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Research update

RLIP76: A novel glutathione-conjugate and multi-drug transporter

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ARTICLE INFO

Article history:
Received 12 August 2008
Accepted 2 October 2008

Keywords:
RLIP76
Cancer
Drug-resistance
Glutathione-conjugate transport
Xenografts

ABSTRACT

RLIP76, a stress-responsive, multi-functional protein with multi-specific transport activity towards glutathione-conjugates (GS-E) and chemotherapeutic agents, is frequently over-expressed in malignant cells. Our recent studies suggest that it plays a prominent anti-apoptotic role selectively in cancer cells. We have previously shown that RLIP76 accounts for up to 80% of the transport of GS-E and blocking the RLIP76-mediated transport of GS-E in cells results in the accumulation of pro-apoptotic endogenous electrophiles and on-set of apoptosis. Here we demonstrate that when RLIP76 mediate transport of GS-E is abrogated either by anti-RLIP76 IgG or accumulation of 4-hydroxynonenal (4-HNE) and its GSH-conjugate (GS-HNE) occurs and a massive apoptosis is observed in cells, indicate that the inhibition of RLIP76 transport activity at the cell surface is sufficient for observed anti-tumor activity. RLIP76 is linked with certain cellular functions including membrane plasticity and movement (as a primary 'effector' in the Ral pathway, perhaps functioning as a GTPase activating protein, or GAP), and as a component of clathrin-coated pit-mediated receptor-ligand endocytosis—a process that mediates movement of membrane vesicles.

1. Introduction

RLIP76 (Ral-interacting protein) is a stress-responsive, multi-specific membrane transport protein, which accounts for the majority of glutathione-electrophile conjugates (GS-E) transport in mammalian cells including human, is a critical component of stress-response in cultured cells and provides protection from stressors including heat, oxidant chemicals, chemotherapeutic agents, UV irradiation and X-irradiation [1–8]. Human RLIP76 (655 aa), rat RalBP1 (647 aa) and mouse RIP1 (648 aa), are highly homologous, having 88% identity in nucleotides and 92% amino acid similarity, and have been shown to be functionally identical [9]. All three contain a Ral-binding domain (aa ~390–445) that binds Ral-GTP through the effector domain of Ral. Residue 49N in the effector domain of Ral is critically important for binding [10]. A Rho/Rac GAP homology domain (aa ~210–357), also present in all three, and is homologous (35% identity) with the Rho-GAP domain of breakpoint cluster region protein (BCR) which exhibits Rho-GAP activity towards Rac and cdc42 [11]. RLIP76, RalBP1 and

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Abbreviations: RLIP76 (RalBP1), Ral-interacting protein; GSH, glutathione; GS-E, glutathione-electrophile-conjugates; DNP-SG, dinitrophenyl S-glutathione; DOX, doxorubicin; 4HNE, 4-hydroxy-t-nonenal; IOVs, in-side-out vesicles; MDR, multi-drug-resistance; Pgp, P-glycoprotein; MRP1, multi-drug-resistance associated protein; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; POB1, partner of RalBP1; AP-2, adaptor protein.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.10.006

RIP1 display definite but relatively weak GTPase activating (GAP) activity towards Rac1 and cdc42, but not towards Ral [12]. Other common characteristics of RLIP76, RalBP1 and RIP1 include their sub-cellular distribution largely in the membrane and particulate fraction. Taken together, studies of structure, function and protein binding of RLIP76, RalBP1 and RIP1 indicate that these are closely similar orthologues with likely to serve very similar if not identical functions across these species. In this article, to avoid confusion we uniformly refer to this protein and to its mouse (RIP1) [13] or rat counterpart (RalBP1) [10] collectively as RLIP76 [2,14]. The Pgp and MRP families of proteins have been extensively reviewed [15–19] and hence they are not covered in this article. In the present article, we review the molecular identity, functional and structural characteristics of RLIP76 and discuss their physiological role in the multi-drug-resistance of cancer cells.

