Doxorubicin transport by RALBP1 and ABCG2 in lung and breast cancer

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Abstract. RALBP1 (RLIP76) is the major transporter of doxorubicin (DOX) in lung cancer cells, and that the difference in sensitivity of small cell lung cancer (SCLC) cells to DOX is due to differential phosphorylation by PKCα. Our recent studies have suggested that RALBP1 present in MCF-7 breast cancer cells has significantly lower specific activity for transport of DOX than wild-type recombinant protein, and its level of expression is significantly lower than that in lung cancer cells. In the present study, we have explored whether or not this is a generalized phenomenon for breast cancer, and have compared the relative contributions of RALBP1 and the ABC-family transporter, ABCG2 to total DOX transport activities in two SCLC (H1417 and H1618), two non-small cell lung cancer (NSCLC) (H358 and H520), and three breast cancer (T-47D, MDA-MB231, and MCF-7) cell lines. Results of these studies show lower protein expression and specific activity of RALBP1 in all three breast cancer cell lines as compared with lung cancer cell lines. Furthermore, we demonstrate that RALBP1 contributes only a minor fraction of DOX transport activity in breast cancer cell lines, suggesting that greater DOX sensitivity of breast cancer may be related to lower RALBP1 transporter activity and that the transport mechanisms involved in multi-drug resistance of lung and breast cancer are distinct.

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Abbreviations: RALBP1 (RLIP76), Ral-interacting protein; DOX, doxorubicin; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; GSH, glutathione; GS-E, glutathione-electrophile conjugate; DNP-SG, dinitrophenyl S-glutathione; MRP, multi-drug resistance associated protein

Key words: doxorubicin, glutathione-conjugates, drug-resistance, ABCG2, RALBP1, siRNA

Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most widely used anticancer drugs, effective in a wide variety of human cancers (1-3). Its efficacy, like that of other anticancer drugs, is hampered by innate and acquired drug-resistance in cancer cells. Multiple mechanisms of DOX-resistance have been elucidated. Although alterations in cellular antioxidant defenses (4), topoisomerases (5), and in stress- and apoptosis signaling pathway proteins (6) are found in many DOX-resistant cells, drug-efflux mechanisms which create a DOX-accumulation defect appear to be the most common alteration (6,7). The ABC-family of multi-specific transporters including P-glycoprotein (Pgp, ABCB1), multidrug-resistance protein (MRP1, ABCC1), and multiple other MRP-related transporters have been shown to mediate DOX-transport and resistance (8-10). Pgp was the first identified among these transporters (11), and differed from MRP and related transporters in that the latter transporters use not only the weakly cationic anthracycline and vinca alkaloids as substrates, but can also transport glutathione-conjugates (GS-E) of endogenous and exogenous alkylating agents (including a number of chemotherapy drugs). Though the ABC-transporters play a significant role in protecting many cancer cell types from DOX, *in vitro* as well as clinical studies indicate the involvement of other DOX-transporters (11,12).

We have identified RALBP1 (RLIP76) as a unique non-ABC transporter of xenobiotics and metabolites, and have shown through cell-culture and mouse gene-interruption studies it to be the predominant transporter of DOX as well as of glutathionylated metabolites of endogenous and exogenous alkylating agents (13-25). Our recent studies have shown that RALBP1 is the major drug transporter in lung cancer cells and that the relatively greater resistance to DOX in NSCLC as compared with SCLC is due to greater activity of RALBP1 attributed to increased PKCα-mediated phosphorylation of T^{297} in NSCLC (23,24). In these studies, we also demonstrated that DOX-resistance mediating effects

Table I. Comparison of transport characteristics of lung and breast cancer cells.

Parameters	Lung cancer	Breast cancer		
	H358[a]	MCF7	T-47D	MDA-MB231
Transport specific activity in crude membrane in-side-out vesicles (pmol/min/mg)				
DOX	188±18	38±3	37±3	40±4
DNP-SG	698±58	104±9	99±11	115±12
RALBP1 Protein (μg/10^8 cells)	38±3	12±1	12±1	13±1
RALBP1 (% of total crude protein)	0.6±0.01	0.21±0.01	0.21±0.01	0.22±0.01
ATPase specific activity (nmol/min/mg)				
Basal	161±25	54±7	50±6	62±8
DOX-stimulated	359±41	95±14	92±10	97±12
DNPSG-stimulated	337±34	105±17	98±8	117±14
Transport specific activity in purified reconstituted vesicles in artificial liposomes (pmol/min/mg)				
DOX	28,400±3100	8110±1100	7760±870	8440±730
DNP-SG	126,600±16,700	31,810±4200	30,290±2800	34,920±3630

[a]Data for this cell line are similar to that reported for other lung cancer cell lines (28,32). The values are presented as mean ± SD from 3 separate experiments with 3 replicates (n=9).

of PKCα are primarily due to the phosphorylation of RALBP1, since PKCα had no effect in either RALBP1$^{-/-}$ MEFs, or in lung cancer cells expressing RALBP1-mutants which cannot be phosphorylated. RALBP1 in the SCLC cells was predominantly in the unphosphorylated form, and had approximately half transport rate as compared with NSCLC (23,24).

