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RLIP76 IN DEFENSE OF RADIATION POISONING

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Abstract

Purpose—To determine the role of RLIP76 in providing protection from radiation and chemotherapy. In the present report, we used RLIP76 to refer to both the mouse (Ralbp1) and the human (RLIP76) 76-kDa splice variant proteins (RLIP76) for convenience and to avoid confusion. In other reports, Ralbp1 refers to the mouse enzyme (encoded by the Ralbp1 gene), which is structurally and functionally homologous to RLIP76, the human protein encoded by the human RALBP1 gene.

Methods and Materials—Median lethal dose studies were performed in RLIP76^{-/-} and RLIP76^{+/+} C57B mice after treatment with a single dose of RLIP76 liposomes 14 h after whole body radiation. The radiosensitivity of the cultured mouse embryonic fibroblasts and the effects of buthionine sulfoximine (BSO), amifostine, c-jun N-terminal kinase (JNK), protein kinase B (Akt), and MAPK/ERK kinase (MEK) were determined by colony-forming assays. Glutathione-linked enzyme activities were measured by spectrophotometric assays, glutathione by dithiobis-2-nitrobenzoic acid (DTNB), lipid hydroperoxides by iodometric titration, and aldehydes and metabolites by thiobarbituric acid reactive substances and liquid chromatography-mass spectrometry (LCMS).

Results—RLIP76^{-/-} mice were significantly more sensitive to radiation than were the wild-type, and RLIP76 liposomes prolonged survival in a dose-dependent manner in both genotypes. The levels of 4-hydroxynonenal and glutathione-conjugate of 4-hydroxynonenal were significantly increased in RLIP76^{-/-} tissues compared with RLIP76^{+/+}. RLIP76^{-/-} mouse embryonic fibroblasts were markedly more radiosensitive than RLIP76^{+/+} mouse embryonic fibroblasts, despite increased glutathione levels in the former. RLIP76 augmentation had a remarkably greater protective effect compared with amifostine. The magnitude of effects of RLIP76 loss on radiation sensitivity was greater than those caused by perturbations of JNK, MEK, or Akt, and the effects of RLIP76 loss could not be completely compensated for by modulating the levels of these signaling proteins.

Conclusion—The results of our study have shown that RLIP76 plays a central role in radiation resistance.

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Conflict of interest: S. Awasthi is founder, stakeholder, and chairman of the Scientific Advisory Board, Terapio, Inc.; J. Singhal, S.S. Singhal, S. Yadav, and Y.C. Awasthi are stakeholders in Terapio, Inc., which has licensed intellectual property regarding RLIP76.

Keywords

RLIP76; Ralbp1; Radiation-resistance; Embryonic fibroblasts

INTRODUCTION

Gene disruption of the mouse RLIP76 gene in C57B mice causes stepwise increases in tissue lipid peroxidation levels, as well as stepwise increases in radiation sensitivity (1). Because multiple functions have been attributed to RLIP76 (2-5), in the present study, we tested the hypothesis that RLIP76 functions to regulate cellular membrane lipid peroxidation-derived alkenals and their glutathione (GSH) conjugates through its activity as an adenosine triphosphate-dependent transporter. Partial evidence for this hypothesis has been put forth in previous studies demonstrating a major loss of glutathione-electrophile conjugate (GS-E) transport in crude membrane vesicles prepared from tissues of RLIP76^{-/-} mice compared with RLIP76^{+/+} mice (1).

In the present report, we used RLIP76 to refer to both the mouse (Ralbp1) and the human (RLIP76) 76-kDa splice variant proteins (RLIP76) for convenience and to avoid confusion. In other reports, Ralbp1 refers to the mouse enzyme (encoded by the Ralbp1 gene), which is structurally and functionally homologous to RLIP76, the human protein encoded by the human RALBP1 gene.

