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Increased Expression of Cdc2 Inhibits Transport Function of RLIP76 and Promotes Apoptosis

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Abstract

RLIP76 is a stress-responsive glutathione-electrophile-conjugates (GS-E) and drugs transporter which is over-expressed in different types of cancers. Cdc2 is a cell-cycle check point control kinase which has been shown to bind to RLIP76 during mitosis, such that endocytosis is inhibited. In present studies, we have purified cdc2 and examined its effect on the transport-activity of RLIP76 reconstituted into artificial liposomes. Both doxorubicin (DOX) and dinitro-phenyl S-glutathione (DNP-SG) transport were inhibited by cdc2 in a concentration dependent manner. Liposomal delivery of cdc2 to H358 cells caused apoptosis, resulted in an increased intracellular doxorubicin-accumulation and decreased rate of efflux from the cells. In the present communication, we propose that the accumulation-deficient drug-resistance mediated by RLIP76 can be modulated by inhibition of RLIP76 transport-activity by cdc2.

Keywords

RLIP76; Cdc2; apoptosis; glutathione-conjugate; doxorubicin; transport-inhibition; drug-resistance

1. Introduction

An incomplete picture of transport mechanisms responsible for drug-resistance due to accumulation-defects has led to a wide array of opinions due to inconsistent or conflicting data regarding the relative significance of a multitude of transport mechanisms. Animal and human clinical data indicate that the ABC-transporters P-glycoprotein (Pgp), MRP1 and related transporters are clearly able to mediate drug-accumulation defects in cultured malignant cells, but correlations with pathology, clinical resistance and outcomes in lung cancer are poor, and attempts at improving therapeutic efficacy by targeting these have not been successful [1,2]. Our findings have supplied a missing piece of the puzzle to the understanding of multi-specific transport mechanisms, a stress-responsive non-ABC, high capacity transporter, which must have had significant confounding effect in studies of ABC-transporters. Taken together with studies by others [3–6] linking RLIP76 with key stress-response, cell-cycling, and endocytosis

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proteins, a novel integrated signaling model emerges in which RLIP76 plays a key effector role in different signaling pathways through its GS-E transport activity [7–12].

RLIP76, is a protein cloned previously by others as a Ral-effector and Ral-GAP that bridged the Ras and Ral pathways, and displayed GAP activity towards Rho/Rac G-proteins [3–5]. Concomitantly, recent studies by other investigators indicate that RLIP76 plays a crucial role in clathrin-coated pit mediated receptor/ligand pair endocytosis, particularly as related to TGF- β , EGF, and insulin [3–6,13,14]. Other investigators have shown that binding of cdc2 to RLIP76 is essential to shut off endocytosis during mitosis [6]. A direct implication of our findings is that the execution of endocytosis, mitosis, and apoptosis is regulated by signaling pathways such as Ral, Ras, Rho/Rac and cdc2 by regulation of the rate of efflux of physiological pro-apoptotic GS-E by RLIP76, which couples ATP-hydrolysis, with GS-E transport [6–8]. Additional results by other investigators have also shown that dissociation of RLIP76 from membrane upon binding with cdc2 results in its translocation to the mitotic spindle where it is purported to function as a motor for spindle movement [6,15]. These studies suggest a more general hypothesis that RLIP76 functions as a modular ATPase that provides energy to different cellular protein by binding to different adaptor proteins.

The role of RLIP76 in intracellular signaling, through removal of intracellular GS-E, appears to be as a signal terminating mechanism. Recently, we have shown that POB1 binds to and inhibits the transport activity of RLIP76, and enhances DOX accumulation and cytotoxicity as well as inhibition of endocytosis [12]. The central importance of RLIP76 in critical cancer-related signaling pathways is emphasized by recent findings that the termination of endocytosis during mitosis occurs as a result of formation of a complex between RLIP76, cdc2 and cyclin B1. This complex results in dissociation of RLIP76 from the membrane and its translocation to the mitotic spindle where it may play a role as a molecular motor for spindle movement during anaphase [6].