Whereas the clinical utility of DOX in lung cancer is limited to small cell lung cancer (SCLC) because of inherently greater resistance of non-small cell lung cancers (NSCLC), DOX remains a first line drug of choice for treatment of most breast cancers in adjuvant as well as metastatic settings (1,2). Our recent studies showing lower expression as well as specific activity of RALBP1 in the MCF-7 breast cancer cell line as compared with lung cancer cell lines suggest that the relative sensitivity of breast cancer to DOX could be due to decrease in RALBP1-mediated efflux of this drug. In the present study, we tested this hypothesis by comparing the contributions of RALBP1 and ABCG2 in the transport of DOX and GS-E between lung and breast cancer cell lines.

Materials and methods

Materials. Sources for reagents for tissue culture, protein purification, and transport studies were the same as previously reported (13,14). DOX was obtained from Adria Laboratories, Columbus, OH. [^{14}C]-DOX (specific activity 57 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Source of anti-RALBP1 IgG used were the same as previously described (14). Anti-ABCG2 antibodies (anti-BCRP) were purchased from R&D Systems, MN. The MRP-specific inhibitor, MK571 was purchased from Biomol Research Laboratories, PA. [^{3}H]-DNPSG was prepared as described (14).

Cell lines and culture. Human SCLC lines H1417, H1618, NSCLC lines H358 (bronchio alveolar), H520 (squamous cell carcinoma), T-47D, and MCF-7 (estrogen-dependent breast cancer) and MDA-MB231 (estrogen-independent breast cancer) cell lines were studied. All the cell lines (except MCF-7) were grown in the RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) P/S solution, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate. MCF-7 cell line was kindly provided by Dr E. Schneider, Wadsworth Cancer Center, Albany, NY, and were grown in DMEM containing 10% FBS and 1% P/S. The cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

RALBP1 siRNA preparation. RALBP1 siRNA was designed as described previously (24), and synthesized by Dharmacon Research Laboratory. The targeted cDNA sequence (AAGA AAAAGCCAATTCAGGAGCC) corresponds to aa 170-176 (nt 508-528). The corresponding sense and antisense siRNA sequences are GAAAAAGCCAAUUCAGGAGCCdTdT and GGCUCCUGAAUUGGCUUUUUCdTdT, respectively. The sequence of the scrambled siRNA in the sense and antisense directions are GUAACUGCAACGAUUUCGAUGdTdT and CAUCGAAAUCGUUGCAGUUACdTdT, respectively. Transfection of siRNA duplexes was performed using trans-messenger transfection reagent kit (Qiagen) and assay for silencing 48 h after transfection.

Drug sensitivity. Bronchio-alveolar human lung carcinoma (H358) and human breast carcinoma (MCF-7) cells ($2x10^4$) were plated into the wells of a 96-well microtiter plate. For determination of the effect of RALBP1 depletion using siRNA, cells were incubated for 3 h with 2 μg/well RALBP1 siRNA in transmessenger transfection reagent (Qiagen). Cells were

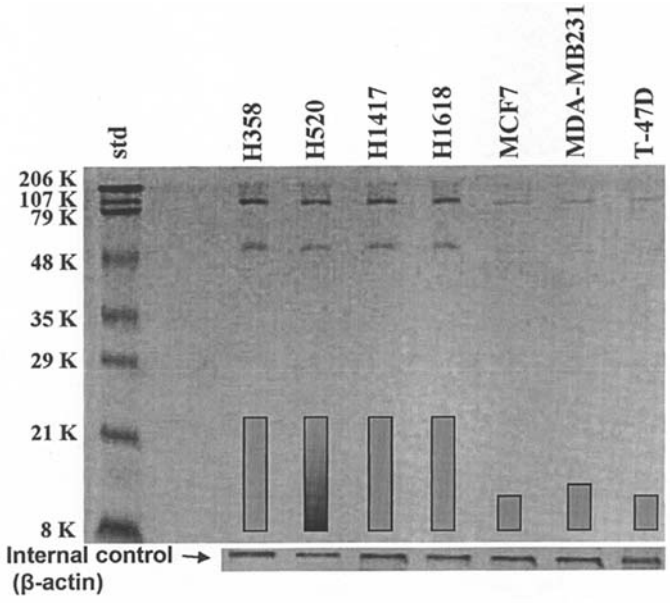

Figure 1. Comparison of RALBP1 levels in human lung and breast cancer cells. Aliquots of crude detergent extracts of the membrane fractions of human lung (H358, H520, H1417, H1618) and breast (MCF-7, MDA-MB231, T-47D) cancer cell lines, containing 200 μg protein were used for SDS-PAGE and Western blotting against anti-RALBP1 IgG as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody and developed with 4-chloro-1-napthol as chromogenic substrate. Results were quantified by scanning densitometry of the full-length RALBP1 protein band near 109 kDa. ß-actin was used as internal control.

washed with PBS, followed by 24 h of incubation at 37˚C in medium then varying concentrations of DOX were added. MTT assay was carried out 24 h later as previously described with 8 replicate wells per measurement, and 3 separate experiments to determine % cell survival (26).

Ouchterlony double immuno-diffusion assay. Immunological cross-reactivity of RALBP1 with ABCG2 (BCRP) was tested by Ouchterlony double immuno-diffusion assay (27) as previously described (28).

Preparation of liposomes containing purified recombinant RALBP1. Purification and authentication of human RALBP1 protein and subsequent preparation of control and RALBP1-liposomes has been described previously in detail (14,19).

Purification of RALBP1 from cell. Cells were pelleted at 700 x g. The pellets were suspended in PBS and aliquots were taken for determining cell number and viability by counting trypan blue dye excluding cells in a hemocytometer. Cells were pelleted and washed with balanced salt solution. Affinity purification of RALBP1 from lung and breast cancer cell lines was carried out in a manner identical to that described previously (28). Functional reconstitution of purified RALBP1 into artificial liposomes and transport studies in RALBP1 liposomes were carried out as described (13,14). Protein estimation, SDS-PAGE and Western blot analyses were performed as previously described (28).

