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POB1 over-expression inhibits RLIP76-mediated transport of glutathione-conjugates, drugs and promotes apoptosis☆

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Abstract

RLIP76 (RALBP1) is a Ral-binding nucleotidase which functions as an energy-dependent transporter for glutathione (GSH)-conjugates as well as structurally unrelated xenobiotics. Partner of RALBP1 (POB1), also referred to as REPS2, was identified as the human RLIP76-binding protein, which contains a coiled-coil C-terminal region that binds with the RLIP76. Recent studies show that over-expression of POB1 in prostate cancer cells induces apoptosis. In present studies, we have purified POB1 and one of its deletion mutants $POB1^{1-512}$ (lacking the RLIP76-binding domain), and examined their effect on the transport activity of RLIP76. Both doxorubicin and a model GSH-conjugate, dinitrophenyl-*S*-glutathione (DNP-SG), transport were inhibited by POB1 in a concentration-dependent manner but not by $POB1^{1-512}$, lacking RLIP76-binding site. Liposomal delivery of recombinant POB1 to H358 (NSCLC) cancer cells caused apoptosis in a concentration-dependent manner, whereas the POB1 mutant deficient in RLIP76-binding site did not exert this effect. Augmentation of cellular POB1 resulted in increased intracellular DOX-accumulation as well as decreased rate of efflux from cells. These results show for the first time that POB1 can regulate the transport function of RLIP76 and are consistent with our previous studies showing that inhibition of RLIP76 induces apoptosis in cancer cells through the accumulation of endogenously formed GSH-conjugates.

Keywords: RLIP76; RALBP1; POB1; REPS2; Apoptosis; Transport-inhibition; Glutathione-conjugate; Glutathione *S*-transferase; Doxorubicin; Drug resistance

RLIP76 (RALBP1), a Ral-binding GTPase activating protein, belongs to a subfamily of Ras superfamily. It is the first Ral effector to be discovered [1–6] and has been shown to bind with multiple signaling proteins including clathrin adaptor AP2, hsf-1, HSP90, POB1, and cdc2 [1,6–9]. These protein–protein interactions link RLIP76 with several important functions including endocytosis of insulin receptor, EGF receptor, and transferrin receptors, mitosis signaling, mitotic spindle movement, neurotransmitter exocytosis in exocyst complex, and filopodia formation [10–13]. In addition, RLIP76 functions as a multispecific transporter that catalyzes ATP-dependent transmembrane movement of not only anionic glutathione conjugates but also of many structurally unrelated amphiphilic xenobiotics [14–21]. It has been shown that the inhibition of transport activity of RLIP76 by coating the lung cancer cells with anti-RLIP76 IgGs results in apoptosis due to accumulation of GSH-conjugates of endogenous electrophiles includ-

☆ *Abbreviations:* RLIP76 (RALBP1), Ral-interacting protein; POB1, partner of RALBP1; DOX, doxorubicin; GSH, glutathione; GSTs, glutathione *S*-transferases; DNP-SG, dinitrophenyl *S*-glutathione; NSCLC, non-small cell lung cancer.

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ing 4-hydroxynonenal (4-HNE) leading to the inhibition of glutathione *S*-transferases (GSTs) and increase in the intracellular concentration of 4-HNE which is known to cause apoptosis.

Screening for the RLIP76-binding proteins using the yeast two-hybrid system yields REPS1 and REPS2 in humans that are known to contain the RLIP76-binding coiled-coil domain at the C-terminal of each protein [5,22–24]. REPS2, also known as POB1 (partner of RALBP1), is located on the human X-chromosome at Xp22 and was identified as a 521 amino acid protein [5,25]. Recently, another form of REPS2 has been obtained using hypothalamus cDNA clone containing an open reading frame encoding a protein of 659 amino acid residues [24,25]. This protein contains 139 additional amino acid residues at the N-terminal end. Using specific POB1 antibodies, both forms of POB1 have been detected (78 and 58 kDa) in prostate cancer cell lines [24]. Whether these forms are splice variants or arise due to posttranslational processing is not known. POB1 contains the C-terminal coiled-coil protein–protein interaction domain that is involved in RLIP76 binding [24]. It has been proposed to regulate the functions like endocytosis of EGF receptor, insulin receptor, and transferrin receptor via binding with POB1 (partner of RLIP76) [8] and μ2, the medium chain of AP2 complex [6,8], involved in filopodia formation [13]. Recently, it has been shown that over-expression of POB1 and its binding with RLIP76 induce apoptosis in prostate cancer cells [24,25]. Since inhibition of RLIP76 transport activity leads to apoptosis in lung cancer cells [16], it is possible that POB1-induced apoptosis of prostate cancer cells could be due to impairment of transport activity of RLIP76 by POB1 binding.

