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Determinants of differential doxorubicin sensitivity between SCLC and NSCLC

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Abstract **Doxorubicin (DOX) transport activity of Ral-interacting protein (RLIP76) in non-small cell lung cancer (NSCLC) is approximately twice that of in small cell lung cancer (SCLC). Since protein-kinase-C (PKC)α mediated phosphorylation of RLIP76 causes doubling of the specific activity of RLIP76, and NSCLC cells are known to have greater PKCα activity, we examined the contribution of PKC mediated phosphorylation of RLIP76 towards intrinsic DOX-resistance in human NSCLC. Expression of a deletion mutant RLIP76$^{delPKC\alpha\text{-}sites}$ followed by depletion of the wild-type RLIP76 using a siRNA targeted at one of the deleted regions resulted in generation of cells expressing only the mutant protein, which could not be phosphorylated by PKCα. DOX-transport activity of the mutant RLIP76 purified from NSCLC and SCLC was similar and comparable to that of RLIP76 purified from the wild-type SCLC. However, this activity was significantly lower than that of RLIP76 purified from the wild-type NSCLC. After siRNA mediated depletion of PKCα, DOX-transport activities of RLIP76 purified from SCLC and NSCLC were indistinguishable. Depletion of PKCα inhibited the growth of NSCLC more than SCLC cells (70 ± 3% vs. 43 ± 5%, respectively). PKCα-depletion lowered the IC_{50} of NSCLC cell lines for DOX to the same level as that observed for SCLC. RLIP76$^{-/-}$ mouse embryonic fibroblasts (MEFs) were significantly more sensitive to DOX as compared with RLIP76$^{+/+}$ MEFs (IC_{50} 25 vs. 125 nM, respectively). However, PKCα-depletion did not affect DOX-cytotoxicity towards RLIP76$^{-/-}$ MEFs, as opposed to RLIP76$^{+/+}$ MEFs which were sensitized by 2.2-fold. These results demonstrate that RLIP76 is a primary determinant of DOX-resistance, and that PKCα mediated accumulation defect and DOX-resistance in NSCLC is primarily due to differential phosphorylation of RLIP76 in SCLC and NSCLC.**

Keywords: Protein-kinase-C; Lung cancer; Doxorubicin; Drug-resistance; RLIP76; siRNA

1. Introduction

Doxorubicin (DOX) is a highly effective anthracycline chemotherapeutic agent belonging to the natural products class of antineoplastic agents. It is a highly active agent in the chemotherapeutic treatment of a number of human malignancies including breast cancer, bladder cancer, multiple myeloma, lymphoma, and small cell lung cancer (SCLC) [1,2]. In contrast to its high activity against small cell lung cancer, it is relatively ineffective against non-small cell lung cancer (NSCLC), which represents about 4/5 of all lung cancers. Even in SCLC, acquired resistance to DOX is common, accounting for the frequent failure to cure SCLC with DOX-based chemotherapy regimens. The resistance mechanisms responsible for inherent DOX-resistance of NSCLC and acquired DOX-resistance in SCLC have been the subject of numerous investigations [3]. The inherently lower cellular accumulation of DOX in NSCLC as compared with SCLC has been frequently attributed as a major cause of DOX-resistance in NSCLC; increased protein-kinase-C (PKC) activity is closely linked with DOX-accumulation defective resistance. The mechanism through which PKC can influence cellular DOX-accumulation remains unclear since regulation of ABC-transporter activity by PKC has not been established. PKCα has been shown to phosphorylate and stimulate the activity of Ral-interacting protein (RLIP76) [4–6], a novel non-ABC-transporter of DOX [7].

RLIP76 is a multifunctional modular protein, which was initially cloned as a Ral binding protein and was originally predicted to be a Ral-effector [8] but the exact actions of this effector were not known. We cloned Ral-interacting protein (RLIP76) independently in search of ATPases capable of and being stimulated by glutathione electrophile conjugates (GS-E), and have demonstrated that it is a stress-responsive protein capable of providing protection to cells from a variety of oxidative stressors including heat, UV-irradiation, X irradiation, and oxidant chemicals and indeed does fulfill the original prediction of RLIP76 being an effector protein [9–11]; these stressors are all known to cause formation of increased electrophilic lipid peroxidation byproducts which are metabolized to GS-E. GS-E are potent inhibitors of enzymes involved in glutathione-linked oxidative stress defense, and our studies clearly show that the transport activity of RLIP76 is important for the protective function of RLIP76. Recent studies by other investigators, showing that RLIP76 is a regulator of the Rho/Rac subfamily of G-proteins, known regulators of xenobiotic