Transport studies in RALBP1-liposomes. Transport studies of ^{14}C-DOX and ^{3}H-DNPSG in reconstituted vesicles were performed by the method as described (14), using 250 ng protein per 30 μl reaction mixture. ATP-dependent uptake of 14-[^{14}C]-DOX (specific activity 8.7x10^{4} cpm/nmol) or ^{3}H-DNPSG (specific activity 3.9x10^{3} cpm/nmol) was determined by subtracting the radioactivity (cpm) of the control without ATP from that of the experimental containing ATP, and the transport of DOX or DNPSG was calculated in terms of pmol/min/mg protein. Liposomes prepared without addition of RALBP1 were used for controls.

Preparation of crude membrane inside-out vesicles (IOVs). Crude membrane vesicles (inside-out vesicles, IOV) were prepared from the breast (T-47D, MDA-MB231, and MCF-7) and lung (H358) cancer cell lines using established procedures for the human erythrocytes (13) and K562 cells (14).

Transport studies in IOVs. Transport studies in IOVs were performed by the same method as described previously (13) except that instead of the no-protein proteoliposome, we used heat-inactivated IOVs as negative controls. Effect of MK571 (MRP1 inhibitor) on transport activity was measured as follows: Aliquots of either reconstituted RALBP1 liposomes containing 250 ng protein or crude membrane IOVs containing 20 μg protein, was incubated separately with buffer alone or varying concentrations of MK571 (1-100 μM) for 30 min at 37˚C. ATP-dependent transport of DOX or DNP-SG in these vesicles was measured according to the method as described (13,14).

Results

Comparison of DOX-transport capacity between lung and breast cancer cell lines. The total ATP-dependent transport activity towards DOX was nearly 5-fold greater in crude-membrane IOVs from lung cancer cells (H358) as compared with breast cancer cells (MCF-7, T-47D and MDA-MB231) (188±18 vs. 38±2 pmol/min/mg crude protein); Likewise, total DNP-SG transport activity was also greater in membranes from H358 as compared with MCF-7, T-47D and MDA-MB231 cells (698±58 vs. 106±11 pmol/min/mg crude protein) (Table I). The significantly greater total DOX-transport capacity in H358 cells as compared with MCF-7, T-47D and MDA-MB231 cells may be an underlying reason for greater DOX-sensitivity of breast as compared with NSCLC.

Comparison of RALBP1 expression in several lung and breast cancer cell lines. RALBP1 protein expression in crude membrane fractions from two NSCLC (H358 and H520), two SCLC (H1417 and H1618), two estrogen-dependent breast cancer (MCF-7 and T-47D) and one estrogen-independent breast cancer (MDA-MB231) cell lines was compared to examine whether or not breast cancer cells, in general, have lesser abundance of RALBP1 as compared to lung cancer cells. Results of these studies showed that RALBP1 levels were similar in SCLC and NSCLC cell lines, significantly lower levels were seen in all three breast cancer cell lines (Fig. 1).