Lipid-derived alkenals and their GSH conjugates are obligate products of physiologic lipid peroxidation and have been shown to directly regulate a number of cellular signaling events, including apoptosis, proliferation, transformation, and gene expression (6). Because pathologically high levels of lipid peroxidation are an inevitable consequence of radiation (7, 8), it is reasonable to assume that known pro-apoptotic moieties such as 4-hydroxynonenal (4HNE), derived from lipid peroxidation, could be playing a direct role in mediating the apoptosis caused by radiation. 4HNE is metabolized primarily to the glutathione conjugate of 4HNE (glutathionyl-HNE [GS-HNE]) through a reversible reaction catalyzed by glutathione S-transferase (GSTs). GS-Es, such as GS-HNE, are excellent substrate inhibitors of GSTs; their accumulation in cells due to increased formation or reduced removal will inhibit the rate limiting enzyme of mercapturic acid synthesis and impair the metabolism of (potentially genotoxic) electrophiles through this pathway. Because the presence of relatively high concentrations of GSH and GST in cells ensures that the GSH-adducts formation from α,β -unsaturated lipid alkenals is near equilibrium, any process that impairs the rate of removal of the GS-E will result in increased cellular concentration of the pro-apoptotic alkenals, such as 4HNE. Thus, GSH-conjugate efflux mechanisms of cellular membranes should serve an anti-apoptotic function by preventing accumulation of 4HNE and other alkylating alkenals (9).

In the present study, we have explored the implications of this model further by comparing the sensitivity to X-irradiation between RLIP76^{+/+} and RLIP76^{-/-} mice and by evaluating the effects of pharmacologic augmentation of RLIP76 in these animals. The mechanisms for these effects and gene-dose relationships were explored in mouse embryonic fibroblasts (MEFs).

METHODS AND MATERIALS

Animal studies

Mice from colonies of RLIP76^{+/+} and RLIP76^{-/-} were used according to an Institutional Animal Care and Use Committee-approved protocol with assistance from trained animal facility personnel. RLIP76 liposomes were administered by intraperitoneal injection.

Method for preparing MEF cultures

Twelve-week-old C57BL/6 mice born of RLIP76^{+/-} × RLIP76^{+/-} mating were genotyped by polymerase chain reaction strategy on mouse tail DNA using forward, reverse, and long terminal region primers (1). Embryo fibroblast lines were prepared from RLIP76^{+/+}, RLIP76^{+/-}, and RLIP76^{-/-} mice on the 13th or 14th day of pregnancy, as described previously (10).

Preparation of RLIP76 liposomes

Recombinant human RLIP76 was purified, authenticated, and reconstituted into artificial cholesterol:asolectin liposomes, as described previously (2). For control liposomes, the addition of purified RLIP76 protein was omitted.

Colony-forming assay

The MEF cells (1×10^5 cells in 500 μ L) were irradiated at 0 (control), 100, 200, 500, and 1,000 cGy (6×10^6 volt-photon/min) for 1.25 min at the Texas Cancer Center (Arlington, TX), and aliquots of 50 or 100 μ L were added to 60-mm Petri dishes containing 4 mL culture medium. After 14 days, adherent colonies were fixed, stained with 0.5% methylene blue for 30 min, and counted using the Innotech Alpha Imager HP.

Determination of enzyme activities

The GSH levels and enzyme activities for GST, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and γ -glutamyl cysteine synthetase (11) were determined in 28,000g supernatants of 10% homogenate (12). Lipid hydroperoxide and thiobarbituric acid reactive substances were determined in whole crude homogenates by established methods as used by us previously (12).

Radiation

Whole animal X-irradiation was administered using a Varian Clinac linear accelerator (2100C; 6-MeV photon beams) with a dose range of 50-1,000 cGy. We placed the mice in their cage on top of a 1.5-cm super flab bolus, isolating them to one side of the cage and centering the field of treatment on them. They were irradiated with one-half of the dose from the anterior and the other one-half from the posterior, by rotating the accelerator gantry 180°.

