initially cloned [55] using the yeast two-hybrid screening method for cloning Ral-A binding proteins [55, 57–60]. Ral-A is a membrane associated GTPase belonging to the ras family of proteins. It is distinct from the subfamily which includes H-ras and K-ras in that it shares 58% homology with the prototypic ras protein, p21, and is regulated by distinct GTPase-activating proteins (GAP) as well as guanine nucleotide exchange factors (GEFs). Ral proteins appear to function as downstream targets of ras signalling through activation of ral-GEF by Ral, and its subsequent interaction with ral-GTP [61–63]. Ral-A has also been purified from human erythrocyte membranes as a calmodulin-binding protein [60], thus linking it with calcium-dependent signaling mechanisms. Ral may also be involved in membrane signaling pathways through its binding with phospholipase D, an enzyme which hydrolyses phosphatidylcholine to phosphatidic acid [60]. The binding of RalBP1, the rat homologue of RLIP76, to Ral appears to recruit RalBP1 to membranes. This may be relevant to the transport activity of RLIP76. Further studies are needed to determine whether there is a link between these putative functions of RLIP76 and its functionality as a xenobiotic transporter. RLIP76-mediated transport of endogenous and exogenous GS-E and other drugs may be of high physiological and clinical significance. The ability of RLIP76 to mediate GS-E transport could be relevant to leukotriene-mediated signalling in chemotaxis. As already mentioned, the transport of chemotherapeutic drugs could contribute to the multidrug resistance of human cancers to chemotherapy. This is particularly relevant because MRP and Pgp are insufficient to explain all forms of transport-mediated multidrug resistance.

The establishment of the identity of DNP-SG ATPase with RLIP76 does not necessarily rule out the presence of other GS-E transporters in erythrocytes and the mechanisms shown in Fig. 1 may not represent all aspects of GSSG and GS-E transport in erythrocytes. As discussed earlier in this article, mechanisms for GS-E transport are heterogenous. Since DNP-SG ATPase/RLIP76 does not seem to mediate GSSG transport [13] it is logical to conclude that GSSG and GS-E transport is mediated by

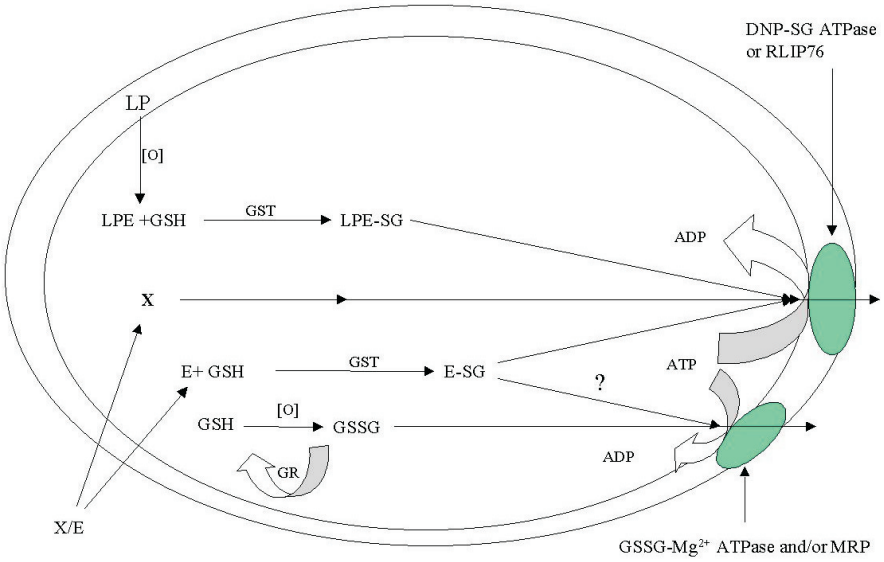

Figure 1. ATP-dependent efflux of glutathione S-conjugates in erythrocytes.

X, non-electrophilic xenobiotics; E, electrophilic xenobiotics; O, oxidation; LP, membrane lipids; LPE, electrophilic products of lipid peroxidation, such as 4-hydroxy nonenal and lipid epoxides; E-SG and LPE-SG, the GSH-conjugates of electrophilic xenobiotics and lipid peroxidation products, respectively; GST, glutathione *S*-transferase; GR, glutathione reductase.

distinct transporters. On the other hand, studies [24] showing relatedness between the GSSG and GS-E transport cannot be ignored. MRP can utilize both GSSG and GS-E as substrates and the presence of MRP in erythrocytes has been shown. MRP has been suggested to be the GS-E export pump of erythrocyte membranes [64]. However, immunoprecipitation studies in our laboratory (unpublished data) with antibodies raised against rec-RLIP76 indicate that the major GS-E transporter in erythrocytes is DNP-SG ATPase/RLIP76. It is possible that MRP may represent the shared mechanism of GS-E and GSSG transport in erythrocytes. The possible relationship between GSSG, Mg^{2+}-ATPase and MRP must, therefore, be explored. Such studies will lead to a better understanding of the molecular basis of GSSG and GS-E transport in erythrocytes.

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