Cdc2 (cdk1), a catalytic subunit of protein kinase complex, M-phase promoting factor (MPF) has critical regulatory functions during mitosis as well as in apoptosis [6,16]. These properties of cdc2 are of potential interest, because it has recently been shown to bind with RLIP76, regulate endocytosis, and translocate RLIP76 to the mitotic spindle, where it may bind to sites known to bind ATPase which provide energy for this process. Cdc2 is known to be phosphorylated by PKC as well as tyrosine-kinase, which is important for its catalytic activity [6,17]. The RLIP76-binding domain of cdc2 has not been identified, but it is known to bind to RLIP76 at aa 481–625 [6]. Our present studies have demonstrated that cdc2 inhibits RLIP76 transport activity in concentration dependent manner and enhances DOX accumulation and cytotoxicity. In summary, our findings indicate that RLIP76 is a common effector protein for regulating cellular GS-E levels, and that multiple signaling proteins may regulate cell proliferation, differentiation, motility, and apoptosis in part through modulating RLIP76 transport activity.

2. Materials and methods

2.1. Materials

^{14}C -DOX (specific activity 44.8 Ci/mmol) was purchased from NEN Life Sciences (Boston, MA). Polyclonal anti-cdc2 IgG was purchased from the EMD Biosciences (San Diego, CA). Polyclonal rabbit-anti-human rec-RLIP76 IgG as well as pre-immune IgG were prepared and purified as described previously [7]. FITC-labeled Annexin V conjugate and TUNEL fluorescence detection kit were purchased from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively.

2.2. Cell lines and Cultures

Human NSCLC H358 (bronchioalveolar) from ATCC (Manassas, VA), was used in these studies. Cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂ in RPMI-1640 medium supplemented with 10 % (v/v) heat-inactivated FBS and 1 % (v/v) P/S solution.

2.3. Cloning, Prokaryotic Expression, and Purification of cdc2

The 925 bp full-length cDNA of cdc2 was a gift from Dr. van den Heuvel, Massachusetts General Hospital Cancer Center, MA. Techniques for restriction enzyme digestion, ligation, transformation and other standard molecular biology manipulations were based on methods described by Sambrook et al [18]. The sequence of the cdc2 was confirmed by DNA sequencing. Following verification of the sequence of cdc2, the pET30a(+) plasmid containing the full-length cdc2 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. Rec-cdc2 was purified by metal-affinity chromatography over Ni-NTA super-flow resin (Qiagen) with slight modifications as described previously for POB1 [12].

2.4. Cloning, Prokaryotic Expression, and Purification of RLIP76

Purified RLIP76 protein (1965 bp; 655 aa) was obtained from *E. coli* BL21(DE3) expressing the pET30a(+) plasmid containing full-length cDNA corresponding to the sequence of RLIP76. The purification was carried out using DNPSG-affinity resin as described previously and purity was confirmed by SDS-PAGE and Western blot analyses [7].

2.5. Functional reconstitution of purified rec-RLIP76 into artificial liposomes and transport studies

Purified RLIP76 was dialyzed against reconstitution buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 1 mM EGTA, 100 mM KCl, 40 mM sucrose, 2.8 mM BME, 0.05 mM BHT, and 0.025% polidocanol) and reconstituted into artificial liposomes according to our published method [7]. ATP-dependent transport of ¹⁴C-DOX and ³H-DNPSG in the rec-RLIP76 proteoliposomes was performed by rapid-filtration technique using the protocol described by us [7]. Functional reconstitution of purified rec-cdc2 protein into artificial-liposomes was also performed in a manner similar to rec-RLIP76. Efficiency of delivery for proteoliposomes has been established previously [12].