We have studied the effect of recombinant POB1 on the transport activity of RLIP76 in isolated systems as well as in lung cancer cells. POB1 inhibited RLIP76 transport activity through specific binding to RLIP76 mediated by a domain encompassing aa 513–659. Increasing cellular POB1 resulted in decreased efflux of DOX from intact cells, increased cellular DOX-accumulation, and marked potentiation of DOX-cytotoxicity.

Materials and methods

Reagents

cDNA of POB1 (2200 bp) and polyclonal rabbit-anti-POB1 IgG were kindly provided by Prof. Leen J. Blok, Erasmus University Rotterdam, The Netherlands. Bacterial strains (DH5α and BL21(DE3)) were purchased from Invitrogen Life (Carlsbad, CA). pET30a(+), the T7 promoter based expression vector, was purchased from Novagen, (Madison, WI). Restriction enzymes, thermophilic DNA polymerase (Vent polymerase), and DNA ligase were from New England Biolabs (Beverly, MA). dNTPs were from Applied Biosystems (Foster city, CA). HPLC-grade oligonucleotides were synthesized by Biosynthesis, (Lewisville, TX). Doxorubicin (adriamycin) was obtained from Adria Laboratories (Columbus, OH). [Glycine-2-^{3}H]GSH (3000 Ci/mmol) was purchased from Pharmacia Biotech (Piscataway, NJ). [^{14}C]DOX (specific activity 44.8 Ci/mmol) was purchased from NEN Life Sciences (Boston, MA). Polyclonal rabbit-anti-human rec-RLIP76 IgG as well as pre-immune IgG were prepared and purified as described previously [17], and the sources of other chemicals used in this study were same as described previously [14–18].

Methods

Cloning and prokaryotic expression of POB1. The 2200 bp full length long version cDNA of POB1 was a gift from Dr. Blok. The cDNA of POB1 was used as a template for PCR amplification of the POB1 coding sequence. The upstream (5′-GGCGGATCCATGGAGGCGGCAGC GGC-3′) and downstream (5′-CCGCTCGAGTCACAACACAGTGA CCGGAC) primers were designed to introduce a *Bam*H1 restriction site (underlined) immediately upstream of the initiator codon and *Xho*I site (underlined) immediately downstream of the stop codon of POB1 open reading frame. The PCR amplification was performed under following incubation conditions; DNA template 500 ng, primers 30 pmol each, dNTPs 2.5 μM, thermopol buffer 1×, BSA 1×, and Vent polymerase 2.5 U. PCR cycles were at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s; 60 °C for 30 s and 1 min at 72 °C, and a final extension at 72 °C for 7 min. PCR product was purified by using Qiagen PCR purification kit and digested with *Bam*HI/*Xho*I restriction enzymes. The cleaved PCR products were ligated into pET30a(+) previously digested with the same restriction enzymes. The ligated products were expressed into the DH5α competent cells and plasmid DNA was purified from the overnight culture of single colony using Qiagen DNA purification kit. Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described by Sambrook et al. [26]. The sequence of the POB1 was confirmed by DNA sequencing. Following verification of the sequence of POB1, the pET30a(+) plasmid containing the full length POB1 was used to transform *Escherichia coli* strain BL21(DE3) and protein was expressed in *E. coli* BL21(DE3) grown at 37 °C after induction with 0.4 mM IPTG.