2. RLIP76 is a non-ABC multi-functional protein

RLIP76 is a multi-functional modular protein found ubiquitously from drosophila to humans [10,13,14,20,21]. It is encoded in humans on chromosome 18p11.3 by a gene with 11 exons and 9 introns [22]. RLIP76 is a 76 kDa protein product of this gene, but splice-variants including a 67 kDa peptide and longer 80 or 102 kDa peptide, cytocentrin have been identified [9,23]. It was cloned originally as a Ral-binding GTPase-activating protein (GAP), and predicted to be a Ral-effector, through a yeast two-hybrid screen [14,24,25]. We cloned RLIP76 independently, in the search for GS-E and chemotherapy drug transporters, from a human bone-marrow cDNA library using polyclonal antibodies raised against purified human erythrocyte putative membrane transporter, DNPSG-ATPase [2,26,27]. The identity of RLIP76 with DNPSG-ATPase was demonstrated by showing that both the human and recombinant protein could be purified by the same GS-E affinity chromatography method [1,2,26]. Tissue-purified and recombinant RLIP76 have been demonstrated to function in isolated *in vitro* systems as well as *in vivo* studies to be an ATP-dependent transporter of GS-E as well as of the amphiphilic anti-cancer drugs such as doxorubicin (DOX), colchicine, vincristine, vinblastine and vinorelbine [1–6,26–28]. Studies demonstrating the marked enhancement of vinorelbine efficacy in lung and colon cancer xenografts by concomitant depletion or inhibition of RLIP76 have confirmed the *in vivo* relevance of these observations [8].

RLIP76 is a modular protein containing a GAP domain [14], protein–protein interaction domains [29], antennapedia homeodomain homologous sequences, leucine-zipper domain, and consensus sequences for protein and tyrosine-kinase phosphorylation and N-myristoylation [30–33]. RLIP76, a novel R-Ras effector, links R-Ras to adhesion-induced Rac activation through a GTPase cascade that mediates cell spreading and migration [34]. GAP activity of RLIP76 has been demonstrated towards Rho/Rac G-proteins that are known to regulate cell membrane plasticity, endocytosis, cell motility, and xenobiotic and stress-responses [14,35–37]. RLIP76 has been shown to bind to a number of important signaling proteins including Ral, clathrin adaptor AP2 [20], Hsf-1, HSP90 [38], partner of RalBP1 (POB1) [39–41], and CDK1 (cdc-2) [42]. These studies with human RLIP76 as well as its mouse (RIP1) and rat (RalBP1) homologs have linked it with a bewildering array of functions including clathrin-coated-pit-mediated receptor-ligand endocytosis of signals including those from insulin and TGF-β receptors, mitosis signaling through CDK1, mitotic spindle movement, and neurotransmitter exocytosis in exocyst complex [9,25,42–45]. The mechanisms through which RLIP76 participates in these functions have been unclear.

Augmenting cellular RLIP76 through stable transfection or through liposomal delivery confers resistance to DOX [2,5]. The GS-E transport function has been shown in several models of cultured cells to be a mechanism for providing protection from diverse stressors including heat, oxidants, UVA, and X-rays, all of which are known to increase cellular GS-E formed from reactive electrophilic intermediates of membrane lipid-oxidation [1,30–32,36,37,46–48]. Most remarkably, acute inhibition of RLIP76 transport activity using specific antibody that recognizes a cell-surface epitope of RLIP76, causes apoptosis [30,48]. Our findings showing that RLIP76 is an ATP-dependent transporter of GS-E and certain xenobiotics, suggest a novel paradigm for signaling of apoptosis, mitosis, cell motility, chemotaxis, endocytosis, exocytosis, stress-defenses and drug-resistance, in which RLIP76 is the chief-regulator of levels of cellular GS-E which are critical chemical signals in common for all of these processes.

This hypothesis predicts that RLIP76 should be a primary determinant of cellular resistance to the stress in the form of chemicals, heat or irradiation. To test this hypothesis, we generated C57B mice which carry heterozygous (+/−) or homozygous (−/−) deletion of the RLIP76 gene. These mice were commissioned from Lexicon Genetics and were created using Cre-Lox technology [49], which can selectively suppress genes. From RLIP76$^{+/-}$ animals, obtained from Lexicon Genetics, we established colonies of RLIP76$^{+/+}$, RLIP76$^{+/-}$ and RLIP76$^{-/-}$ mice by segregation and mating of animals based on genotyping by PCR on tail tissue as described [6]. Western-blot analysis of mouse tissues using anti-RLIP76 antibodies confirmed decreased RLIP76 levels in the RLIP76$^{+/-}$ mouse, and its absence in tissues from the RLIP76$^{-/-}$ mouse as demonstrated by us [6,50].