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Abbreviations: RLIP76 (RALBP1), Ral-interacting protein; DOX, doxorubicin; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; GS-E, glutathione electrophile conjugate; PKC, protein-kinase-C

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metabolism and resistance [12], combined with our studies showing that RLIP76 is a multispecific transporter, suggest that RLIP76 protects cells from apoptosis caused by drug/toxin/oxidant stresses by preventing cellular accumulation of toxic-drugs and GS-E of endogenous and exogenous electrophilic toxins through its transport activity [7,13].

Our recent studies in panels of SCLC and NSCLC have shown that RLIP76 contributes approximately 2/3 of total DOX-transport activity with the remainder being contributed to by ABC transporters [14]. Similarly, RLIP76 was also found to be the predominant GS-E transporter of the model substrate LTC4 in cancer cells [15]. Greater than 80% loss in liver and heart tissue of total DOX and GS-E transport activity in RLIP76 knockout mice [16] strongly supports the hypothesis that RLIP76 can contribute substantially to total DOX-transport capacity in cancer cells. Sequence analysis of RLIP76 indicates the presence of four potential PKCα phosphorylation sites [6]. Since increased PKC activity is correlated with increased DOX-resistance and decreased cellular DOX-accumulation, and because RLIP76 can be activated through phosphorylation by PKCα [4–6], the present studies were performed to test the hypothesis that cellular RLIP76 levels and transport activity, and the regulation of RLIP76 by PKCα is a significant determinant of inherent DOX-resistance in NSCLC.

2. Materials and methods

2.1. Materials

Sources for reagents for tissue culture, protein purification, and transport studies were the same as previously reported [17]. [^{14}C]-DOX (specific activity 57 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Source of anti-RLIP76 IgG used in these studies were the same as previously described [9]. PKCα was obtained from Molecular Probes, Oregon. Polyclonal rabbit-anti-human PKCα IgG was purchased from Biosource (Camarillo, CA).

2.2. Method for preparing MEF cultures

Twelve weeks old C57BL/6 mice born of heterozygous × heterozygous (RLIP76$^{+/-}$ × RLIP76$^{+/-}$) mating were genotyped by PCR strategy on mouse tail DNA using forward, reverse and long terminal region (LTR) primers. These mice were commissioned from Lexicon Genetics and were created using Cre-Lox technology. Embryo fibroblast lines were prepared from RLIP76$^{+/+}$, RLIP76$^{+/-}$ and RLIP76$^{-/-}$ mice on the 13th or 14th day of pregnancy according to the method as described previously [18].

2.3. Cell lines and cultures

Human SCLC lines, H182, H1417, H1618, NSCLC lines H226 (squamous cell carcinoma), H1395, H2347 (adenocarcinoma), and H358 (bronchio alveolar) cell lines were used in present studies. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in 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.

2.4. Drug sensitivity assay

Cell density was estimated by counting trypan blue excluding cells in a hemocytometer, and 20 000 cells/160 μl medium were plated into each well of 96-well flat-bottomed microtiter plates. After 24 h, an aliquot of medium without drug (control) or containing different concentrations of drug was added to the wells and IC_{50} of drug was measured 96 h later by MTT assay as previously described [19]. Eight replicate wells were used in each point in each of three separate measurement of IC_{50}. Measured absorbance values were directly linked with a spreadsheet for calculation of IC_{50}, defined as the drug concentration that reduced formazan formation by 50%. For determination of the effect of PKCα siRNA #8, cells were incubated for 3 h with 2 μg/well PKCα siRNA in Transmessenger Transfection Reagent (Qiagen). Cells were then washed with PBS and MTT assay was performed 48 h after treatment of siRNA as described above.

2.5. Purification of RLIP76 from SCLC and NSCLC

Cells were pelleted at 700 × 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. Affinity purification of RLIP76 from human lung cancer cell lines was performed in a manner identical to that described previously [6]. Functional reconstitution of purified RLIP76 into artificial liposomes and transport activity in RLIP76 liposomes was measured as described previously [14,17].