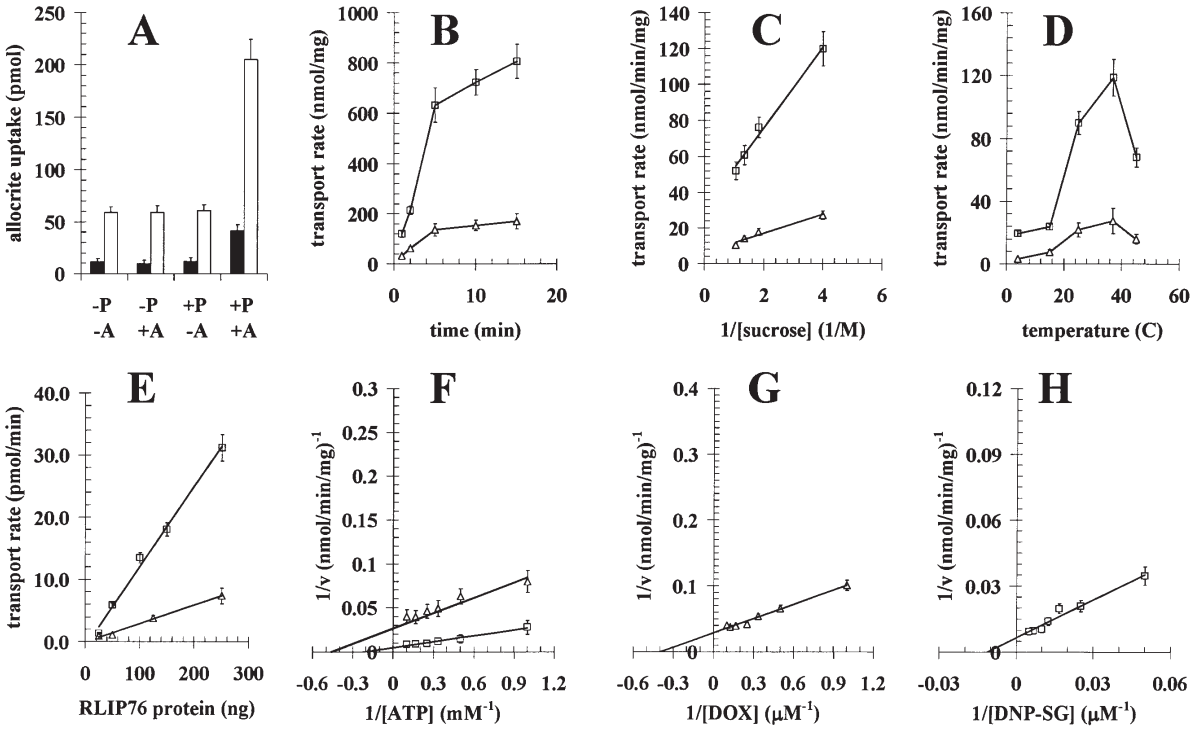

Figure 2. Kinetics of DOX and DNPSG transport by RALBP1 purified from H358 cells. Transport studies were carried out as described in Materials and methods. The uptake of radio-labeled ^{14}C-DOX (triangles) and ^{3}H-DNP-SG (squares) by control- or RALBP1-proteoliposomes was compared in the presence or absence of ATP. The proteoliposomes prepared from H358 RALBP1, were incubated at 37˚C with either DOX (3.6 μM, black bar) or DNP-SG (100 μM, white bar) for 5 min prior to addition of buffer without or with ATP, and uptake was measured 5 min after ATP addition. Filtered aliquots (30 μl) contained 62.5 μg lipid (4:1::asolectin:cholesterol) and 0.25 μg protein (either purified RALBP1 or crude protein). Total radioactivity retained by 0.45 μm filters in 96-well plates was determined after solubilizing filters in scintillation fluid, converted to pmol using the specific activity of DOX ($8.7x10^4$ cpm/nmol) and DNP-SG ($3.9x10^3$ cpm/nmol). Each point represents an average and SD calculated from 12 measurements [control (-P) or RALBP1-proteoliposomes (+P), without (-A) or with ATP (+A), in triplicate]. Uptake in RALBP1-liposomes with ATP is significantly greater than other groups ($p<0.001$) (A). One of the parameters (time, extra-vesicular sucrose, temperature, and vesicle-protein) was varied holding the other constant at standard conditions which include either 3.6 μM DOX or 100 μM DNP-SG, 4 mM ATP, 40 mM extra-vesicular sucrose, 250 ng protein/30 μl aliquot filtered, temperature 37˚C, and 5 min incubation after addition of ATP. Results shown are for time dependence (B), osmolar dependence (C), temperature dependence (D), and RALBP1-protein dependence (E). Transport rates were determined under varying DOX, DNP-SG or ATP concentrations. The kinetics for ATP was performed with either DOX or DNP-SG fixed at 3.6 or 100 μM, respectively, and for either DOX or DNP-SG kinetics, ATP was fixed at 4 mM. Transport rates were plotted vs. drug concentration and analyzed by double reciprocal plots. Results for ATP (F), DOX (G) and DNP-SG (H) kinetics are shown. Each point representing DOX (triangle) or DNP-SG (square) was measured in triplicate by measuring and subtracting uptake observed in control proteoliposomes without or with ATP and RALBP1-proteoliposomes without ATP from that observed in RALBP1-proteoliposomes with ATP (4 groups, each in triplicate) as shown in (A).

Comparison of purified RALBP1 from lung and breast cancer cell lines. RALBP1 was purified by DNP-SG affinity purification method (14) from detergent extracted membrane fractions from a panel of lung and breast cancer cell lines and quantified by ELISA. Lung cancer cells contained >3-fold greater RALBP1 proteins as compared with breast (38±3 vs. 12±1 μg/10^8 cells, respectively). RALBP1 was 0.6% of total membrane protein in the lung cancer cell line, a value identical to that found in previous purifications from a panel of 12 lung cancer cell lines (28). In contrast, the relative abundance of RALBP1 in the breast cancer cell line was only 0.2% of total membrane protein ($p<0.01$). Transport activity of RALBP1 for DOX is a function of its basal ATPase activity which is stimulated by DOX and other allocrites. Purified RALBP1 from H358 was found to have greater basal as well as DOX or DNP-SG stimulated ATPase activity as compared to that from MCF-7, T-47D and MDA-MB231 cells. Similarly, the DOX and DNP-SG transport activity of RALBP1 purified from H358 was significantly greater than that from MCF-7, T-47D and MDA-MB231 cells (Table I). Together these results indicate that the abundance as well as the activity of RALBP1 was several fold higher in lung cancer cells as compared to that in breast cancer cells.

Kinetic characterization of RALBP1 from breast and lung cancer cells. The transport characteristics of RALBP1 purified from MCF-7, T-47D and MDA-MB231 cells were quite similar. Therefore, we exhaustively compared the kinetics of transport of DOX and DNP-SG by RALBP1 purified from H358 (lung cancer cell) and MCF-7 (a representative of breast cancer cell). This was achieved by a series of experiments measuring ATP-dependent DOX or DNP-SG uptake in RALBP1-proteoliposomes prepared from purified protein from H358 (Fig. 2) or MCF-7 cells (Fig. 3). Purified protein was reconstituted into artificial proteoliposomes prepared from soybean asolectin: cholesterol (4:1) with a protein: lipid ratio of 50 μg protein/5 mg lipid per ml reconstitution buffer. This method yields liposomes which have been extensively characterized previously, showing an intra-vesicular volume of 18 μl/ml extra-vesicular volume (by inulin-exclusion study) and median diameter of 0.5 μm by transmission electron microscopy and are suitable for transport studies (29). Western blot