Measurement of 4HNE and GS-HNE in mouse liver

The liquid chromatography-mass spectrometry (LCMS) method for 4HNE and GS-HNE measurement was modified from the previously published high-performance liquid chromatography method (13). A 10% homogenate of liver tissue from RLIP76^{-/-} and RLIP76^{+/-} mice untreated or treated with radiation was prepared in 1 mL final volume, followed by the addition of 2 mL acetonitrile and vortex. After 20,000g centrifugation for 30 min, the supernatant was collected. For the GS-HNE sample preparation, this supernatant was dried under a stream of nitrogen; for the HNE sample preparation, the acetonitrile/buffer supernatant was extracted with 3 mL of dichloromethane. The dichloromethane extract was then dried under nitrogen. The final sample volumes were 100 μ L. A Thermo Fisher Surveyor LC system coupled to a Thermo Fisher LXQ linear ion trap mass spectrometer was used for all separations using a Supelco Ascentis C₁₈ column (25 cm × 2.1 mm, 5 μ m) and a guard cartridge at a flow rate of 0.3 mL/min. The autosampler tray was held at 4°C during analysis, and all sample injections were 20 μ L. The GS-HNE separations were performed using the following gradient program: 75/25 water with 0.1% acetic acid/acetonitrile held for 2 min to 25/75 water with 0.1% acetic acid/acetonitrile at 5 min. The mobile phase for HNE analysis consisted of 60/40 acetonitrile/water with 0.1% acetic acid.

The mass spectrometer was in positive ion mode using selected ion monitoring (SIM). The sheath and auxiliary gases were at 27 and 20 arbitrary units, respectively. The runs were broken into two segments. The capillary temperature for segment 1 (time, 0-4.5 min) was 230° C. Segment 2 began after 4.5 min, where the capillary temperature was changed to 300° C. The other parameters for both segments were as follows: source voltage, 5.00 kV; capillary voltage, 48.0 V; and tube lens offset, 30.0 V. The SIM mass ranges monitored for GS-HNE analysis were 154.7-159.7, 305.8-310.8, 462.0-468.0, and 476.5-481.5. For HNE analysis, the mass spectrometer settings were the same as for segment 2 for the GS-HNE analysis (capillary temperature 300°C) with the addition to the SIM analysis of a mass range of 139.6-144.6 to detect the internal standard (trans-3-non-2-enone, obtained from Aldrich, St. Louis, MO).

Statistical analysis

The Kaplan-Meier method was used to estimate the survival curves for radiation and chemotherapy. The estimated survival curves of the treatment groups were compared using the log-rank test, and the corresponding *p* values were computed.

RESULTS

RLIP76 loss increases radiosensitivity, which reversed with RLIP76 supplementation

RLIP76^{-/-} mice were more sensitive to radiation than RLIP76^{+/+} mice (*p* < 0.001; Fig. 1, upper panels). The median lethal dose of RLIP76^{+/+} mice was 200-300 cGy, and that for RLIP76^{-/-} mice was 50-100 cGy, indicating a dose modification factor of 3-4. The administration of RLIP76 liposomes at a single fixed dose of 200 µg recombinant RLIP76 protein has been previously shown to cause a significant increase in RLIP76 in mouse tissues, including the brain (1). In the present study, when a dose identical to that used in previous studies was administered 14 h after radiation, we observed a remarkable improvement in survival of both RLIP76^{-/-} and RLIP76^{+/+} mice (Fig. 1, lower panels). The protective effect of the liposomes was significant for the RLIP76^{+/+} mice at 300 cGy (*p* < 0.001) and for the RLIP76^{-/-} mice at 100 (*p* < 0.001), 200 (*p* < 0.001), and 300 (*p* < 0.001) cGy. At the 50- and 100-cGy dose, the RLIP76^{-/-} mice treated with RLIP76-proteoliposome had survival identical to that of the RLIP76^{+/+} mice.