2.6. Transport studies in RLIP76-liposomes

Transport studies of DOX in reconstituted vesicles were performed by the method as described previously [9,14], using 250 ng protein per 40 μl reaction mixture. Equimolar concentration of NaCl was substituted for ATP in the controls. ATP-dependent uptake of 14-[^{14}C]-DOX (specific activity 8.5×10^4 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 was calculated in terms of nmol/min/mg protein. Liposomes prepared without addition of RLIP76 were used for controls.

2.7. Stable transfection of RLIP76 and RLIP76$^{delPKC\alpha\text{-}sites}$ in SCLC and NSCLC cells

H1618 (SCLC) and H358 (NSCLC) were transfected with the eukaryotic expression vector alone (pcDNA3.1) or with pcDNA3.1-RLIP76 and the mutants of RLIP76$^{\text{del PKCsite[aa 118–120 (SKK), 297–299 (TEK), 353–355 (SNR) and 509–511 (SEK)]}}$ using lipofectamine 2000 (Invitrogen). RLIP76 lacking the PKCα -site, deletion mutants were made by PCR-based site-directed mutagenesis as described previously [6]. Stable transfectants were selected in the presence of 600 μg/ml G418 for 2 weeks. Several G418-resistant stable clones were selected for further characterization and were maintained in medium containing 300 μg/ml G418. Single clonal stable transfectants was established by sequential dilution into 96-well plate, such that only a single cell was seeded in each well. Expression of RLIP76 and its mutants mRNA in lung cancer cell lines were evaluated by RT-PCR analysis.

2.8. RLIP76 siRNA preparation

We chose the region around the PKC site (nucleotide 1529–1549 starting from 1 AUG codon in the open reading frame) in the C-terminal region of RLIP76 as the target region to design for siRNA to suppress the RLIP76 so that the mutant of RLIP76 lacking the PKC binding site remains unaffected. We searched for 21 nucleotide sequence motif, AA(N19)TT and selected hits with approximately 50% GC contents. The sequence of sense siRNA corresponds to N19. We converted 3′ end of the sense siRNA to TT. The rationale for this sequence conversion was to generate a symmetric duplex with respect to the sequence composition of sense and antisense 3′ overhangs. The antisense siRNA was synthesized as the complement to position 1–19 of the 21-nucleotide motif. The selected siRNA sequence was blast-search (NCBI database) against EST libraries, to ensure that only one gene is targeted. Chemically synthesized siRNA duplex in the 2′ de-protected and desalted forms, was purchased from Dharmacon Research (Lafayette, CO). A 21 nucleotide long scrambled siRNA duplex was used as a control. The scrambled siRNA sequence was not homologous with RLIP76 mRNA in a blast-search against RLIP76. The siRNA duplex was re-suspended in 1× universal buffer, provided by Dharmacon Research Laboratory. The targeted cDNA sequence (AAAAAGAAGAGATTGAACGCC) corresponds to aa 509–511 (nucleotide 1529–1549). The corresponding sense and antisense siRNA sequences are AAAGAAGAGAUUGAACGCCdTdT and GGCGUUCAAUCUCUUCUUUdTdT, respectively. The sequence of the scrambled siRNA in the sense and antisense directions are GUAACUGCAACGAUUUCGAUGdTdT and CAUCGAAAUCGUUGCAGUUACdTdT, respectively. Transfection of siRNA duplexes were performed using Transmessenger Transfection Reagent kit (Qiagen) and assay for silencing 48 h after transfection.

2.9. Human PKCα siRNA preparation

In this study, we prepared siRNA targeting human PKCα. We chose aa 450–456 (nucleotide1348–1366, designated as # 8) and aa 654–660 (nucleotide 1960–1978, designated as # 9) starting from 1 AUG codon in the open reading frame in the C-terminal region of PKCα as the target region to design for siRNA. The selected siRNA sequence was blast-search (NCBI database) against EST libraries, to ensure that only one gene is targeted. Chemically synthesized siRNA duplex in the 2′ de-protected and desalted form was purchased from Dharmacon Research. A 21 nucleotide long non-silencing siRNA duplex was used as a control. The siRNA duplex was resuspended in 1× universal buffer, provided by Dharmacon Research Laboratory. The targeted cDNA sequence for # 8 (GGATTGTTCTTTCTTCATA) corresponds to nt 1348–1366 and for # 9 (GAAGGGTTCTCGTATGTCA) corresponds to nt 1960–1978. The corresponding sense and antisense siRNA sequences for # 8 are GGAUUGUUCUUUCUUCAUAdTdT and UAUGAAGAAAGAACAAUCCdTdT, for # 9 are GAAGGGUUCUCGUAUGUCAdTdT and UGACAUACGAGAACCCUUCdTdT, respectively. The sequence of the non-silencing control siRNA in the sense and antisense directions are UUCUCCGAACGUGUCACGUdTdT and ACGUGACACGUUCGGAGAAdTdT, respectively. Transfection of siRNA duplexes was performed using Transmessenger Transfection Reagent kit (Qiagen) and assay for silencing 48 h after transfection.