Purification of recombinant POB1. Rec-POB1 was purified by metal affinity chromatography over Ni–NTA Superflow resin (Qiagen) with slight modifications as described below. *E. coli* BL21(DE3) expressing POB1 were lysed in 20 mM Tris–HCl containing 250 mM NaCl; 100 μM PMSF, and 5 mM imidazole, pH 7.9, sonicated, and incubated for 4 h at 4 °C with gentle shaking followed by centrifugation at 13,000 rpm for 30 min. The supernatant was mixed with Ni–NTA Superflow resin pre-equilibrated with the same buffer. The resin was incubated overnight at 4 °C with gentle shaking and washed with wash buffer (20 mM Tris–HCl, 300 mM NaCl; 20 mM imidazole, and 100 μM PMSF, pH 7.9) until OD_{280} was zero. The bound protein from the resin was eluted with elution buffer (20 mM Tris; 500 mM NaCl; 400 mM imidazole, and 100 μM PMSF, pH 7.9) and was dialyzed against 10 mM Tris–HCl (pH 7.4), 100 μM EDTA, and 100 μM PMSF containing 0.025% $C_{12}E_9$.

Since polidocanol interfered with Bradford reagent, protein was estimated by the method of Minamide and Bamburg [27]. Western blot analysis was performed by the method of Towbin et al. [28] and polyacrylamide gel electrophoresis (PAGE) was performed in the system described by Laemmli [29].

Cloning, expression, and purification of POB1$^{1–512}$. Deletion mutant of POB1 lacking the RLIP76-binding site, POB1$^{1–512}$ containing the sequence aa 1–512 starting from 1 in the open reading frame was constructed by PCR amplification using the full length POB1 as the template. The primers were: upstream (5′-GGCGGATCCATG GAGGCGGCAGCGGC-3′) containing the *Bam*H1 site (underlined) and downstream (5′-CCGCTCGAGGGTAACAATCCTGACTTGG TA) containing the *Xho*I restriction site (underlined). The PCR amplification was performed at the following incubation conditions; DNA template 500 ng, primers 30 pmol each, dNTPs 2.5μM, ther-

mopol buffer 1×, BSA 1×, and Vent polymerase 2.5 U. PCR cycles were, at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s; 60 °C for 30 s, and 1 min at 72 °C, and a final extension at 72 °C for 7 min. PCR product was purified by using Qiagen PCR purification kit and digested with *Bam*HI/*Xho*I restriction enzymes. The cleaved PCR products were ligated into pET30a(+) previously digested with the same restriction enzymes. The ligated product was expressed into the DH5α competent cells while plasmid DNA was purified from the overnight culture of single colony using Qiagen DNA purification kit. The sequence of the deletion mutant was confirmed by DNA sequencing. Following verification of the sequence of POB1$^{1-512}$, the pET30a(+) plasmid containing the POB1$^{1-512}$ was used to transform *E. coli* strain BL21(DE3) and protein was expressed in *E. coli* BL21(DE3) grown at 37 °C after induction with 0.4 mM IPTG. POB1$^{1-512}$ was purified using a similar protocol as described above for full length POB1.

Functional reconstitution of purified RLIP76 into artificial liposomes and transport studies. Prokaryotic expression and purification of bacterially expressed rec-RLIP76 was performed and its purity was established as described previously [17,18]. Purified RLIP76 was dialyzed against reconstitution buffer (10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA, 100 mM KCl, 40 mM sucrose, 2.8 mM BME, 0.05 mM BHT, and 0.025% polidocanol). An aqueous emulsion of soybean asolectin (40 mg/ml) and cholesterol (10 mg/ml) was prepared in the reconstitution buffer by sonication and 0.1 ml of this mixture was added to 0.9 ml aliquot of dialyzed purified rec-RLIP76 containing 20 μg protein. The reaction mixture was sonicated at 50 W for 30 s. Vesiculation was initiated by addition of 200 mg SM-2 Bio-beads pre-equilibrated in the reconstitution buffer without polidocanol. Vesiculation was carried out for 4 h at 4 °C, followed by removal of SM-2 Bio-beads by centrifugation and the vesicles (RLIP76-liposomes) were collected. Control vesicles (control-liposomes) were prepared using an equal amount of crude protein from *E. coli* not expressing RLIP76. ATP-dependent transport of [^{14}C]DOX and [^{3}H]DNP-SG in the rec-RLIP76 reconstituted proteoliposomes performed by rapid filtration technique using the exact protocol described by us previously [17].

Transport of [^{14}C]DOX and, [^{3}H]DNP-SG, and its inhibition by POB1 and POB1$^{1-512}$. For these experiments, fixed amount of purified rec-RLIP76 (20 μg) was reconstituted in proteoliposomes along with varying amounts (0–80 μg) of either POB1 or its deletion mutant (POB1$^{1-512}$) and transport of [^{14}C]DOX and [^{3}H]DNP-SG was measured in these proteoliposomes as described by us [17]. In one control POB1 proteins were excluded while in other control equivalent amounts of BSA were reconstituted in proteoliposomes.