2.6. Transport of ¹⁴C-DOX and ³H-DNPSG by RLIP76 and its Inhibition by cdc2

For these experiments, fixed amount of purified rec-RLIP76 (250 ng) was reconstituted into proteoliposomes along with varying amounts (0–300 ng) of cdc2, and transport of ¹⁴C-DOX and ³H-DNPSG were measured. In one control, cdc2 protein was excluded while equivalent amount of BSA was reconstituted in liposomes (i.e. control liposomes), and transport of ¹⁴C-DOX and ³H-DNPSG was measured by using a 96 well-plate filtration manifold to separate the extra-vesicular drug from that taken up by the vesicles. Uptake was measured in parallel in RLIP76-proteoliposomes and control liposomes, in absence or presence of 4 mM ATP at a fixed time point of 5 min, at 37 °C.

2.7. Effect of cdc2 on apoptosis by FITC-labeled annexin V conjugate assay

H358 cells (1 × 10⁵ cells/ml) were grown on cover slips. The cells were treated with control or cdc2-proteoliposomes (40 µg/ml final conc.). After 24 h incubation, the medium was removed and cells were washed with cold PBS. Cells were treated with 100 µl of annexin-binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂, pH 7.4) containing 10 µl of annexin V conjugate and incubated for 15 min at room temperature. The cells were washed with the annexin-binding buffer and mounted on the slides using Vectashield mounting media.

Fluorescence micrographs were taken using Zeiss LSM 510 META (Germany) laser scanning fluorescence microscope at 400 × magnification.

2.8. Effect of cdc2 on apoptosis by TUNEL assay

H358 cells (1×10^5 cells/ml) were grown on cover slips. The cells were treated with control or cdc2-proteoliposomes (40 µg/ml final conc.). After 24 h incubation, the medium was removed, and cells were washed with PBS. TdT-mediated dUTP nick end labeling (TUNEL) assay was performed using Promega Fluorescence detection kit [12]. Slides were analyzed under a fluorescence microscope using a standard fluorescein filter set to view the green fluorescence at 520 nm and red fluorescence of propidium iodide at > 620 nm. Fluorescence micrographs were taken using Zeiss LSM 510 META laser scanning fluorescence microscope at 400 × magnification.

2.9. Effect of cdc2-liposomes on ^{14}C -DOX accumulation and efflux

Drug accumulation and efflux studies were performed in H358 cells treated with either control or cdc2-proteoliposomes (40 µg/ml), according to the protocol described by us [9]. The back-added curves of cellular residual DOX vs. time were constructed as described previously [9].

2.10. Drug-Sensitivity Assay

Cell density during the log phase was determined by counting trypan blue excluding cells in a hemacytometer, and 20,000 cells were plated into each well of 96 well flat-bottomed micro-titer plates. After 24 hours of incubation, the cells were treated with control or cdc2-proteoliposomes (final conc. 40 µg/ml). DOX was added and IC_{50} was measured by performing MTT-assay 96 h later as described previously [9]. Eight-replicate wells were used for each point in each of three separate measurements of IC_{50} .

2.11. Colony-Forming Assay

H358 cells (1×10^5 cells/500 µL) were incubated with control or cdc2-proteoliposomes (40 µg/ml final conc.) for 24 h and then aliquots of 50 and 100 µL in 60 mm size Petri-dishes, separately, in a total volume of 4 ml of medium. After 10 days, control and cdc2-proteoliposomes treated cells were stained with methylene-blue for 30 min. and colonies were counted using Innotech Alpha-Imager HP [12].

2.12. Statistical Analysis

All data were evaluated with a two-tailed unpaired student's t test or compared by one-way ANOVA and are expressed as the mean $\pm$ SD. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification of recombinant RLIP76 and cdc2

Recombinant human RLIP76 protein was purified by DNPSG-affinity chromatography [7]. The SDS-PAGE and Western blot against anti-RLIP76 IgG demonstrated largely intact and pure protein at 95 kDa position, confirmed by N-terminal sequencing. In contrast, 38 kDa peptide band, is a truncated C-terminal peptide, could also be detected in SDS-PAGE and this 38 kDa band was recognized by anti-RLIP76 antibodies in Western blot (Fig. 1A and 1B). Ni-NTA affinity purified cdc2 was similarly examined, found free of significant impurities by SDS-PAGE, and recognition by the specific antibodies demonstrated the identity of the purified protein. The purified cdc2 protein was seen in both SDS-PAGE and Western-blot at the expected molecular weight of 32 kDa (Fig. 1C and 1D). No significant contaminant protein

bands were observed in SDS-PAGE and the pattern of bands in Western blot was identical to that seen in SDS-PAGE (Fig. 1).