3. Results and discussion

3.1. Effect of deletion of PKC-phosphorylation sites of RLIP76 in SCLC and NSCLC

In order to investigate differential phosphorylation of RLIP76 by PKCα in the intact SCLC and NSCLC cells, we devised a scheme for depleting wild-type RLIP76 in both cell types after transfection with either empty vector, full length RLIP76 or with RLIP76$^{delPKC\alpha\text{-sites}}$, a construct in which the four potential PKCα phosphorylation sites have been deleted. The siRNA siRLIP76$^{1529–1549}$ used to deplete wild-type RLIP76 was reconstituted in the Transmessenger Transfection Reagent Kit (Qiagen) and added to cells for 3 h, then washed off. After 48 h incubation, remaining cells were harvested and RLIP76 was purified, quantified by ELISA, and reconstituted into artificial liposomes for measurement of ATP-dependent DOX-transport activity. Proteoliposomes were pre-incubated without or with PKCα and 1 mM ATP for 30 min prior to transport measurement. RLIP76 purified from SCLC (H1618) had half the specific activity of that purified from NSCLC (H358). Pre-incubation with PKCα, stimulated the activity of RLIP76 purified from either NSCLC or SCLC. Depletion of RLIP76 using siRLIP76$^{1529–1549}$ resulted in extensive apoptosis in cells transfected with either empty vector, or with full length RLIP76 and no RLIP76 could be purified (Fig. 1). RLIP76 purified from cells transfected with RLIP76$^{delPKC\alpha\text{-sites}}$ without treatment with siRLIP76$^{1529–1549}$ (consisting of both the wild-type and mutant protein) had a slightly lower specific activity and blunted stimulation of activity by PKCα. The specific activity of RLIP76 purified from cells transfected with RLIP76$^{delPKC\alpha\text{-sites}}$ and treated with siRLIP76$^{1529–1549}$ (to deplete wild-type RLIP76) was lower and not significantly affected by PKCα. Remarkably, when wild-type RLIP76 was depleted leaving only the mutant protein, we did not observe apoptosis, indicating that the activity of this mutant, though quite low, is adequate to prevent apoptosis under ambient culture conditions. Most importantly, purified protein from both SCLC and NSCLC expressing exclusively RLIP76$^{delPKC\alpha\text{-sites}}$ (after treatment with siRLIP76$^{1529–1549}$) had roughly equal specific activity, certainly much lower than that in wild-type NSCLC, and unaffected by PKCα (Fig. 1). These findings strongly indicated that PKCα mediated phosphorylation of RLIP76 in NSCLC, and lack thereof in SCLC, is a major contributor to the differences in specific activity of this protein between SCLC and NSCLC.

3.2. Effect of PKCα-depletion on DOX-transport activity of RLIP76 from SCLC and NSCLC

The above postulate predicts that depletion of PKCα should have similar effects on the specific activity of RLIP76 as did the