3. Structural motifs of RLIP76

Primary structure of RLIP76 can be divided into four regions. N-terminal domain (aa 1–209), is rich in antennapedia homeodomain homologous peptides. These peptides are known to be capable of transporting of wide range of substrates across membranes. Two central domains include, one carrying a Rac/cdc42 GAP (through aa 210–357) activity, while the other binds to activated Ral (aa 391–499). A C-terminal coiled-coil domain (aa 500–647) has subsequently been shown to interact with Reps1/POB1, CDK1 and Hsf-1. These structural features link RLIP76 to a confounding array of physiological functions including clathrin-coated-pit-mediated receptor-ligand endocytosis of epidermal growth factor receptor (EGFR), insulin receptor (IR) and transforming growth factor β (TGF-β) as well as mitosis signaling [9,30–33,38–43].

From the sequence alignment studies of RLIP76 using Blast search engine, the following sites were identified as potential

candidate motifs in the primary structure of RLIP76. N-terminal and C-terminal ATP-binding domains at amino acids 69–74 and 418–425, respectively; several N-myristoylation sites were located at amino acids 21–26, 40–45 and 191–196; N-glycosylation site at amino acid 341–344; cyclic adenosine-monophosphate (cAMP) binding site at amino acid 113–116; cAMP-dependent protein kinase phosphorylation sites at amino acids 308–315; leucine zipper pattern at amino acids 547–578; several protein kinase C and casein kinase II phosphorylation sites and trypsin and chymotrypsin cut sites. Two ATP binding sites on N and C-terminal domains [46], PKCα phosphorylation sites [30,51,52], several regions that have high sequence homology with the vector-peptides and membrane-associated proteins, that play an important role in transport and membrane anchorage and a cell-surface domain have being experimentally confirmed through site-directed mutagenesis studies using substitution and deletion mutant analyses [3].

4. Role of RLIP76 in endocytosis

RLIP76 is a novel link between GSH-linked oxidant defense, phase I and II biotransformation, stress signaling, receptor-ligand pair endocytosis and Ral and Rac-1 signaling pathways [14,30–37]. The role of RLIP76 in G-protein signaling includes mainly its functions as an effector protein bridging Ras-Ral pathways. It is regulated by Ral and displays GAP-activity (inhibitory) towards Rho proteins most importantly cdc42, a pro-apoptotic G-protein that exerts an inhibitory effect on Ras. RLIP76 is also an integral component of complexes containing Ral, clathrin-adaptor protein AP2, POB1 and Epsin which are involved in the clathrin-coated-pit-mediated receptor-ligand endocytosis, a mechanism for internalizing receptor-ligand pairs that terminate signaling [42,43].

RLIP76 and POB1 are downstream molecules of small GTP binding protein Ral. The presence of EH-domain in both RLIP76 as well as POB1 suggests them to play a role in clathrin-dependent endocytosis [35]. This hypothesis has been seen to be true by both proteins being involved in receptor-mediated endocytosis together with Epsin and EPS15. In the course of endocytosis, RLIP76, POB1, Epsin and EPS15 form a complex with alpha-adaptin of AP2. During the mitotic phase of the cell, complex formation is reduced by phosphorylation of Epsin by p34cdc2. Phosphorylation inhibits the complex formation by inhibiting the binding of POB1 to EH domain [9,53]. C-terminal domain of RLIP76 is involved in EGF-receptor endocytosis via an interaction with POB1 [54].

In the event of endocytosis, Ras activation activates Ral-GEFs which in turn activate Ral. Activated Ral associates with Ral effector RLIP76 and recruited it to the membrane [20]. RLIP76 sorts out the plasma membrane clathrin adaptor AP2 complex from the trans-golgi network clathrin adaptor AP1 complex. AP2 works exclusively at plasma membrane while AP1 works at golgi network. RLIP76 binds specifically with mu2 subunit of the AP2. Requirement of GTPases at various stages of a coated pit cycle has been demonstrated in endocytosis assays in semi-intact cells [20,55]. Role of dynamin clearly established the requirement of GTP binding proteins at the stage of membrane docking of AP2 and the role during formation of coated pits remains to be elucidated. Equilibrium between GTP and GDP-bound Ral could regulate the association of AP2 with the membrane. Trapping Ral in its GTP bound form stabilizes the mu/RLIP76 binding with membranes and thus disrupts the dynamic cycles of AP membranes interactions, i.e. association and dissociation that are required for coated pits assembly and disassembly [20]. Therefore Ral in its GTP bound from inhibits the endocytosis.

During mitotic phase, POB1, RLIP76 and Epsin are phosphorylated by p34cdc2. Phosphorylation of RLIP76 does not effect binding with POB1 or vice versa. Receptor-mediated endocytosis is completely arrested during mitosis by disassembly of its complex with POB1 and alpha adaptin. Phosphorylated RLIP76 may associate with centrosome. It is being hypothesized that during mitotic phase RLIP76/POB1 may interact with different proteins and function in assembly of mitotic apparatus as they are involved in assembly of receptor and endocytotic proteins during the interphase [29,35,42,53].