3.2. Cdc2 inhibits the DOX and DNPSG transport activity of purified rec-RLIP76 in artificial-liposomes

Other investigators have reported specific binding of cdc2 to RLIP76 [6], but any effect of this binding on RLIP76 transport-activity was not known. In previous studies, we had demonstrated that POB1 and Hsf-1 inhibit the DOX transport activity of RLIP76 in a saturable manner and that a maximum of about 50 and 40 % inhibition was achievable with POB1 and Hsf-1, respectively [12]. Because cdc2 has been shown to bind RLIP76 in a similar region (aa 481–625), we hypothesized that cdc2 would also inhibit the transport activity of RLIP76. To test this postulate, we used purified rec-RLIP76 reconstituted into artificial asolectin:cholesterol liposomes for measurement of ATP-dependent transport activity using ^{14}C -DOX and ^3H -DNPSG, without or with addition of purified cdc2 protein to the transport medium. ^{14}C -DOX and ^3H -DNPSG uptake into the proteoliposomes in the absence of ATP was subtracted from that in the presence of ATP to obtain ATP-dependent uptake. Increasing ratio of albumin added to the transport reaction did not affect ATP-dependent ^{14}C -DOX and ^3H -DNPSG transport catalyzed by RLIP76. In contrast, cdc2 caused a saturable inhibition of ^{14}C -DOX and ^3H -DNPSG transport with maximal inhibition of about 35 % (Fig. 2A–D). The nature of transport inhibition is consistent with distinct but perhaps overlapping binding sites for POB1 (aa 499–655), Hsf-1 (aa 440–655) and cdc2 (aa 481–625), all being present in the C-terminal region [6,8]. These striking findings predict that simultaneous augmentation of POB1, Hsf-1 and cdc2 in cells would very effectively inhibit RLIP76 transport activity in cells.

3.3. Effect of cdc2 on accumulation, efflux, and cytotoxicity of DOX in H358 NSCLC

Because inhibition or depletion of RLIP76 has been shown previously to selectively target certain cancer cell types for apoptosis and sensitize them to chemotherapy drug-toxicity [10, 11,19], the present observations suggested that augmenting the level of cdc2 could increase the efficacy of chemotherapeutic drugs by increasing cellular accumulation through inhibited efflux. We tested for this possibility using the well characterized H358 cell line that is typical for other lung cancer cell lines and is relatively resistant to DOX [20]. H358 cells were incubated with ^{14}C -DOX for varying time intervals and drug-uptake in cells was quantified. Cells were pre-treated for 24 h with control or cdc2-proteoliposomes. Result of these drug accumulation studies showed that the uptake was increased substantially when cdc2 protein was added (Fig. 3A). To confirm that the increased accumulation was due to inhibited transport, drug efflux studies were carried out in cells pre-treated in the same manner. The drug efflux curve was steepest with control liposomes, affected markedly slowed when cdc2 protein was present (Fig. 3B). The decreased efflux and increased accumulation caused by cdc2, translated into increased sensitivity to DOX in MTT cytotoxicity assay done at 96 h after drug exposure (Fig. 3C). Taken together, these findings demonstrated that cdc2 is effective inhibitor of DOX efflux in these cells known to express RLIP76, and that the greater drug accumulation translates to greater cytotoxicity.