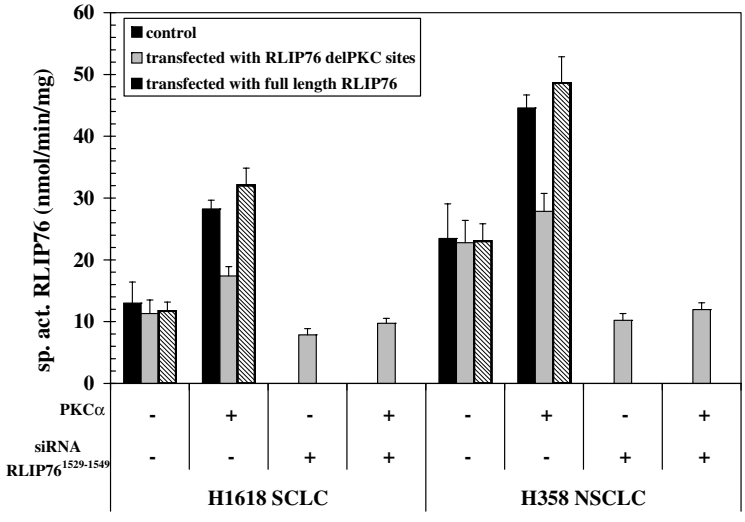

Fig. 1. Effects on the specific activity for DOX-transport of expression of PKCα phosphorylation sites deletion mutant RLIP76 in SCLC and NSCLC cell lines. RLIP76 was purified from H1618 SCLC and H358 NSCLC cells transfected with either vector alone (black bar), full length RLIP76 (hatched bars) or RLIP76$^{delPKC\alpha\text{-sites}}$ (gray bars), treated for 3 h with either siRNA RLIP76$^{1529–1549}$ (which should selectively deplete wild-type RLIP76, and not RLIP76$^{delPKC\alpha\text{-sites}}$) or corresponding scrambled control siRNA, followed by harvesting cells at 48 h for protein purification. Specific activity shown from ($n = 12$). DOX-transport activities obtained from studies in artificial liposomes into which each protein was reconstituted separately. Missing black or hatched bars are for the control cells and those cells transfected with wild-type full length RLIP76, all of which undergo apoptosis upon depletion of wild-type RLIP76, and no RLIP76 protein can be purified.

expression of a PKCα-phosphorylation deficient mutant. To test this postulate, we purified and measured DOX-transport activity of RLIP76 from SCLC and NSCLC cell lines subject to pre-treatment with siRNA for depletion of PKCα. Depletion of PKCα by siRNA # 8 and # 9 (Fig. 2A and B, respectively) was accomplished using a Qiagen Transmessenger Transfection reagent kit. PKCα level was generally higher in NSCLC as compared with SCLC, and complete depletion was demonstrated for the three SCLC and four NSCLC cell lines within 48 h by Western blot analyses (Fig. 2, panels A and B). Purified RLIP76 from cells treated either with PKCα siRNA # 8 or control scrambled siRNA was reconstituted into artificial liposomes and specific activity towards DOX-transport was measured. After PKCα-depletion, the specific activity of RLIP76 from all three SCLC and four NSCLC cell lines subjected to PKCα depletion were indistinguishable (Fig. 3A), validating the assertion that the higher specific activity of RLIP76 in NSCLC is largely due to greater activity of PKCα in NSCLC.

3.3. The effect of PKCα depletion on DOX-cytotoxicity in SCLC and NSCLC

To see how this difference in specific activity of RLIP76 translated into effects on cell growth as well as DOX-cytotoxicity, we compared cell growth and IC_{50} of DOX between cells treated with PKCα siRNA # 8 or scrambled siRNA. PKCα-depletion caused decrease in cell growth in SCLC by 43 ± 5% ($n = 3$), whereas in NSCLC, this effect was ~70 ± 3% ($n = 4$) (Fig. 3B).The effect of PKCα-depletion on DOX-cytotoxicity was much more marked in NSCLC as compared with SCLC, as DOX-resistance of NSCLC was reduced to the level found in SCLC (Fig. 3C). PKCα depletion in SCLC sensitized DOX only slightly, presumably though other pathways regulated by PKCα. Taken together, these studies showed that PKCα is a primary determinant of the specific activity of RLIP76, and that DOX-resistance correlates well with the specific activity for DOX-transport by RLIP76.

3.4. Requirement of RLIP76 for PKCα mediated DOX-resistance

If DOX-resistance conferred by PKCα was mediated primarily through phosphorylation of RLIP76, PKCα-depletion should not sensitize cells lacking RLIP76 to DOX. We thus compared the effects of PKCα depletion on cell growth and DOX-cytotoxicity in $RLIP76^{+/+}$ vs. $RLIP76^{-/-}$ MEFs. PKCα-depletion inhibited the growth of $RLIP76^{+/+}$ MEFs 43 ± 11%, but $RLIP76^{-/-}$ MEFs were unaffected (Fig. 4A).