Drug sensitivity assay. Cell density during the log phase was determined by counting trypan blue excluding cells in a hemocytometer, and 20,000 cells were plated into each well of 96-well-flat bottomed microtiter plates. After 24 h of incubation, the cells were treated with control-liposomes or liposomes containing recombinant full length POB1 or POB1$^{1-512}$ (final concentration 40 μg/ml). DOX was added and IC_{50} was measured by performing MTT assay 96 h later as described previously [30]. Eight replicate wells were used for each point in each of three separate measurements of IC_{50}.

Effect of full length POB1 and POB1$^{1-512}$ on apoptosis by TUNEL assay. H358 cells were grown on the coverslips. The cells were treated with equal amounts of liposomes reconstituted with 40 μg/ml (final concentration) recombinant full length POB1 and POB1$^{1-512}$ protein. After 24 h incubation, the medium was removed and cells were washed with PBS four times. TUNEL assay was performed using Promega fluorescence detection kit. Fluorescence micrographs were taken using Zeiss LSM 510 META (Germany) laser scanning fluorescence microscope at 400× magnification.

Effect of POB1-liposomes on [^{14}C]DOX accumulation in lung cancer cell. H358 cells were harvested and washed with PBS, and aliquots containing 5×10^6 cells (in triplicate, for each time-point) were inoculated into fresh medium. After overnight incubation, the cells were pelleted and resuspended in 80 μl medium containing 4 μg either control, POB1 or POB1$^{1-512}$ liposomes, and incubated at 37 °C for 24 h. After 24 h incubation, 20 μl of 14-[^{14}C]DOX (final 3.6 μM, specific activity 8.5×10^4 cpm/nmol) was then added to the medium and incubated for 5, 10, 20, and 30 min at 37 °C. Drug uptake was stopped by rapid cooling on ice. Cells were centrifuged at 700*g* for 5 min at 4 °C and the medium was completely decanted. Radioactivity was determined in the cell pellet after washing twice with ice-cold PBS.

[^{14}C]DOX efflux studies. The NSCLC (H358) cells were harvested, and washed with PBS, and aliquots containing 5×10^6 cells (in triplicate) were inoculated into fresh medium. After overnight incubation, the cells were pelleted and resuspended in 80 μl medium containing 4 μg of either control, POB1 or POB1$^{1-512}$ liposomes, and incubated at 37 °C for 24 h. After 24 h incubation, 20 μl of 14-[^{14}C]DOX (final 3.6 μM, specific activity 8.5×10^4 cpm/nmol) was then added to the medium and incubated for 60 min at 37 °C. Cells were centrifuged at 700*g* for 5 min, after which the supernatant was completely removed and the cell pellet was washed with PBS twice. The pellet was immediately resuspended in 1 ml PBS. Fifty microliters aliquots (clear supernatant) were removed every min for radioactivity counting for 15 min. The back-added curves of cellular residual DOX vs. time were constructed as described previously [31].

Results and discussion

Purification of recombinant POB1, POB1$^{1-512}$, and RLIP76

Full length POB1 cDNA as well as a truncated cDNA encoding POB1$^{1-512}$ were used to create a His-tagged construct, which was expressed in *E. coli*, and corresponding proteins were purified by Ni–NTA affinity chromatography. SDS–PAGE confirmed the purity of these proteins, which showed single predominant bands at 78 and 52 kDa for POB1 and POB1$^{1-512}$, respectively, which were recognized by anti-POB1 antibodies in Western blots (Figs. 1A and B). Purity of recombinant RLIP76 was also established by SDS–PAGE and Western blot analyses against anti-RLIP76 (Fig. 1C) where it showed characteristic bands of purified RLIP76, which included a predominant band near 95 kDa, and several faint bands at lower M_r, corresponding to internal peptides derived from RLIP76 [17], all of which were recognized by anti-RLIP76 antibodies in Western blots.