5. RLIP76 in stress resistance

The stress inducible nature of RLIP76 has being demonstrated in several different malignant cell lines, with various types of stresses, such as chemical (e.g. doxorubicin, naphthalene), oxidative (ROS), radiation (UV, X-ray) or heat [6,30,36,37,50]. It has been experimentally established that RLIP76 is required for PKCα-mediated cell proliferation pathways [51,56] as well as for protection through heat-shock proteins (HSPs) [38,41].

Depletion of PKCα causes ~30% decrease in rate of proliferation of mouse embryonic fibroblast cells (MEFs). This decrease is absent in RLIP76 gene knockout ($RLIP76^{-/-}$) MEFs. PKCα stimulate DOX-resistance by increased efflux, in $RLIP76^{+/+}$ MEFs while $RLIP76^{-/-}$ MEFs lack this phenotype completely. These results lead to the definitive conclusion that RLIP76 is downstream of PKCα and is required for PKCα-mediated signal proliferation [52,56].

During normal physiological conditions, expression of HSPs is regulated by sequestering the transcription factor Hsf-1 by an interaction with RLIP76 [38,41]. During heat shock, under the activation of Ral, Hsf-1:RLIP76 complex dissociates and hence the expression of heat-shock proteins are transcriptionally activated by Hsf-1. However despite the induction of HSPs, $RLIP76^{-/-}$ mice are markedly sensitive to radiation, which could be overcome by intra-peritoneal administration of RLIP76 in proteoliposomes [6,50]. That is, HSPs (known to be radiation protective proteins) also cannot function in the absence of RLIP76.

6. RLIP76 is necessary for PKCα-mediated DOX-resistance

Non-small cell lung cancer (NSCLC) cells are inherently more resistant to chemotherapeutic agent DOX as compared to small cell lung cancer (SCLC) [23,47,51,52,56]. Since RLIP76 is a key transporter responsible for drug-accumulation defect consisting of several PKCα-phosphorylation sites, we investigated the role of PKCα-activity on transport properties of RLIP76. The

enhanced transport activity of RLIP76 by PKCα-mediated phosphorylation, lead us to the hypothesis, differential PKCα-mediated phosphorylation as a cause for the variations of drug-resistance observed in NSCLC and SCLC. Higher expression of PKCα in NSCLC as compared to SCLC as well as, the decrease of IC_{50} value of NSCLC for DOX, to the level of SCLC, by depletion of PKCα confirmed our hypothesis to be true. Although RLIP76$^{-/-}$ MEFs were significantly more sensitive to DOX as compared to RLIP76$^{+/+}$ MEFs (IC_{50} 25 vs 125 nM), depletion of PKCα only affected the DOX-sensitivity of RLIP76$^{+/+}$ MEFs. These experimental data confirmed that RLIP76 in necessary for the PKCα-mediated drug-resistance signaling [51,52,56]. Recent studies by others have identified a large number of differentially phosphorylated forms of RLIP76 [57].

The studies using recombinant-RLIP76 proteoliposomes in the presence of PKCα indicated enhanced transport of both endobiotics (LTC_4) as well as xenobiotics (DNP-SG) [4]. In order to answer the question whether PKCα-mediated phosphorylation of RLIP76 as a cause for differential drug-resistance observed in NSCLC and SCLC, we determined the expression of PKCα in several NSCLC and SCLC cell lines. Western-blot analysis using antibodies against PKCα, with equal amount of crude protein from either cell lines indicated a higher expression of PKCα in all NSCLC cell lines as compared to SCLC cell lines [51,52]. Depletion of PKCα by siRNA, in several NSCLC and SCLC cell lines, showed a decrease in the drug-resistance (IC_{50} for DOX) of NSCLC cell lines to the level observed with SCLC cell lines. Same phenomenon was observed in DOX-transport studies using proteoliposomes reconstituted with purified RLIP76 from PKCα depleted NSCLC and SCLC. These results directly indicate that PKCα play a major role towards differential DOX-resistance of NSCLC and SCLC [51,52,56].