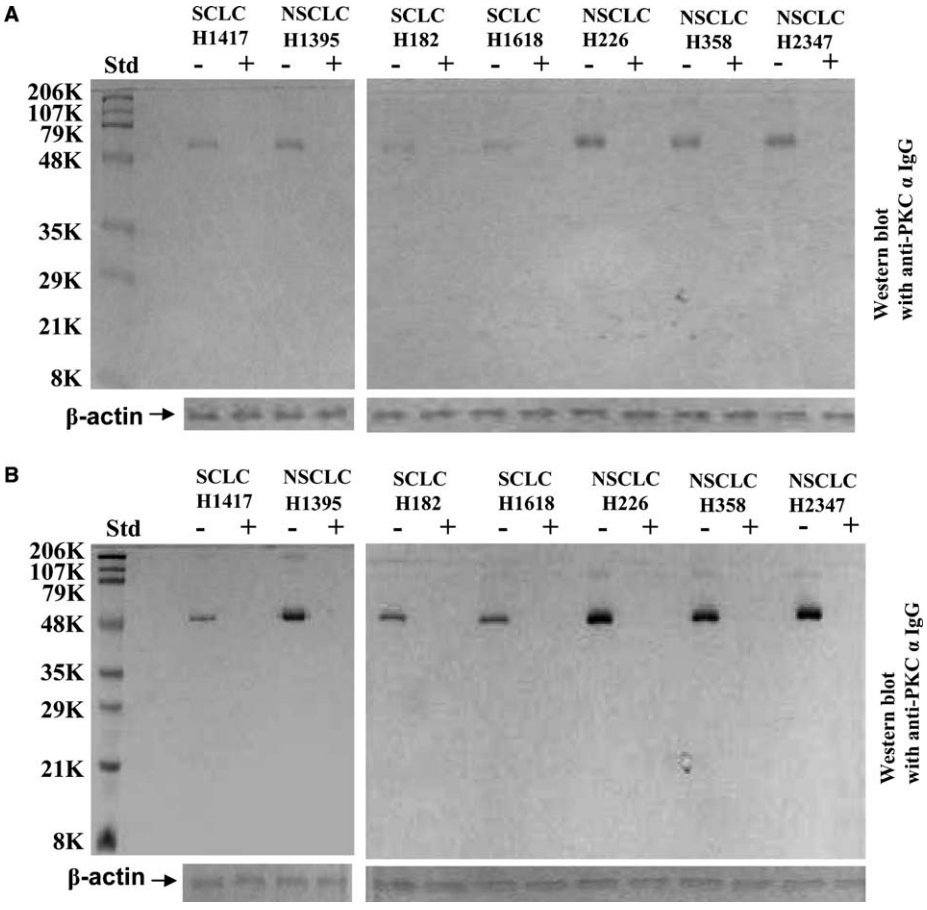

Fig. 2. Depletion of PKCα in SCLC and NSCLC by siRNA. H182, H1417 and H1618 SCLC and H226, H358, H1395 and H2347 NSCLC cell lines were treated with PKCα-siRNA # 8 (+) or corresponding scrambled siRNA (–) (panel A) and PKCα-siRNA # 9 (+) or corresponding scrambled siRNA (–) (panel B) (Dharmacon) using Transmessenger Transfection Reagent (Qiagen) for 3 h followed by washing with PBS. Cells were then allowed to grow in fresh complete medium for 48 h before preparation of cell homogenate for Western blotting. Protein (100 μg/lane) from crude homogenate from all cell lines was applied to SDS–PAGE, immuno-blotted to nitrocellulose membranes, and Western blot analyses were performed using rabbit-anti-human-PKCα antibodies (Biosource, CA) as primary antibody, and HRP conjugated goat-anti-rabbit secondary antibody. Blots were developed using the chromogenic substrate, 4-chloro-1-naphthol. β-actin was used as loading control.