Inhibition of DOX and DNP-SG transport by POB1

ATP-dependent transport of DOX and DNP-SG in proteoliposomes reconstituted with RLIP76 has been demonstrated [15,17–20]. In present studies, purified RLIP76 was reconstituted into proteoliposomes along with different amounts of POB1 or POB1$^{1-512}$ (0–80 μg) and ATP-dependent transport of DOX as well as DNP-SG was determined in these proteoliposomes. Results presented in Fig. 2A show inhibition of DOX and DNP-SG transport activity of RLIP76 in a concentration-dependent manner by recombinant POB1, but not by POB1$^{1-512}$ or albumin. Maximum inhibition (~50% for DOX-transport, and 68% for DNP-SG transport)

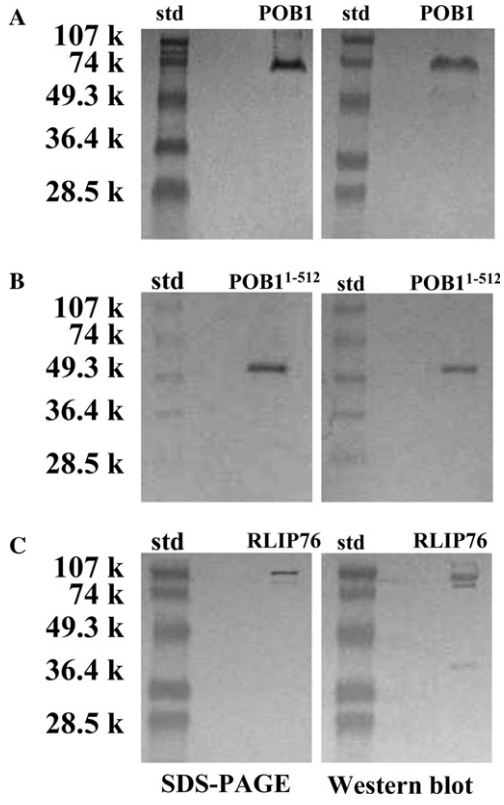

Fig. 1. Expression and purification of recombinant POB1, POB1$^{1–512}$, and RLIP76. Ni–NTA Superflow resin (Qiagen) purified rec-POB1 (A) and rec-POB1$^{1–512}$ (B), and DNP-SG Sepharose-affinity purified rec-RLIP76 (C) were applied to SDS–PAGE (10 μg each) and subjected to Western blot analyses against rabbit-anti-POB1 (primary antibodies for POB1 and POB1$^{1–512}$) and rabbit-anti-RLIP76 IgG. SDS–PAGE was stained with Coomassie brilliant blue R250 and Western blots were developed using horseradish peroxidase-conjugated goat-anti-rabbit-IgG as secondary antibody and 4-chloro-1-naphthol as chromogenic substrate.

was reached near a 1:1 molar ratio of POB1/RLIP76 (Fig. 2A). Higher molar ratio of POB1/RLIP76 (2/1 or 4/1) did not further inhibit RLIP76 transport activity (Fig. 2B). These results suggest that maximal inhibition of RLIP76 catalyzed transport is achieved at 1:1 stoichiometric binding of POB1 to RLIP76. In previous studies, we have observed that transport by RLIP76 of DOX or DNP-SG is unaffected by 2 mM GSH [20], a characteristic in contrast to GSH-stimulated activity of ABC transporters. In the present proteoliposome system, we also found no effect of 2 mM GSH on POB1-mediated inhibition of RLIP76 (Fig. 2B). These findings highlighting the distinct nature of transport by RLIP76 as compared with ABCC1 indicate that POB1 binding to RLIP76 does not confer the property of GSH-regulated transport.

Effect of POB1 on DOX-sensitivity

We have previously shown that inhibition of RLIP76-mediated ATP-dependent transport induces apoptosis in lung cancer cells and increases sensitivity of these cells to DOX. In order to examine whether the inhibition of RLIP76-mediated transport by POB1 in cells affected their DOX-sensitivity, we determined the effect of incorporation of POB1 and its deletion mutants in cells on their sensitivity to DOX. Previous studies [17] indicate that proteins encapsulated in proteoliposomes can be effectively delivered inside these cells. Therefore, H358 (NSCLC) cells were treated with control-liposomes or liposomes containing recombinant POB1 or POB1$^{1–512}$ (final concentration 40 μg/ml) to overload these cells with either protein. DOX was added and IC_{50} was measured by performing MTT assay 96 h later as described previously [30]. Treatment with POB1 liposomes increased the sensitivity of the H358 cells to DOX by about 3-fold, whereas POB1$^{1–512}$ had no significant effect on DOX-sensitivity. (Fig. 2C). Since POB1$^{1–512}$, which does not inhibit DOX transport, has no effect on the sensitivity of the cells to DOX, these results indicate that inhibition of RLIP76-mediated DOX-efflux by POB1 sensitizes these cells to DOX through increased intracellular accumulation of the drug. We have previously shown that inhibition of RLIP76 through other mechanisms results in increased DOX-accumulation in lung cancer cells [14–16]. Lack of any effect of BSA on the transport of DOX or the sensitivity of cells to DOX rules out nonspecific binding effect of protein(s) on these parameters.