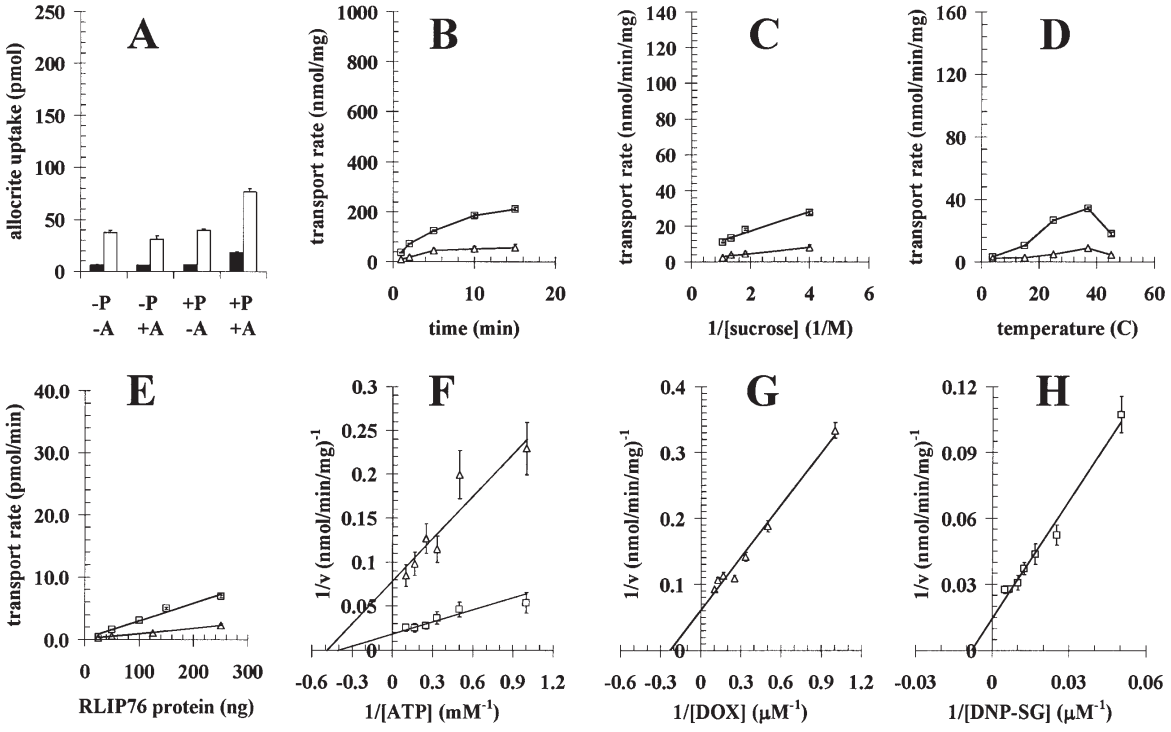

Figure 3. Kinetics of DOX and DNPSG transport by RALBP1 purified from MCF-7 cells. Transport studies were carried out as described in Fig. 2. The uptake of radio-labeled ^{14}C-DOX (triangles) and ^{3}H-DNP-SG (squares) by control- or RALBP1-proteoliposomes was compared in the presence or absence of ATP. The proteoliposomes were prepared from MCF-7 RALBP1.

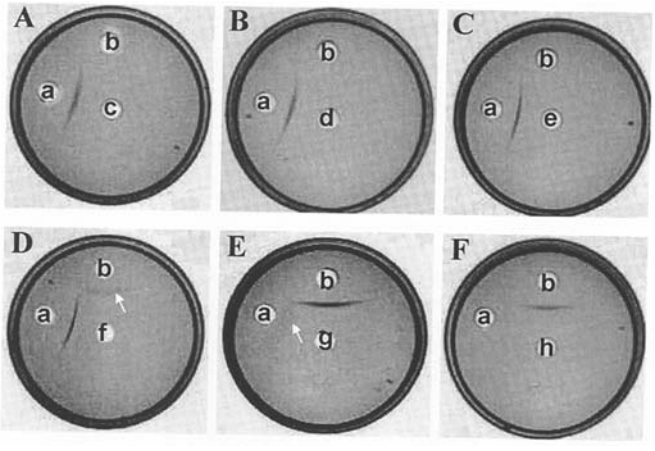

Figure 4. Immunological characterization of RALBP1 from lung and breast cancer cells. RALBP1 was examined for immunological cross-reactivity with anti-ABCG2 in Ouchterlony double immuno-diffusion assay. In all panels, peripheral wells contained antibodies: anti-RALBP1 IgG (a) and anti-ABCG2 IgG (b). The central well contained antigens: purified rec-RALBP1 (c), purified H358-RALBP1 (d), purified MCF7-RALBP1 (e), crude membrane fractions of H358 (f), crude membrane fractions of MCF7 (g) and crude membrane fractions of MCF-7 pretreated with RALBP1 siRNA (h).

analysis of the pellet and supernatant fractions of 104,000 x g centrifugation of RALBP1-proteoliposomes revealed that >95% of total RALBP1 was found within the pellet fraction indicating excellent incorporation of the protein within vesicles. Each transport measurement was performed by quantifying [^{14}C]-DOX or [^{3}H]-DNP-SG uptake by control proteoliposomes or RALBP1-proteoliposomes without or with 4 mM ATP in the transport buffer (triplicate measurement for each of 4 groups) as described in detail previously for rec-RALBP1 (14).