The role of RLIP76 in PKCα-mediated resistance signaling was analyzed by determination of IC_{50} for DOX in RLIP76$^{+/+}$ and RLIP76$^{-/-}$ MEFs in the presence and absence of PKCα. RLIP76$^{-/-}$ MEFs were significantly sensitive to DOX as compared to RLIP76$^{+/+}$ MEFs. Depletion of PKCα did not affect the DOX-sensitivity of RLIP76$^{-/-}$ MEFs. On the other hand PKCα depletion sensitized RLIP76$^{+/+}$ MEFs by 2.2 fold. These results demonstrate that the effect of PKCα in mediated drug-resistance requires the presence of functional RLIP76; that is, the primary function of PKCα with respect to DOX-resistance in NSCLC is to keep RLIP76 in its active phosphorylated state. The complete reversion of the resistance phenotype from NSCLC to that of SCLC upon depletion of PKCα and the complete inability of PKCα to affect resistance in RLIP76$^{-/-}$ MEFs strongly support this conclusion.

7. Identification of membrane anchoring domains of RLIP76 and cellular localization

The cellular distribution of RLIP76 is of utmost importance due to its multi-functional nature as an ATP-dependent transporter, effector in Ras-Ral signal pathways, mitosis and receptor-mediated endocytosis [2,9,20,30,42,45]. Though our previous studies indicate RLIP76 to be associated with the membrane, particularly N-terminal domain, other investigators suggest a cytosolic distribution attached to cytoskeleton [38,46]. In the absence of transmembrane helices in the primary structure of RLIP76, we selected several regions with high sequence homology to vector-peptides that are known to be capable of transmembrane transport of allocrites as candidate regions for membrane anchorage [30]. A series of deletion mutants of

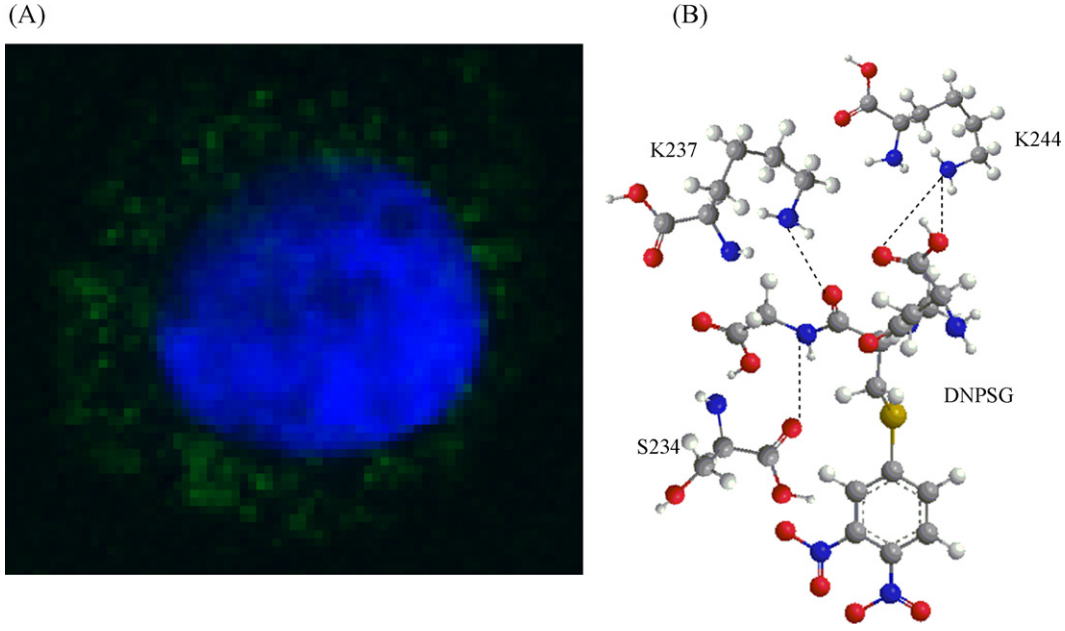

Fig. 1 – Membrane localization of RLIP76. Immuno-histochemistry studies using antibodies against RLIP76 on live-unfixed H226 cells indicated a membrane associated pattern. NSCLC H226 cells grown on sterilized cover-slips were incubated with anti-RLIP76-IgG as the primary-antibodies followed by FITC-conjugated goat-anti-rabbit-IgG as the secondary-antibodies. DAPI was used as a nuclear counter-stain. The cells were analyzed by Zeiss LSM 510 META laser-scanning fluorescence microscopy (panel A). Interactions of RLIP76 with glutathione-conjugates (panel B).

RLIP76 were constructed and effects of mutations on expression of the protein between cytosol and membrane as well as on transport properties were studied. The results of the study indicate aa 154–219 as important in membrane anchorage and that membrane anchorage is required for its transport function [3]. Immuno-histochemical co-localization of RLIP76 with Herceptin in H358 lung cancer cells transfected with deletion mutant of aa 171–185, in which the wild-type protein has being suppressed by siRNA, indicate aa 171–185 comprises a cell-surface epitope [3].