Effect of POB1 and POB1$^{1–512}$ overloading on apoptosis

Inhibition of RLIP76-mediated transport by anti-RLIP76 IgG has been shown to induce apoptosis in lung cancer cells and this effect has been suggested due to accumulation of the GSH-conjugates in cells [14]. Accumulation of GSH-conjugates leads to inhibition of GSTs and impairs the ability of these enzymes to metabolize 4-HNE, which is known to induce apoptosis [32–34]. Since our results showed that the ATP-dependent transport activity of RLIP76 for DOX as well as DNP-SG, a GSH-conjugate, was inhibited by POB1 in a concentration-dependent manner, we investigated whether overloading the cells with POB1 induced apoptosis. To test this hypothesis, H358 cells were treated with equal amounts of proteoliposomes reconstituted with either recombinant full length POB1 or POB1$^{1–512}$ protein. Final concentration of recombinant POB1 or POB1$^{1–512}$ in proteoliposome was 40 μg/ml. Results of apoptosis measurement performed by TUNEL assay using Promega fluorescence detection kit (Figs. 2D–F) showed that the full length POB1 caused apoptosis in cells (Fig. 2F) while the POB1$^{1–512}$, which did not inhibit transport properties of RLIP76, did not have any significant apoptotic effect (Fig. 2E). These results are consistent with our previous studies showing that inhibition of RLIP76 results in increased accumulation of glutathione-conju-