Increased DOX and DNP-SG-uptake was demonstrable only in the presence of both ATP and RALBP1-proteoliposomes and not in control proteoliposomes without or with ATP, or in RALBP1-proteoliposomes without ATP (Figs. 2A and 3A). The uptake of both allocrites with respect to time approximated linearity for the first 5 min, and was fit to a single compartment model of uptake (Figs. 2B and 3B). DOX and DNP-SG uptake by vesicles was sensitive to osmolarity, decreasing with increasing sucrose concentration (Figs. 2C and 3C). Optimal temperature for transport was found to be 37˚C, with progressive inactivation above 45˚C (Figs. 2D and 3D). In proteoliposomes reconstituted with varying amounts of protein (between 5 and 50 μg/ml), the uptake was linear with respect to protein, indicating that transport rate was linearly related to the amount of RALBP1 used for assay (Figs. 2E and 3E). The transport of DOX and DNP-SG was saturable with respect to ATP (Figs. 2F and 3F) as well as the transported allocrite (Figs. 2G and H and 3G and H). For H358, DOX-transport, K_m of ATP was 2.4 mM and for DOX, 2.6 μM; for DNP-SG transport, K_m for ATP was 3.6 mM and for DNP-SG, 80 μM. For MCF7 DOX-transport, K_m of ATP was 2.8 mM and for DOX, 4.2 μM; for DNP-SG transport, K_m for ATP was 2.9 mM and for DNP-SG, 110 μM.

These results show that the specific ATP-hydrolysis-dependent transmembrane anti-gradient transport of DOX as well as DNP-SG is catalyzed by RALBP1 purified from both MCF-7 as well as H358 cells, and demonstrate for the first time that RALBP1 purified from cancer cell lines behaves similarly

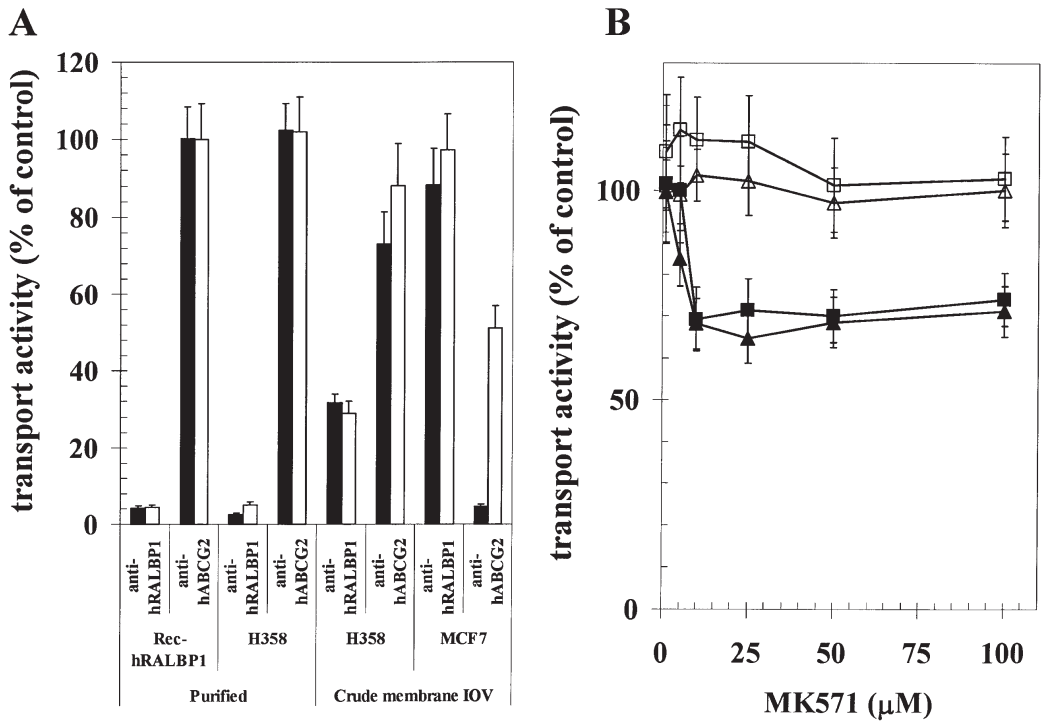

Figure 5. Effect of anti-RALBP1, anti-ABCG2, and MK571 on DOX and DNP-SG transport in breast and lung cancer cells. Transport activity towards DOX (black bars) or DNP-SG (white bars) was determined in the presence of anti-RALBP1 or anti-ABCG2 (67 μg/ml final concentration of antibody) in either artificial proteoliposomes reconstituted with RALBP1 (purified from either recombinant source or H358 NSCLC cell line) or crude membrane IOVs from H358 cells or MCF-7 breast cancer cells. Results presented are mean and SD from 3 determinations, normalized to control activity measured in the presence of an equal concentration of pre-immune serum (A). The effect of MRP-specific inhibitor MK571 on DOX (triangle) or DNP-SG (square) transport by either RALBP1 purified from H358 and reconstituted into artificial liposomes (white symbols) or IOVs prepared from crude membranes of H358 cells (black symbols) (B). Results presented are mean and SD from 3 determinations each at 6 concentrations of MK571 between 1 and 100 μM.

to either recombinant or tissue purified RALBP1 previously studied (13,14). Collectively, these results show that RALBP1 is in greater abundance and has higher specific activity for transport of DOX as well as GS-E in H358 cells as compared with MCF-7 cells.