Multiple roles of RLIP76 such as ATP-dependent transporter, role as an effector protein in signaling pathways, mitosis, membrane ruffling and receptor-mediated endocytosis all indicate RLIP76 to be associated with the membrane (Fig. 1A). Primarily it is expressed in the plasma membrane or nuclear membrane and also in cytoplasm, allied with the cytoskeleton. Previous studies have shown that N-terminal domain of RLIP76 (N-RLIP76$^{1-367}$) can be extracted only in the presence of a detergent indicating it to be associated with the membrane whereas C-terminal domain of RLIP76 (C-RLIP76$^{410-655}$) is found in both aqueous and the detergent fractions [2,46]. The rat and mouse homologues of RLIP76, known as Ralbp1 [10] and RIP1 [13], respectively, appear to be associated with the membrane by our studies as well as studies by other investigators [10,13,58,59]. Immuno-histochemistry studies using antibodies against different regions of RLIP76 in human lung cancer cells lines indicate the existence of cell-surface epitope [3,48]. Human lung cancer cells incubated with anti-RLIP76 antibodies were checked for the sensitivity to DOX. Considerable decrease in IC_{50} values towards DOX upon exposure to antibodies suggests the existence of cell-surface epitope [48].

In search for the potential GS-E binding site of RLIP76 by sequence homology with well-known GS-E binding proteins using the IBM Bioinformatics Group Multiple Sequence Alignment algorithm, several amino acids have been found to be the potential candidates. Both drug sensitivity and uptake studies indicated that substitution of S234, K237 and K244 with alanine markedly affects the transport function of RLIP76 and the binding of the GS-E with the mutants of RLIP76 as compared to the wild type protein (Fig. 1B).

8. RLIP76 as a novel link in stress defense and signaling pathways

RLIP76 is bound to clathrin, the AP2 adaptor protein and is shown to play a vital role in endocytosis [30,35,42–45]. However, its functional role in endocytosis had not been studied in RLIP76-deficient cells or tissues. Herein, we report that RLIP76 loss causes an almost complete cessation of clathrin-dependent endocytosis of EGFR, which is restored fully by wild-type RLIP76. Remarkably, mutant RLIP76 proteins selected for decreased affinity for glutathione-conjugates displayed proportionately the same loss in endocytosis capacity as in other functional measures including substrate-stimulated transport activity, or the ability to confer drug-accumulation defect and drug-resistance phenotype. The rate of clathrin-dependent endocytosis was found to be directly proportional to the ATPase and transport activity of RLIP76. These studies demonstrate that GS-E transport activity of RLIP76 is necessary for clathrin-dependent endocytosis. These fundamental observations give an insight about the functioning of the endocytosis apparatus, and its regulation by the mercapturic-acid pathway.

9. Heat-shock responses

Heat-shock (stress) proteins [Hsp] are a family of proteins that vary in size (10–110 kDa) and perform two essential functions within the cell. At homeostasis Hsp can behave as 'chaperones' assisting proper folding of and proper compartmentalization of other proteins. Hsp can unfold and refold improperly folded proteins into the proper orientation or assist in targeting them for degradation. In a stress-induced environment (temperature, xenobiotics, radiation and oxidative injury) where a higher likely hood of denatured proteins can exist, Hsp can mediate by either re-naturing the protein, degrading the protein, protecting the protein from becoming denatured, or transporting it to a compartment where it can be degraded. All of these actions assist the cell in maintaining its integrity [60]. It is known that many Hsp are regulated by Heat-Shock Factor 1 (Hsf-1). Hsf-1 is a transcription factor that forms a ternary-complex with some of the Hsp (inactive form). Upon stress, the Hsp is released and Hsf-1 is allowed to bind to DNA, which up-regulates and increases the Hsp production assisting in relief from the impending stress. It was recently discovered that Hsf-1 forms a complex with RLIP76 [38,41]. Upon stress, the ral signaling pathway is activated and RLIP76 is removed from the complex, which allows Hsf-1 to translocate into the nucleus where it up-regulated the production of stress proteins. Thus, RLIP76 binding to Hsf-1 serves to inhibit Hsf-1 from increasing heat-shock protein RNA transcription. Our results are consistent with this postulate since loss of RLIP76 caused a stepwise up-regulation of heat-shock proteins [41,45].