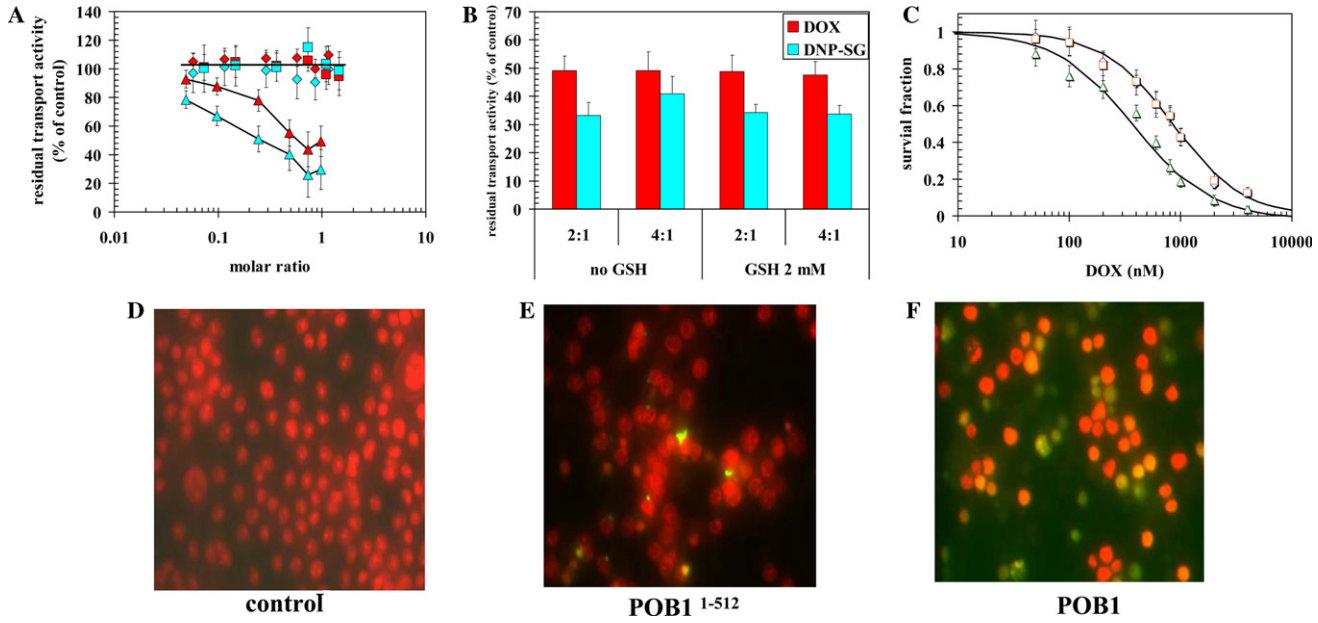

Fig. 2. Effect of POB1 on the transport activity of RLIP76, DOX-sensitivity, and apoptosis. The transport activity of RLIP76 towards DOX (red symbols) and DNP-SG (blue symbols) (A) was measured by using purified recombinant RLIP76 reconstituted into artificial cholesterol/asolectin liposomes as previously described [17]. The effect of POB1 (triangles), POB1$^{1-512}$ (squares) or bovine serum albumin (diamonds) at varying molar ratios was examined by including varying concentrations of these proteins in the transport medium. Transport medium contained RLIP76-proteoliposomes (250 ng protein/30 μl), 100 μM [^{3}H]DNP-SG (specific activity 3264 cpm/nmol) or 3.6 μM [^{14}C]DOX (specific activity 90,585 cpm/nmol), without or with 4 mM ATP (three experiments, each in triplicate). Effect of 2 mM GSH on ATP-dependent transport of DOX (red) and DNP-SG (blue) was examined in the presence of 2- or 4-fold molar excess of POB1 (B). The effect of increased POB1 on DOX-cytotoxicity was examined by incubating cells with proteoliposomes containing either POB1 (triangle), POB1$^{1-512}$ (square) or albumin (diamond) (C) (three determinations, each with eight replicates). The effect of POB1-overloading on apoptosis was examined by TUNEL assay (D–F). H358 NSCLC cells grown on coverslips incubated for 24 h with proteoliposomes containing either albumin (D), POB1$^{1-512}$ (E) or POB1 (F) for 24 h prior to TUNEL assay using Promega fluorescence detection kit and examined using Zeiss LSM 510 META (Germany) laser scanning fluorescence microscope with filters 520 and >620 nm. Photographs taken at identical exposure at 400× magnification are presented. Apoptotic cells showed green fluorescence, characteristic of cell shrinkage.

gates of endogenously generated toxicants such as 4-HNE, and culminates in apoptosis.

Effect of POB1-liposomes on accumulation and efflux of [^{14}C]DOX in lung cancer cells

We conducted drug-uptake and efflux studies with control, purified rec-POB1 or purified rec-POB1$^{1-512}$ liposome (40 μg/ml final concentration) treated intact H358 cells. For uptake studies, cellular DOX accumulation was quantified at varying times after addition of drug to the extracellular medium (Fig. 3A). The total DOX accumulation was markedly increased in rec-POB1 liposome treated cells. These results were consistent with decreased transport of DOX or DNPSG in rec-POB1 liposome treated cells. In experiments designed to measure the efflux of DOX, we loaded cells with liposomes and [^{14}C]DOX by incubating for 60 min, followed by rapid dilution in drug-free medium, and taking sequential aliquots of external medium for radioactivity counting. Cell-associated drug was calculated by back-addition as previously described [31] and plotted with respect to time. The rate of loss of DOX from cells due to efflux was significantly lower for rec-POB1 liposome treated cells as compared with the control cells (Fig. 3B). Furthermore, total cellular DOX accumulation was increased more than 2-fold that seen in cells treated with control liposomes (Fig. 3B).

Previous studies of interactions of POB1 and RLIP76 have primarily focused on the integral role of these proteins in down-regulation of insulin–insulin receptor signaling though clathrin-coated pit-mediated receptor–ligand endocytosis [5,8]. The established ATPase and transport activity of RLIP76, and the previously demonstrated induction of apoptosis by POB1 over-expression, in context of demonstrated specific inhibition of RLIP76 through the same domain interactions identified by others as being important in endocytosis, rules out the role of either protein as simply an adaptor or scaffold. In contrast, our results suggest an integral role of physiological glutathione-conjugate transport in endocytosis. Since RLIP76 is clearly up-regulated by oxidative and other stresses, our findings link glutathione-conjugate transport in oxidative-stress-induced insulin-resistance. In this model, increased RLIP76 in response to oxidative stress would increase the rate of