Relative contribution of RALBP1 and ABCG2 to the total transport of DOX- and DNP-SG in lung and breast cancer cells. ABCG2 plays a significant role in DOX-transport and resistance in breast cancer (30), and its role in the transport of drugs in cancer cells has also been suggested (31). Since our previous studies indicate that RALBP1 is the predominant transporter of DOX as well as GS-E in lung cancer cells, we quantitated the relative contributions of RALBP1 and ABCG2 in the transport of DOX and DNP-SG (a model GS-E allocrite) in breast and lung cancer cells. Specific inhibition of the transport mediated by RALPB1 and MRP by their respective antibodies has been used by us to successfully quantitate RALBP1 and MRP (ABCC1) mediated transport in cells (32). ABCG2 being an ABC transporter is immunologically similar to ABCC1 but its antibodies should not cross-react with RALBP1. This was confirmed by the results of Ouchtertony double immuno-diffusion experiments (Fig. 4) which showed that anti-ABCG2 antibodies did not recognize RALBP1 and vice versa. These results also showed that in crude homogenates of MCF-7 cells, both ABCG2 and RALBP1 were present. A weaker precipitin line of anti-RALBP1 as compared to that with anti-ABCG2 antibodies (Fig. 4) suggested lesser abundance of RALBP1 in MCF-7 cells as compared to ABCG2 which was consistent with the results presented in Table I. In the homogenates of MCF-7 cells, in which RALBP1 expression was suppressed, anti-RALBP1 did not recognize any protein. Together these results confirmed that ABCG2 and RALBP1 were immunologically distinct, the antibodies used in these experiments were specific, and that both RALBP1 and ABCG2 were expressed in MCF-7 cells, the latter being more abundant.

Experiments were designed to study the inhibition of transport by these antibodies in (a) reconstituted proteoliposomes with purified RALBP1 from recombinant source (14), (b) RALBP1 purified from H358 and, (c) membrane vesicles (IOVs) prepared from H358 and MCF7 cells. Results of these studies presented in Fig. 5A, show that the majority of the transport in proteoliposomes reconstituted with rec-RALBP1 or that purified from H358 was inhibited by anti-RALBP1 antibodies. In contrast, anti-ABCG2 antibodies had no significant effect on the transport activity in these proteoliposomes. These results demonstrated that majority of DOX transport in H358 was mediated by RALBP1 and ABCG2 played an insignificant role in the efflux of this drug from H358 cells.

Anti-ABCG2 antibodies did not inhibit any noticeable transport activity in proteoliposomes prepared from either recombinant RALBP1 or that purified from H358 lung cancer cells. However, anti-ABCG2 antibodies inhibited ~90% of the DOX transport in crude vesicles prepared from MCF-7. It is interesting to note that the extent of inhibition of the transport of DNP-SG by these antibodies in MCF-7 cells was

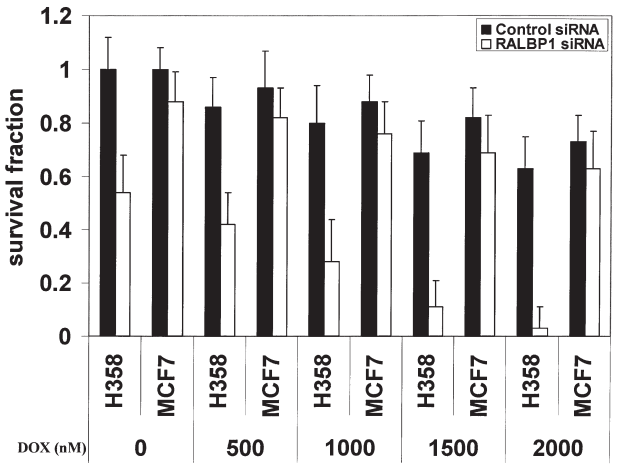

Figure 6. Effects of the siRNA of RALBP1 on DOX sensitivity of lung and breast cancer cells. Bronchio-alveolar human lung carcinoma (H358) and human breast carcinoma (MCF-7), $2x10^4$ cells each were plated into the wells of a 96-well microtiter plate. For determination of the effect of RALBP1 depletion using siRNA, cells were incubated for 3 h either with 2 μg/well RALBP1 siRNA (white bars) or scrambled siRNA (black bars) in trans-messenger transfection reagent. Cells were washed with PBS, followed by 24 h of incubation at 37˚C in medium then varying concentrations of DOX were added. MTT assay was carried out 24 h later with 8 replicate wells per measurement, and 3 separate experiments to determine survival.

much less than that of the DOX transport. These results suggest that while ABCG2 contribute to the majority of the DOX transport in MCF-7 cells, there may be additional transport mechanisms for the efflux of DNP-SG or GS-E. Anti-RALBP1 inhibited only a minor fraction of the activity for the transport of DOX as well as DNP-SG in IOVs prepared from MCF-7 cells, further indicating the presence of a GS-E transport mechanism in breast cancer cells (Fig. 5A) which is distinct from RALBP1 or ABCG2.

Differential inhibition of ABCG2 and RALBP1 by MK571. MK571 is a known specific inhibitor of ABCC1 and ABCG2 (33) but its effects on RALBP1 are not known. We compared the inhibitory effect of MK571 on DOX or DNP-SG transport in either crude-membrane vesicles from H358 or proteoliposomes reconstituted with purified RALBP1 from H358 or recombinant source (Fig. 5B). MK571 had no effect on DOX- or DNP-SG transport in artificial proteoliposomes reconstituted with purified RALBP1. Maximum inhibition of DOX or DNP-SG transport in crude membrane vesicles prepared from H358 by MK571 was achieved at 10 μM MK571. Higher concentrations did not cause more than ~30% inhibition of total transport. These results are consistent with those of previous studies showing that in lung cancer cells, ABC transporter contributes to ~30% of the efflux of DOX or DNP-SG.