Results of recent studies by Mivechi [36] have shown that RLIP76 is a primary regulator to Hsf-1, the transcription factor considered the master controller of the heat-shock response. Numerous heat-shock proteins and other chaperones are transcriptionally up-regulated by Hsf-1 in response to chemical oxidant-stressors as well as radiant-stressors that have in common augmentation of cellular lipid-peroxidation levels. In RLIP76$^{-/-}$ mice, heat-shock protein (Hsp1α, Hsp40, Hsp105, mammalian stress protein 1, stress-induced phosphor-protein 1 and insulin-like growth factor binding protein 5) levels have been shown by us to be increased 2–5 fold by real-time Q-PCR [45]. We do not know whether this pathway of modulating heat-shock protein by RLIP76 by depletion or inhibition is operational in any particular cancer cells. If so, it is possible that cells with increased Hsf-1 levels should have a relatively lower RLIP76 transport activity due to inhibition, thus the effects of RLIP76 depletion could be exacerbated. In RLIP76$^{-/-}$ MEFs, transfection with wild-type RLIP76 should function to suppress Hsf-1 activity to the base line level seen in RLIP76$^{+/+}$ MEFs whereas Hsf-1-binding-deficient mutants of RLIP76 should not be able to suppress Hsf-1 [41].

10. Effect of RLIP76 disruption on GS-E and DOX-transport

We generated RLIP76$^{+/+}$ and RLIP76$^{-/-}$ mice colonies, based on genotyping [6,50]. Western-blot analysis of mouse tissues using anti-RLIP76 IgG confirmed detectable level of RLIP76 in RLIP76$^{+/+}$ mouse tissues and its absence in RLIP76$^{-/-}$ mice tissues [6]. Replacement of RLIP76 in RLIP76$^{-/-}$ mouse tissues was examined in animals given 400 μg/0.2 ml purified RLIP76 liposomes, i.p. Whereas Western-blot and immuno-histochemistry showed no immuno-logically detectable RLIP76 in RLIP76$^{-/-}$ animals given control-liposomes, RLIP76 was clearly detected in mouse tissues at 24 h after a RLIP76-liposomes dose [6]. Consistent with the observed function of RLIP76 as a transporter of GS-E and DOX in cell culture studies [47], GS-E and DOX-transport in membrane vesicles was found to be decreased in RLIP76$^{-/-}$ mice tissues. A greater than 80% loss of GS-E and DOX-transport activity was seen in the RLIP76$^{-/-}$ mouse tissues. Delivery of RLIP76 to RLIP76$^{-/-}$ animals via a liposomal delivery system completely reversed transport mechanisms [6].

11. RLIP76 loss results in accumulation of 4HNE and GS-HNE in mouse tissue

In RLIP76$^{-/-}$ mice, the transport of DNP-SG, a model substrate used for GS-E transport studies, is impaired as indicated by loss of ~80% transport activity for DNP-SG in the IOVs prepared from the plasma membrane fraction of tissues [6,30]. Lipid-peroxidation products including lipid-hydroperoxides and aldehydes were also increased in the tissues of these mice implying that GS-E formed physiologically from lipid-hydroperoxides would also accumulate in tissues of RLIP76$^{-/-}$ mice. Recently, we have addressed this question by directly measuring tissue levels of 4HNE and its GSH-conjugate (GS-HNE) using LCMS. Homogenates were prepared from freshly excised liver tissue from RLIP76$^{+/+}$ and RLIP76$^{-/-}$ mice and subjected to extraction for either 4HNE or the GS-HNE using the protocols described by us [50]. The results of these studies showed that both, 4HNE as well as GS-HNE were increased by about three-fold in the RLIP76$^{-/-}$ mouse liver tissue. These studies demonstrated for the first time that loss of RLIP76 results in the accumulation of endogenously generated electrophiles and their GSH-conjugates *in vivo* [50]. The significance of RLIP76-mediated transport of GS-HNE necessitates a robust and facile analytical method to determine intracellular GS-HNE and HNE levels.

12. POB1

Yeast two-hybrid screening with RLIP76 as bait has yielded POB1 (Partner of RLIP76/RalBP1), also an endocytosis protein [61]. Both proteins are found in signal transduction complexes for membrane tyrosine-kinase receptors (such as insulin and EGF-R) and are phosphorylated [25]. Interestingly, POB1 has also been shown to be a pro-apoptotic protein when expressed in prostate cancer cells [39], which have very high RLIP76 content [7]. Reasoning that this may be due to an inhibitory effect of POB1 on the transport activity of RLIP76, we tested the effects of POB1 on the transport activity and drug-resistance mediating effects of RLIP76. POB1 bound specifically to RLIP76 through the C-terminal domain of RLIP76 and inhibited transport activity as well as RLIP76 mediated drug-resistance [40].