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

Our previous studies have shown loss of GS-E transport capacity by ~70% in RLIP76^{-/-} mouse tissues. These tissues also had significantly increased total tissue aldehydes and hydroperoxides, implying that GS-E formed physiologically from lipid hydroperoxides would also accumulate in tissues of RLIP76^{-/-} mice. We tested this by measuring the tissue levels of 4HNE and GS-HNE using an LCMS method. The extraction efficiency and recovery compared with internal standards was quantitative. Both 4HNE and GS-HNE were increased by about threefold in the RLIP76^{-/-} mouse liver tissue (Fig. 2). These results showed that the loss of RLIP76 results in the accumulation of endogenously generated electrophiles and their GSH conjugates *in vivo*. In RLIP76^{+/+}, GS-HNE levels increased in response to irradiation, but these levels were lower than those seen in RLIP76^{-/-} mice even without irradiation. Unlike in the wild-type, HNE and GS-HNE did not change significantly in the liver of irradiated RLIP76^{-/-} mice. These findings are explained by the histologic finding of extensive tissue edema and cell loss already evident by 24 h in the liver of irradiated RLIP76^{-/-} animals.

RLIP76 loss confers radiosensitivity in MEFs

To determine whether the radiation sensitive phenotype of the homozygous knockout mouse was due to radiation sensitivity at a cellular level, we compared the radiosensitivity of RLIP76^{+/+}, RLIP76^{+/-}, and RLIP76^{-/-} MEFs. The results shown in Fig. 3A show that

RLIP76^{+/+} mice were the most radioresistant, followed by RLIP76^{+/-} and RLIP76^{-/-} mice. The GSH levels in MEFs were depleted to 45% of control by 72-h exposure to 0.1 mM buthionine sulfoximine (BSO) or to 15% by 24-h exposure to 1 mM BSO. No effect on the radiosensitivity was observed at 100, 200, or 500 cGy irradiation in any of the MEFs (data not presented). At 1,000 cGy, a small, but significant, radiosensitization by GSH depletion was observed only in the RLIP76^{-/-} mice and only with 1 mM BSO (Fig. 3B). The radioprotective effect of 4 mM amifostine was compared with that of RLIP76 augmentation by liposomal delivery to cells using RLIP76 liposomes. At all radiation doses (100, 200, 500, or 1,000 cGy), the effect of pretreatment with 4 mM amifostine was less than the effect of RLIP76 gene loss (representative data shown for the 1,000 cGy dose, Fig. 3C). In contrast, supplementing cells with RLIP76 liposomes (at a concentration previously shown to increase cellular RLIP76 by threefold) (2, 14), resulted in complete reversal or radiosensitivity to the level of the control. The combined effects of RLIP76 and amifostine suggested an additive effect of radioprotection, significant by analysis of variance across all dose levels ($p < 0.05$).

Effect of RLIP76 loss and radiation on Jun N-terminal kinase, extracellular signal-regulated kinase, p38, and Akt

Other stress-protective mechanisms involved in radioresistance were investigated in *in vivo* studies of MEF from RLIP76^{+/+} and RLIP76^{-/-} mice. Western blot analyses confirmed the complete loss of RLIP76 protein in RLIP76^{-/-} and partial loss in RLIP76^{+/-} (Fig. 4A). The antiapoptotic protein BAD was unaffected. In contrast, BCL_{XL} was significantly lower in RLIP76^{-/-} MEFs. Caspase-3 cleavage assessed by measuring levels of pro-caspase-3 was greater in RLIP76^{-/-} cells (Fig. 4B). However, by an enzyme-linked immunosorbent assay (ELISA) for measuring total caspase activation, it appeared that RLIP76 loss did not, by itself, significantly affect total caspase activation. In contrast, 500 cGy irradiation did increase total caspase activity in both RLIP76^{+/+} and RLIP76^{-/-} MEFs, and the degree of increase was significantly greater in RLIP76^{-/-} MEFs ($p < 0.05$; Fig. 4C).