3.4. Effect of cdc2 on apoptosis and colony-forming activity

In previous studies, we have demonstrated that RLIP76 inhibition, in the absence of cytotoxic drugs, causes apoptosis in lung and several other types of cancer cells, while sparing non-malignant cell types [10]. The mechanism of apoptosis has been postulated to be through the direct toxicity of lipid-hydroperoxide metabolites that accumulate upon the inhibition of RLIP76, as well as through effects on stress-defense pathway proteins including JNK and AP1, and that apoptosis proceeds through a caspase dependent mechanism [21]. In present studies, we tested the effects of cdc2 in causing apoptosis as determined by appearance of annexin V

on the cell surface (an early event in apoptosis), and by the TUNEL assay which reports the appearance of DNA-fragmentation (a late event). Cdc2 caused the appearance of apoptosis by both assays (Fig. 4A and B). Results of Annexin V and TUNEL studies demonstrated that cdc2-proteoliposomes did cause apoptosis whereas the control liposomes caused no apoptosis. These studies show that RLIP76 is required for the majority of total apoptotic effect of cdc2, and in context of the effect of cdc2 on transport activity, these findings are most consistent with the idea that the apoptotic activity of cdc2 is mediated through its effects on inhibition of transport. Because of the relatively subjective nature of such measurements, we quantified these effects by colony forming assays on cells treated with no liposomes, control liposomes or cdc2-proteoliposomes. The control-liposomes did not significantly affect colony forming capacity, in contrast, cdc2-proteoliposomes had a significant ($P < 0.005$) effect (~ 40 % reduction in colony formation) as compared with controls (Fig. 4C).

4. Discussion

Present studies demonstrate for the first time that cdc2 inhibits the transport activity of RLIP76. Taken in context of known functions and well defined binding interactions of RLIP76 with cdc2, these findings have particular significance with respect to the view of the signaling mechanisms involved. RLIP76 is found both in the cytosol and in the plasma membrane, and that specific cdc2-binding to RLIP76 in membrane fraction inhibits the transport activity of RLIP76. The consequences of this inhibition were demonstrated clearly in terms of drug efflux, drug accumulation, cytotoxicity, apoptosis and colony-forming activity. These data suggest that cdc2 interactions with RLIP76 may also function to modulate RLIP76 activity. Studies in other's labs and our, indicate that stable transfection and constitutive expression of cdc2 is not possible in cancer cell lines because of apoptosis caused by cdc2 over-expression [16]. In the H358 cells, augmentation of cdc2 caused increased apoptosis. The occurrence of apoptosis were documented and quantified by immuno-histochemical TUNEL and annexin V assays (Fig. 4). These results show for the first time that cdc2 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 GS-E [12]. Relevance of these findings generally to cancer, and specifically to lung cancer drug resistance are based not only in observations by others of the seminal roles of RLIP76 interacting pathways in carcinogenesis and cancer pharmacology, but also because of the relatively promiscuous nature of RLIP76 with respect to substrate specificity such that numerous amphiphilic chemotherapy drugs can function as transported substrates, as well as competitive inhibitors of its physiological function, GS-E efflux.

Our recent studies showing significantly higher expression of RLIP76 in cancer cells as compared with normal cells, and a good correlation between RLIP76 expression, total transport activity and resistance to DOX, and relative sensitivity to apoptosis triggered by RLIP76 depletion through siRNA or antisense, suggest that there may be sufficient cancer-specific expression and cancer-specific toxicity to warrant consideration of RLIP76 as a therapeutic target for cancer therapy [10,11]. Identification of a cell surface domain (aa¹⁷¹⁻¹⁸⁵) which, when targeted at the cell surface results in inhibition of transport activity, increased cellular drug-accumulation, and apoptosis, may considerably ease the task of development of specific small molecule inhibitors through either molecular modeling or library screening for ligand binding this epitope. Cdc2 and RLIP76 are involved in clathrin coated pit-mediated receptor/ligand endocytosis, a mechanism necessary to terminate signaling initiated by binding of various ligands including TGF, EGF and insulin to their corresponding receptors [6,8]. Cdc2 confers drug accumulation and sensitivity through inhibiting the transport activity of RLIP76. Present studies have demonstrated that inhibitory effects of cdc2 on RLIP76 transport activity translates to increase in apoptosis, drug accumulation, drug sensitivity and decrease in drug efflux. The marked apoptotic effect of cdc2 augmentation in lung cancer cells suggests a novel

targeted therapy in which liposomally encapsulated cdc2 could be used clinically as a therapeutic agent.