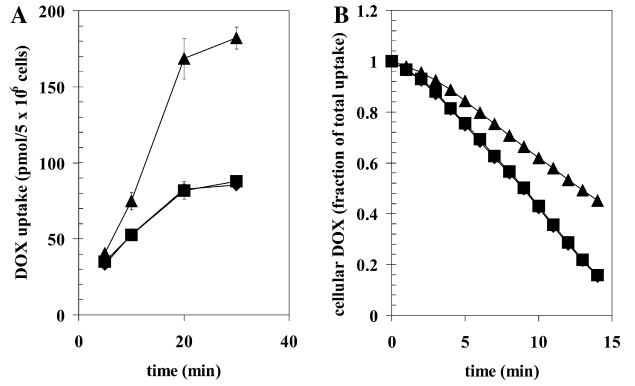

Fig. 3. Effect of POB1-liposomes on cellular DOX-accumulation and efflux from intact cells. The cellular accumulation of DOX was determined by incubating H358 cells (5×10^6) with control-liposomes (diamond), purified rec-POB1$^{1-512}$ liposomes (square), and purified rec-POB1 liposomes (triangle) (40 μg/ml final concentration) at 37 °C. Subsequently, 20 μl of 14-[^{14}C]DOX (final 3.6 μM, specific activity 8.5×10^4 cpm/nmol) was added and cells were incubated for varying time periods (5–30 min) at 37 °C, washing off extracellular drug and determining cell-associated radioactivity. The values, mean and standard deviations ($n = 9$) of DOX uptake in terms of pmol/5×10^6 cells are presented (A). For intact cell efflux studies (B), attached cells were loaded with liposomes and [^{14}C]DOX by incubating for 60 min, washing off extracellular drug with PBS, followed by rapid dilution in drug-free buffer. Aliquots of buffer were removed at 60 s intervals, and at the end of the study, total residual radioactivity in the dilution buffer was measured. The back-added curves of cellular residual DOX vs. time were constructed as described previously [31].

down-regulation of insulin–insulin receptor complex, decreasing signaling time and contributing to insulin-resistance. Since RLIP76 and POB1 are also involved in down-regulation of EGF-R and TGFβ-R [6,8], these proteins could regulate responsiveness to endogenous ligands, or even sensitivity to exogenous ligands, such as Herceptin or Erbitux which are used in breast and colon cancer chemotherapy. The multiple potential mechanisms through which RLIP76 could function to thwart chemotherapy or antibody therapy efficacy are noteworthy, and suggest that targeting RLIP76 for inhibition could be a novel and effective clinical strategy for enhancing the efficacy of these therapies. We have previously shown the protective role of RLIP76 and the pro-apoptotic effects of RLIP76 inhibition in K562 myeloid leukemia cells exposed to stresses, including DOX [32,33]. Present findings corroborate previous findings of POB1-mediated apoptosis in prostate cancer and demonstrate that this mechanism of RLIP76-regulation is operative in lung cancer.

Previous studies have shown that increased RLIP76-expression in response to stress is accompanied by increased capacity of cells for the efflux of glutathione-conjugate (GS-HNE) of the model alkenal, 4-HNE, which is known to cause apoptosis [32,33]. Inhibition of this efflux by blocking RLIP76 by anti-RLIP76 antibodies promotes apoptosis. It is likely that apoptosis in prostate cancer cells over-expressing POB1 is also due to the inhibition of RLIP76-mediated transport of GS-HNE. In general, these findings appear to be consistent with a model in which intracellular accumulation of 4-HNE results in alkylation of DNA, apoptosis and necrosis, and that in this model, RLIP76 functions as a defense against stress-induced apoptosis by regulation of intracellular 4-HNE, an obligate pro-apoptotic product of oxidant or radiant stress [32,33]. It appears that POB1 acts as a regulator of transport activity of RLIP76 which may be crucial for the maintenance of 4-HNE homeostasis in cells because it has been demonstrated that while higher concentrations of 4-HNE cause apoptosis [32,33], lowering of 4-HNE concentrations below the basal levels leads to differentiation and transformation of cells [34,35]. Thus, regulation of RLIP76 transport activity by POB1 may serve not only an important role in signaling but may also be relevant to the mechanisms of drug resistance. Further studies are needed to clearly define these roles of POB1.

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