Unphosphorylated extracellular signal-regulated kinase (ERK) was unchanged, but p-ERK1/2 was greater in RLIP76^{-/-} than in RLIP76^{+/+} MEFs and p-Jun N-terminal kinase (JNK) was significantly greater in RLIP76^{-/-} than in RLIP76^{+/+} MEFs (Fig. 4B). By Western blot assay, at all points ≤ 48 h after 200 cGy radiation, p-ERK and p-JNK were greater in RLIP76^{-/-} MEFs (Fig. 4D). By ELISA at 24 h after 500 cGy radiation, pJNK was increased in both RLIP76^{+/+} (1.7-fold, $p < 0.02$) and RLIP76^{-/-} (2.3-fold, $p < 0.01$; Fig. 4E), and the degree of increase in pJNK in RLIP76^{-/-} cells was significantly greater than that seen in RLIP76^{+/+} cells ($p < 0.01$), indicating that loss of RLIP76 results in slightly increased JNK and pJNK and that radiation activated JNK to a greater degree in RLIP76^{-/-} than in RLIP76^{+/+} MEFs. The loss of RLIP76 had no significant effect on either p38 or p-p38 in unirradiated cells (Fig. 4F); however, radiation was slightly increased in p38 and p-p38 in the RLIP76^{+/+} cells ($p = \text{NS}$). In contrast, irradiated RLIP76^{-/-} cells had a significant increase in p-p38 compared with similarly irradiated RLIP76^{+/+} cells ($p < 0.01$).

Slightly increased p-protein kinase B (p-Akt) was seen in unirradiated RLIP76^{-/-} cells compared with RLIP76^{+/+} cells (1.5-fold by intensity; Fig. 4G). This result from MEF studies was confirmed by measurements in tissue homogenates of liver, heart, and kidney tissue from mice of both genotypes (Fig. 4H). Greater Akt activation occurred after radiation in a dose-dependent manner in RLIP76^{+/+} cells; at the greatest dose, the greater sensitivity of RLIP76^{-/-} cells was evident with near complete loss of cells, as well as p-Akt signal (Fig. 4G). RLIP76 loss significantly affected the activation of stress and apoptosis pathway proteins. These changes were consistent with previous studies (1).

Radioresistance interactions between RLIP76 and Akt, JNK, or ERK

We addressed the possibility that the radiosensitivity of RLIP76 knockout MEFs was due primarily to changes in the stress resistance mechanism Akt, JNK, and ERK by examining the transient transfection of RLIP76^{-/-} cells with either constitutively active (ca) or dominant negative (dn) Akt, dominant negative JNK, or dominant negative MAPK/ERK kinase (MEK), as well as constitutively active (ca) Akt or MEK. The cells were treated with radiation at doses of ≤ 500 cGy, and colony-forming assays were performed to assess growth inhibition (Fig. 5). The radiosensitive nature of RLIP76 knockout MEFs was confirmed ($p < 0.001$). The radiosensitivity of RLIP76^{-/-} MEF cells was unaltered with transfection with empty vector. The dnAkt transfection did not sensitize RLIP76^{+/+} cells, but it did sensitize RLIP76^{-/-} ($p < 0.05$). In contrast, caAkt protected both RLIP76^{-/-} and RLIP76^{+/+} MEFs (Fig. 5A). Transfection with dnJNK protected the RLIP76^{+/+} MEFs, but it sensitized the RLIP76^{-/-} MEFs (Fig. 5B). Both dnMEK and caMEK had similar effects of sensitizing radiation-induced growth inhibition, more remarkably in RLIP76^{-/-} than in RLIP76^{+/+} (Fig. 5C). However, none of these interventions could abrogate the difference in radiosensitivity between RLIP76^{+/+} and RLIP76^{-/-}. In this regard, the differential effect of dnJNK was most remarkable, because in the presence of RLIP76, depletion of JNK clearly protected the cells, but in the absence of RLIP76, its depletion sensitized cells further. These findings with respect to JNK were perhaps not surprising given the close link of JNK signaling to the mercapturic acid pathway through GST- π , which is known to activate JNK (15). It is possible that the regulation of the concentration of GST inhibitory glutathione conjugates (such as GS-HNE) plays a role in determining whether JNK activation results in pro- or antiapoptotic effects.