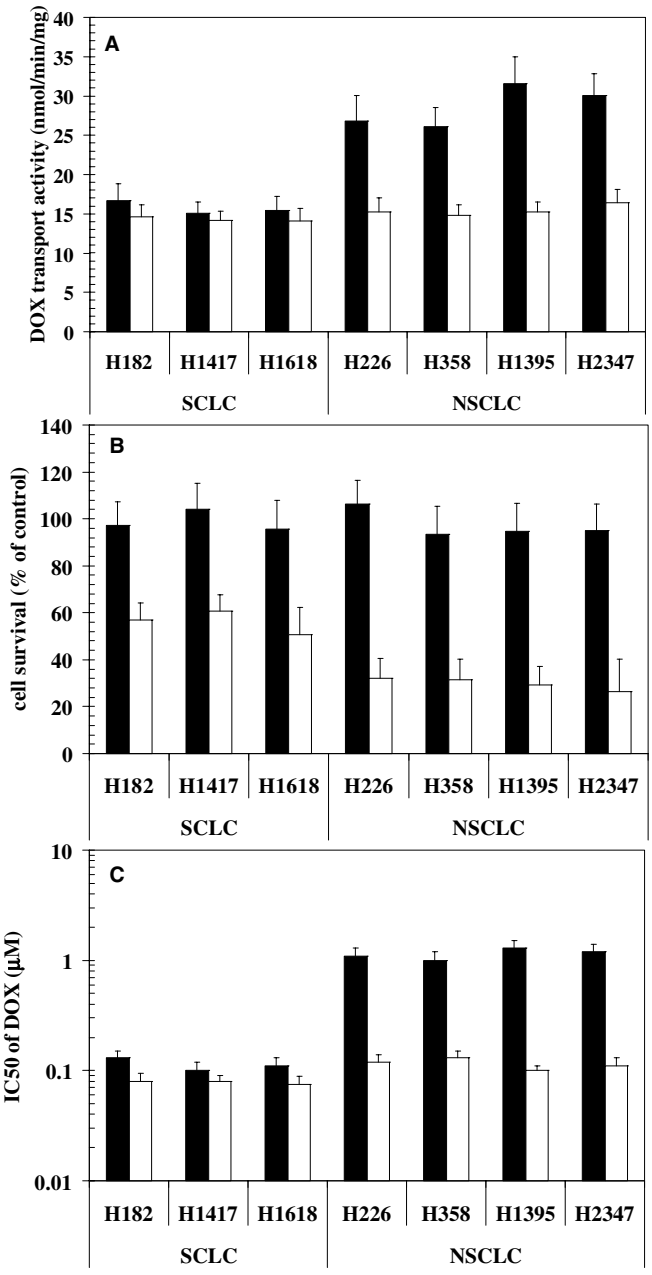

Fig. 3. Effect of PKCα-depletion on cell survival, RLIP76 activity and DOX IC_{50} in SCLC and NSCLC H182, H1417 and H1618 SCLC and H226, H358, H1395 and H2347 NSCLC cell lines were treated with PKCα-siRNA # 8 (open bars) or corresponding scrambled siRNA (filled bars) (Dharmacon) using Transmessenger Transfection Reagent (Qiagen) for 3 h followed by washing with PBS. Cells were then allowed to grow in fresh complete medium for 48 h prior to preparation of cell homogenate from which RLIP76 was purified to homogeneity using dinitrophenyl *S*-glutathione affinity chromatography as previously described [6]. Purified RLIP76 fractions were reconstituted into artificial cholesterol:asolectin liposomes as previously described [9] and specific activity of DOX-transport was measured using ^{14}C-DOX (sp. act. 8.5×10^4 cpm/nmol) (panel A). For cell growth studies in the absence of DOX, cells were treated with PKCα siRNA # 8 or scrambled siRNA in the same manner as for panel A, followed by inoculation of cells at 2×10^4 cell/well into 96 well plates. Viable cell density was determined at 96 h by addition of MTT (panel B). For DOX-cytotoxicity studies, the same procedure was followed as in panel B, but DOX 0.01 to 2 μM was included in the 96 well plate (eight replicates per concentration). The IC_{50} values were obtained from fitting a modified Hill equation to data obtained from MTT assay (panel C). The values presented in all panels are means and S.D. from three experiments each. Control siRNA treated (filled bars); PKCα siRNA treated (open bars).

Furthermore, RLIP76$^{-/-}$ MEFs were clearly an order of magnitude more sensitive to DOX as compared with the wild-type RLIP76$^{+/+}$ MEFs (Fig. 4B and C). Though PKCα-depletion clearly sensitized the RLIP76$^{+/+}$ MEFs to DOX, this sensitization was not to the level of that seen with RLIP76$^{-/-}$ MEFs. Most remarkably, PKCα-depletion had no effect on the cytotoxicity of DOX in RLIP76$^{-/-}$ MEFs, indicating that RLIP76 is required to see the DOX-sensitizing effect of PKCα-depletion.