The human POB1 also referred to as REPS2, protein is highly conserved in mammals where it has been suggested to function as a molecular scaffold recruiting proteins involved in vesicular traffic and linking them to the actin cytoskeleton remodeling machinery [62]. POB1 was identified as a 657 amino acid protein, contains the C-terminal coiled-coil protein–protein interaction domain that is involved in RLIP76 binding and was found highly expressed in androgen-dependent prostate cancer cell lines [25,39], is down regulated during prostate cancer progression [63]. It has been shown that over-expression of POB1 and its binding with RLIP76 induce apoptosis in prostate cancer cells [39].

13. Conclusions

Studies summarized in this review provide strong evidence for the role of RLIP76 as an effector protein which catalyzes the efflux of GS-E, a process which is a crucial component of diverse cellular signaling pathways including mitosis, proliferation, differentiation, apoptosis, and endocytosis. The linkage of RLIP76 to the Ral and Ras pathways and in particular to the Rho/Rac pathway, which is known to control stress-responses, is also of fundamental significance and similar links have not been found for other transporters. Although clear evidence has been provided for the interaction of RLIP76 with these pathways, mechanistic explanations regarding how RLIP76 is involved in mediating a diverse array of functions has previously been far from clear. Through its protein–protein binding motifs, it has clearly been shown to bind important signaling proteins including Ral, AP2 clathrin-adaptor protein [30,53], POB1 [39,40,62,63], CDK1 [42], and Hsp90 as well as Hsf1 [38,41], the directionality of signaling has been far from clear, and have left open questions as to whether these proteins are regulating some effector function of RLIP76, or whether RLIP76 is functioning as a regulator of these signaling proteins. In this regard, it should be noted that when originally cloned, RLIP76, was predicted to be an effector protein [10,13,14]. Our previous and present studies have clearly demonstrated an effector function for RLIP76, as an active nucleotidase which is capable of coupling ATPase-activity with trans-membrane movement of several allocrites [23,30,33,46–48,64,65]. Previous studies indicating that RLIP76 also functions in mitotic spindle apparatus movement [9,30], in membrane movement during endocytosis and exocytosis [30,42–45], taken together with observations by us and by others that the C-terminal domain of RLIP76 is found both in membrane as well as cytosol, that it contains an active ATPase domain, strongly support the proposal by us as well as others that RLIP76 is a modular protein containing multiple domains which may perform distinct functions at distinct intracellular sites [20,21,30–32]. Thus, the findings that RLIP76 functions in the mitotic spindle, endocytosis, exocytosis, and transport are not mutually exclusive or contradictory, and indicate different domains of RLIP76, which function in ATPase, protein–protein

binding, GAP-activity, and membrane insertion, may be serving distinct functions in different cellular compartments. Recently, RLIP76 has also been proposed to be involved in the mechanism of pharmaco-resistance [66] and RLIP76 antibodies increased intracellular levels of 4HNE, decreased levels of GSH and activate C-Jun NH_2 kinase signaling (JNK), thus inducing oxidative stress-mediated cell apoptosis [67]. In lung cancer cells, RLIP76 was shown to be a major transporter of DOX and also GS-E, representing about two-thirds of the transport-capacity. Pgp and multi-drug-resistance protein (MRP) were responsible for the remaining transport activity [47,68].

14. Significance

RLIP76 is a fundamental link between biochemical pathways of GSH-linked metabolism of xenobiotics and stress-defense signaling pathways. It represents a stress-resistance effector that plays a pivotal role in defending normal cells from poisons, and cancer cells from apoptosis. Thus augmentation of RLIP76 in normal cells that are stressed or injured may be a pharmacological method for treatment of poisoning and wounds, and controlled depletion or inhibition of RLIP76 should naturally target malignant cell that rely on the mercapturic-acid pathway to protect themselves from apoptosis. The rate regulatory role of RLIP76 in endocytosis has many unexplored implications with respect to this mechanism that functions to terminate signaling initiated by ligand-receptor binding. RLIP76 plays a central role in radiation and chemotherapy-resistance through its activity as a multi-specific ATP-dependent transporter.

Acknowledgements

This work was supported in part by National Institutes of Health Grants CA 77495 and CA 104661, Cancer Research Foundation of North Texas, Institute for Cancer Research and the Joe & Jessie Crump Fund for Medical Education.

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