Loss of RLIP76 causes accumulation of lipid hydroperoxides and alkenals in MEFs

Lipid hydroperoxides, which are markers of oxidative stress, reflecting the oxidation of membrane lipids, were increased in a stepwise fashion from RLIP76^{+/+}, to RLIP76^{+/-} and RLIP76^{-/-} MEFs (Fig. 6A). Similarly, thiobarbituric acid reactive substances, which represent oxidized substances originating from membranes and carbohydrates, were also increased in a stepwise fashion (Fig. 6B). These findings have, for the first time, demonstrated that MEF cells lacking RLIP76 have inherently greater levels of lipid-hydro-peroxides, as well as reactive aldehydes that are formed from decomposition of lipid hydroperoxides. These results are consistent with the prediction of our model that loss of GSE transport activity caused by loss of RLIP76 would result in accumulation of their precursor lipid hydroperoxides and their degradation products.

Loss of RLIP76 causes profound changes in glutathione and antioxidant enzymes

Glutathione was increased significantly and in a stepwise fashion from RLIP76^{+/+}, to RLIP76^{+/-}, to RLIP76^{-/-} ($p > 0.01$; Fig. 6C); however, all the major GSH-linked enzyme defenses, including GST, glutathione peroxidase, γ -glutamyl cysteine synthetase, glutathione reductase, and glucose-6-phosphate dehydrogenase (Fig. 6D-H) were decreased ($p < 0.05$). Thus, increased GSH levels could not compensate for RLIP76 loss, perhaps because of the loss of activity of the GSH-linked enzymes.

DISCUSSION

The initiation of lipid peroxidation by hydroxyl radicals is a common early event in cell injury and cell death mediated by ionizing radiation. A large number of chemical toxins also accelerate lipid peroxidation through direct and indirect mechanisms. Lipid hydroperoxy radicals are sufficiently stable to diffuse significant distances under aqueous conditions and cause DNA scission or alkylation (16,17). Downstream, lower energy lipid-derived reactive oxygen species (primarily α,β -unsaturated alkenals) are weaker electrophiles (i.e., 4HNE) and thus form reversible Michael adducts with nucleophilic sites in DNA (18) and function as small molecule

signals between oxidative events in the membrane and transcriptional control for stress responses particularly through activator protein-1 (19-21). The ability of alkenals such as 4HNE to cross-link protein through Schiff's bases with amino groups in protein, as well as Michael adducts with sulfhydryls, renders them able to bind and denature proteins and other macromolecular structures (18). Small increases in 4HNE signal proliferation and differentiation. In contrast, large acute increases can trigger apoptosis through depletion of GSH and by directly affecting other apoptosis signaling proteins (6).

The transport activity of RLIP76 toward endogenous GS-E of the products of lipid peroxidation thus acts as a physiologic antiapoptotic mechanism. In this model, RLIP76 should act as an antiapoptotic agent toward both chemical oxidants (*i.e.*, anthracyclines) and radiant oxidants (X-rays) (1,19,20). The present results with MEFs from knockout mice have provided strong support for the present chemical models of signaling and apoptosis. RLIP76 couples adenosine triphosphatase activity with movement of molecules across the membrane. A splice variant of RLIP76, cytocentrin, is an effector involved in mitotic spindle movement (22). Strong evidence from studies by other investigators has shown that RLIP76 is an integral protein located at the cusp of endocytotic vesicles and integrally involved in mediating the movement of membrane vesicles during endocytosis (3-5,23). Evidence from other investigators that RLIP76 regulates heat shock factor-1-mediated heat-shock protein expression (24), corroborated by our results in knockout animal tissues (1), provides additional data for the assertion that one or more of several RLIP76 binding proteins (*i.e.*, heat shock factor-1, cdc-2, clathrin adapter activating protein 2, cdc42, ralB) could similarly modulate stress responses by modulating the transport activity of RLIP76 (7). These three diverse activities of RLIP76 might have in common the adenosine triphosphatase activity and movement. Thus, a general function of RLIP76 could be to transform chemical energy to movement in context of different macromolecular cellular machinery. Our findings, thus, have direct implications that could help to elucidate the molecular nature of diverse physiologic and toxicologic mechanisms.