Results of present studies demonstrate that RLIP76 is a primary determinant of DOX-sensitivity, and that the DOX-resistance mediating effects of PKCα are mediated primarily through phosphorylation and stimulation of RLIP76 transport activity. These findings offer a novel explanation for the previ-

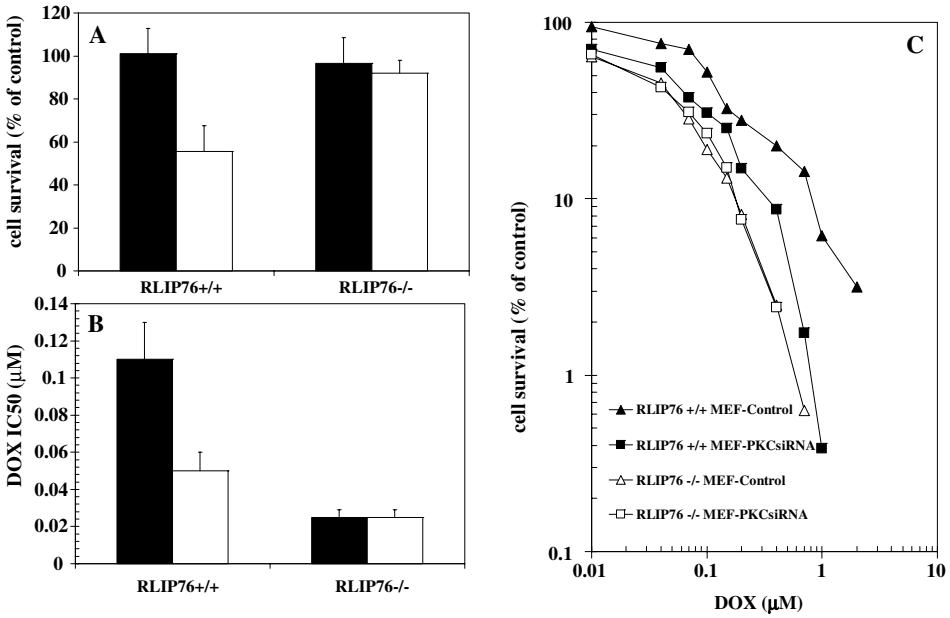

Fig. 4. Effect of PKCα-depletion on cell survival and DOX IC_{50} in $RLIP76^{+/+}$ and $RLIP76^{-/-}$ MEF cells. MEFs were cultured from the fetus at 12–13 day gestation as previously described [18]. For cell growth studies in the absence of DOX, cells were treated with PKCα siRNA # 8 (open bars) or scrambled siRNA (filled bars) in the same manner as for Fig. 3 panel A, followed by inoculation of cells at 2×10^4 cell/well into 96 well plates. Viable cell density was determined at 96 h by addition of MTT (panel A). For DOX-cytotoxicity studies, the same procedure was followed as in Fig. 3 panel C, DOX 0.01 to 2 μM was included in the 96 well plate (eight replicates per concentration). The IC_{50} values were obtained from fitting a modified Hill equation to data obtained from MTT assay (panel B). The cytotoxicity curves for $RLIP76^{+/+}$ and $RLIP76^{-/-}$ MEFs treated either with scrambled or PKCα-siRNA # 8 are presented (panel C). The values presented in all panels are means and S.D. from three experiments each. Control siRNA treated (filled bars); PKCα siRNA treated (open bars).

ously unexplained observation of DOX-accumulation defects in PKC-overexpressing cells, and support previous studies in which PKC activity has been proposed as a major determinant of the inherent DOX-accumulation defect and resistance of NSCLC [20]. Since RLIP76 is also involved in other signaling pathways, our findings do not rule out the possibility that PKCα-depletion interferes with other signaling functions of RLIP76 in the Ral and Rho/Rac/cdc42 pathways [7]. The closely related roles of PKC and RLIP76 are supported by studies showing that modulation of membrane structure and plasticity, the Ras/Ral-GEF/Ral pathway, and Rho-family G-proteins that regulate xenobiotic metabolism and stress–response are directly affected by both PKC and RLIP76 [7].

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