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Abbreviations

RLIP76 (RALBP1)	Ral-interacting protein
POB1	partner of RLIP76
DOX	doxorubicin
GSH	glutathione
GS-E	glutathione-electrophile conjugates
NSCLC	non-small cell lung cancer

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Figure 1. Purification of RLIP76 and cdc2 from transformed *E. coli*
 DNPSG-affinity purified rec-RLIP76 (5 µg) (**panels A and B**) and Ni-NTA Super-flow resin (Qiagen) purified rec-cdc2 (5 µg) (**panels C and D**) were applied to SDS-PAGE (**panels A and C**) and subjected to Western-blot analyses (**panels B and D**) against anti-RLIP76 IgG and anti-cdc2 IgG as primary antibody. SDS-PAGE was stained with Coomassie Brilliant Blue-R250 and Western-blot were developed using horseradish peroxidase-conjugated goat-anti-rabbit-IgG as secondary antibody.

Figure 2. Inhibition of DOX and DNP-SG transport in RLIP76-proteoliposomes by cdc2
 ^{14}C -DOX (panels A and B) and ^3H -DNPSG (panels C and D) transport activity was measured as ATP-dependent DOX and DNP-SG uptake in artificial liposomes reconstituted with purified rec-RLIP76. Control included liposomes prepared without RLIP76. Purified cdc2 protein was added in varying ratio to the transport reaction mixture. Results presented are representative from three separate experiments, each with triplicate determinations. •, control-liposomes treatment; ■, cdc2-proteoliposomes treatment; * $p < 0.005$ compared with control-liposomes treatment

Figure 3. Effect of cdc2 supplementation on uptake, efflux and DOX-cytotoxicity

H358 NSCLC cells were treated with control-liposomes (green circles) and proteoliposomes containing 40 $\mu\text{g/ml}$ cdc2 (red squares). Uptake of ^{14}C -DOX (sp. act. 8.9×10^4 cpm/nmol) by cells was determined by incubating the cells for varying periods of time with radio-labeled ^{14}C -DOX, and measuring radio-activity in the cell pellet (**panel A**). For efflux studies, cells were loaded with ^{14}C -DOX for 60 min, followed by rapid dilution in 1 ml buffer. Aliquots (50 μl) of the buffer were taken at 1 min intervals, and cellular drug content was calculated by back-addition from the residual DOX in cells at the end of the experiment (**panel B**). The cytotoxic effects of DOX were measured by MTT assay 96 h after drug-exposure (**panel C**).

* $p < 0.001$ compared between control-liposomes vs. cdc2-proteoliposomes treatment.

Figure 4. Effect of cdc2 on apoptosis and colony-forming efficiency in H358 NSCLC

The effect of cdc2 overloading on apoptosis was examined by TUNEL (**panel A**) and FITC-labeled Annexin V assay (**panels B**). The H358 cells grown on cover-slips incubated for 24 h with control and cdc2-proteoliposomes (40 $\mu\text{g}/\text{ml}$) prior to Annexin V or TUNEL assays and examined using Zeiss LSM 510 META (Germany) laser scanning fluorescence microscope with filters 520 nm and >620 nm. Photographs taken at identical exposure at $400\times$ magnification are presented. Apoptotic cells showed green fluorescence and characteristic of cell shrinkage. Colony-forming assay was performed by staining the cells with methylene blue and the colonies were counted using Innotech Alpha Imager HP (**panel C**). Values are means $\pm$ S.D. of three separate experiments. * $p < 0.005$ compared with no liposomes and control-liposomes treatment.