CONCLUSION

The loss of RLIP76 confers sensitivity to xenobiotics and radiation owing to the loss of a common transport mechanism for GSH conjugates and xenobiotics. These findings indicate that GSH conjugate efflux plays a central role both as an effector and as a key regulator of stress and signaling.

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Fig. 1.

RLIP76 offers protection from radiation toxicity in mice. C57 Black mice RLIP76^{+/+} (circles) or RLIP76^{-/-} (diamonds) were weighed and randomized to radiation groups (50, 100, 200, or 300 cGy whole body X-irradiation) and further randomized and treated by one intraperitoneal injection of 0.2 mL buffer containing either control liposomes (no protein, Upper panels) or RLIP76-liposomes (Lower panels) at 14 h after radiation. RLIP76 liposomes and control liposomes contained identical amounts of phospholipids and cholesterol, but the former had been reconstituted in the presence of purified RLIP76 such that each 0.2 mL contained 200 μ g (2.6 nmol). After radiation, mice were monitored for health and survival twice daily; survival curves presented.

Fig. 2.

Comparison of 4-hydroxynonenal (4HNE) and glutathione conjugate of 4HNE (GS-HNE) levels in liver tissue of RLIP76^{+/+} vs. RLIP76^{-/-} mice. For 4HNE and GS-HNE measurements, 10% (wt/vol) homogenate of liver from RLIP76^{+/+} and RLIP76^{-/-} mice were prepared and assayed by liquid chromatography-mass spectrometry (LCMS) using the LCMS Thermo-LXQ with a C₁₈ reversed phase column and selective ion-monitoring mode (see the “Methods and Materials” section for conditions). One representative chromatograms (of six each) showing GS-HNE in RLIP76^{+/+} and RLIP76^{-/-} (A,D) and 4HNE (B,E) shown. Calibration curves generated using 4HNE and GS-HNE standards, and *t*-3-non-2-enone was used as internal control. Average and standard deviations from three separate measurements of 4HNE and GS-HNE from RLIP76^{+/+} (C) and RLIP76^{-/-} (F) mouse liver tissues from mice without or with 500 cGy whole body irradiation shown.

Fig. 3.

Effects of buthionine sulfoximine (BSO), amifostine or RLIP76 liposomes on mouse embryonic fibroblast radiosensitivity. RLIP76^{+/+} (circles), RLIP76^{+/-} (squares), and RLIP76^{-/-} (triangles) mouse embryonic fibroblasts were irradiated at 1,000 cGy with 6-MeV photons. Cells were inoculated into colony-forming assays immediately after radiation, and colonies were counted at Day 14. Colonies were stained with methylene blue and counted using an image acquisition and analysis system (Alpha Imager HP) (A). Effects of BSO-mediated glutathione (GSH) depletion (0.1 mM BSO for 72 h or 1.0 mM for 24 h) were studied in three genotypes of mouse embryonic fibroblasts irradiated at 1,000 cGy (B). Effects of amifostine (C) determined by pretreatment of cells with 4 mM amifostine for 30 min before radiation. RLIP76 liposomes contained 50 μ g purified RLIP76/mL; control liposomes prepared without RLIP76. Liposomes were added 24 h before radiation. All assays were done in triplicate, and average and standard deviations presented.

Fig. 4.