Differential effects of RALBP1 depletion in breast and lung cancer cells. We have previously shown that lung cancer, ovarian cancer, prostate cancer as well as melanoma, cell lines which overexpress RALBP1, undergo apoptosis upon depletion of RALBP1 (25). In contrast, normal cells such as human endothelial, epithelial, or smooth muscle cells, which do not overexpress RALBP1, are relatively insensitive to RALBP1-depletion-mediated apoptosis (25). RALBP1 depletion or inhibition has also been shown by us previously to enhance DOX-cytotoxicity in cells overexpressing RALBP1 (22). In the present study, we compared the effects of RALBP1-depletion by siRNA on DOX-cytotoxicity. Results of these studies (Fig. 6) show that RALBP1 depletion preferentially sensitizes H358 cells to DOX which are consistent with results presented in preceding section showing that RALBP1 is the major DOX transporter in lung cancer cells. In contrast, breast cancer cells where the major transporter of DOX is ABCG2, RALBP1 siRNA had only minimal effect on the DOX sensitivity of these cells, further confirming only a minor role, if any, of RALBP1 in efflux of DOX from breast cancer cells.

Discussion

Collectively, these findings show that ABCG2 and RALBP1 are functionally and immunologically distinct transporters, both capable of DOX and DNP-SG transport, but expressed differently between lung and breast cancer. Whereas RALBP1 is the predominant DOX-transporter in lung cancer, the DOX-transport activity of ABCG2 predominates in breast cancer. The lower content of RALBP1 in breast cancer cells could be due to differential transcriptional or translational regulation of RALBP1 between lung and breast cancer. These aspects of RALBP1 regulation are at present not yet well defined since the genomic construct has not been studied with respect to transcriptional regulation, and mRNA processing is also not yet defined. It is known that RALBP1 has several splice-variants which are expressed differentially in tissues (34,35). However, the present findings may not be readily explained by differential expression of splice variants because the molecular mass of RALBP1 from breast and lung cancer are similar, and only one major form is present in both cells. Since lower expression of RALBP1 seen in breast cancer cells appeared independent of estrogen-receptor status, it is unlikely that the observed differences between breast and lung are due to estrogen-mediated effects (36-39).

The lower specific activity for transport suggests intrinsic structural differences in the protein obtained from lung vs. breast cells, perhaps as a result of post-translational modification which affect activity or stability of the protein, or perhaps the presence of a co-purified inhibitory factor present only in breast cancer cells. Because the differences observed by us were present both at the level of crude protein and membranes as well as with purified protein, it is unlikely that the observed differences are due to entirely soluble co-purified inhibitors. In this regard, it is known that POB1 (partner of RALBP1) binds specifically to RALBP1, and inhibits its transport activity (20). The readily dissociable nature of POB1 binding makes it unlikely that it co-purified with RALBP1. Furthermore, Western blot analyses of purified protein to check for POB1 did not reveal detectable POB1 contamination (data not presented).

RALBP1 is known to be phosphorylated by both serine/threonine-kinases as well as tyrosine kinases, and may undergo other post-translational modifications including farnesylation, and N-myristoylation (15). Our recent studies showing that the

greater activity of RALBP1 in NSCLC as compared with SCLC is due to differential expression of PKCα (23,24) suggest that differential post-translational modifications could explain the difference between RALBP1 from breast vs. lung cancer. Since the proteolytic susceptibility of RALBP1 is clearly affected by phosphorylation, it is possible that breast RALBP1 is less stable. Differential farnesylation or N-myristoylation could affect the amount of RALBP1 present in the membrane fraction, giving rise to the observation of greater RALBP1 protein in lung cancer cells. However, this is also unlikely since ELISA assays in whole cell homogenates showed the same degree of difference in RALBP1 content between lung and breast cancer cells. Further studies are needed to resolve these issues.

Near complete immuno-precipitation of DOX-transport activity in breast cancer cells by anti-ABCG2 indicates that Pgp is not likely to play any significant role in these cells, a conclusion consistent with pathological studies by others showing that Pgp is unlikely to be a major contributor to DOX-resistance in breast cancer (30,40). Our results suggesting that a GS-E transporter distinct from both RALBP1 and ABCG2 may be present in breast cancer cells serve to point out the complexity of GS-E transport mechanism. Because of the cross-reactivity of ABCG2 with ABCC1, it is expected that ABCC1 will also be immuno-precipitated by anti-ABCG2. Thus, most likely the unidentified GS-E transporter(s) of MCF-7 cells is/are distinct from ABCC1. Identification of this other transporter(s) could have important implications in design of agents to augment alkylating agent therapy in breast cancer. Though the underlying mechanisms remain to be defined, the observation that RALBP1 activity and content of breast cancer cells is lower than that of lung cancer cells, and particularly the finding that ABCG2 is the dominant DOX-transporter in breast cancer cells, serve to clarify the relative role of DOX-transporters and serves to point out that studies of transporters in one histology of cancer do not always apply to others. Clinical implications of these findings could be that targeting RALBP1 is more likely to be of therapeutic value in lung cancer, whereas targeting ABCG2 is more likely to benefit breast cancer patients.

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