Effect of RLIP76 loss on stress signaling pathways in mouse embryonic fibroblasts without and with radiation. Mouse embryonic fibroblasts cultured from RLIP76 $^{+/+}$ and RLIP76 $^{-/-}$ pups at 13 days' gestation and grown in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum for five passages before study. Cells were plated at 4,000/cm 2 and grown for 4 days before assay. Western blot analyses were performed using commercially available antibodies (active motif) according to manufacturer's instructions. Relative levels of RLIP76 protein were compared between mouse embryonic fibroblasts of three genotypes by Western blot analyses (A) with lanes containing 100 μ g crude membrane fraction, primary antibody as anti-RLIP76 IgG with high specificity, as previously shown (2), and secondary antibody as horseradish peroxidase conjugated goat-antirabbit IgG. Blots were developed using 4-chloro-1-naphthol and developed bands were quantified by scanning densitometry. β -Actin was used as internal control. Levels of BCL $_{XL}$, BAD, pro-caspase-3, total extracellular signal-regulated kinase (ERK)2, p-ERK1/2, and p-Jun N-terminal kinase (JNK)1/2 were compared between RLIP76 $^{+/+}$ and RLIP76 $^{-/-}$ mouse embryonic fibroblasts (B). Enzyme-linked immunosorbent assay for total caspases (Immunochemistry Technologies) was performed in RLIP76 $^{+/+}$ and RLIP76 $^{-/-}$ cells 24 h after culturing without or with 500 cGy radiation (C). Time-dependent effects on p-ERK1/2 and p-JNK were examined (D) in mouse embryonic fibroblasts at 0-48 h after 200 cGy with Western blot analyses performed as above. Comparison of JNK and p-JNK (E) and p38 and p-p38 (F) were performed by enzyme-linked immunosorbent assay (active motif). p-Akt was examined in RLIP76 $^{+/+}$ and RLIP76 $^{-/-}$ mouse

embryonic fibroblasts 24 h after 0-1,000 cGy X-irradiation by Western blot analysis (G), with primary antibodies from Upstate Cell Signaling (anti-p-AKT - ser 473). Same kit and antibodies used to analyze p-Akt in membrane fraction obtained from liver, heart, and kidney tissues of RLIP76^{+/+} and RLIP76^{-/-} mice (H).

Fig. 5.

Effects of Akt, Jun N-terminal kinase (JNK), and MAPK/ERK (MEK) modulation in RLIP76^{+/+} and RLIP76^{-/-} mouse embryonic fibroblasts. RLIP76^{+/+} and RLIP76^{-/-} mouse embryonic fibroblasts were transfected with adenoviral vector alone or containing dominant negative Akt, constitutively active Akt (A), dominant negative JNK (B), dominant negative MEK, or constitutively active MEK (C) using the method previously described (25). Cells were radiated at 0, 200, or 500 cGy (6-MeV photons, 1.25 min) and inoculated into colony-forming assays. Colonies were counted using an Alpha Innotech imager 72 h later. Colony numbers were normalized to respective unirradiated controls. Treatment groups identified by symbols using following convention: circles indicate $+/+$; triangles, $-/-$; blue, untransfected; pink, empty vector; red, dominant negative (dn) construct; and green, constitutively active (ca) construct. Measurements performed in triplicate, and standard deviations were $<10\%$ in all cases.

Fig. 6.

Effect of RLIP76 loss on antioxidant defenses in mouse embryonic fibroblasts. RLIP76^{+/+}, RLIP76^{+/-}, and RLIP76^{-/-} mouse embryonic fibroblasts homogenized in 10 mM potassium phosphate buffer. β -Mercaptoethanol (1.4 mM) included in homogenates for enzyme activities and omitted from homogenates used to determine lipid hydroperoxides, thiobarbituric acid reactive substances, and glutathione. Lipid hydroperoxides and thiobarbituric acid reactive substances were determined in whole homogenate, and other measurements were performed in 28,000g supernatant fraction of homogenate. Three measurements were performed, each in triplicate, and average and standard deviations presented. Significant findings denoted as follows: * $p < 0.1$ and ** $p < 0.05$ for comparison of RLIP76^{+/+} vs. either RLIP76^{+/-} or RLIP76^{-/-}; + $p < 0.1$ and ++ $p < 0.05$ for comparison of RLIP76^{+/-} vs. RLIP76^{-/-}.