Involvement of riboflavin kinase expression in cellular sensitivity against cisplatin

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Abstract. Flavin adenine dinucleotide (FAD) is an essential coenzyme for glutathione reductase (GR) which catalyzes the reduction of oxidized glutathione to regenerate the reduced form involved in protection against oxidative stress. Riboflavin kinase (RFK) also known as flavokinase is involved in the first step of bioactivation of riboflavin (RF) to form flavin mononucleotide (FMN) which can be subsequently converted to FAD in an ATP-dependent reaction catalyzed by FAD synthetase (FADS). We investigated the involvement of RFK in cisplatin resistance using human prostate cancer PC3 cells. RFK overexpression renders cells resistant not only to cisplatin but also to hydrogen peroxide (H_2O_2) and diamide. Furthermore, knockdown of RFK expression induced apoptosis. We demonstrated that overexpression of RFK increased the levels of FAD, FMN and total glutathione and the expression of GR and glutathione S-transferase-π (GSTπ). RFK expression is up-regulated in cisplatin-resistant P/CDP6 cells in addition to FAD, total glutathione level, GR and GSTπ. Knockdown of RFK expression also sensitized both PC3 and P/CDP6 cells to cisplatin. Moreover, cellular levels of RFK expression correlate well with Gleason score, known as a good indicator of patient prognosis. The present study suggests that RFK expression is involved not only in cellular protection from oxidative stress but also in malignant progression of prostate cancer.

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Abbreviations: 5-FU, 5-fluorouracil; cDNA, complementary DNA; FAD, flavin adenine dinucleotide; FADS, FAD synthetase; FMN, flavin mononucleotide; GF, galactoflavin; GR, glutathione reductase; GSSG, glutathione disulfide; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; RF, riboflavin; RFK, riboflavin kinase; WST-8, water-soluble tetrazolium salt; γ-GCS, γ-glutamylcysteine synthetase

Key words: RFK, B2-vitamers, cisplatin, oxidative stress, prostate cancer

Introduction

Riboflavin, vitamin B_2 (RF) is a water-soluble vitamin present in a wide variety of foods. Riboflavin kinase (RFK) also known as flavokinase (1) is required for the conversion of riboflavin into its active coenzyme derivatives. Riboflavin is phosphorylated by RFK to generate flavin mononucleotide (FMN), which is adenylated by FAD synthetase (FADS) to generate flavin adenine dinucleotide (FAD). FAD is a coenzyme for glutathione reductase (GR), which mediates the regeneration of reduced glutathione; L-γ-glutamyl-L-cysteinyl-glycine (GSH). Glutathione is the major regulator of the intracellular thiol redox status and effectively scavenges free radicals and other reactive oxygen species (ROS). In these reactions, glutathione is oxidized to form glutathione disulfide (GSSG), which is then reduced to glutathione by the NADPH-dependent GR. If oxidative stress or any other factors limit the GR reaction, then GSSG will accumulate. Glutathione synthesis is primarily regulated by γ-glutamylcysteine synthetase (γ-GCS). Glutathione-S-transferases (GSTs) catalyze the conjugation of glutathione with many endogenous compounds.

The total glutathione level is higher in many multidrug and radiation resistant tumors than in normal tissues (2,3). Elevated levels of γ-GCS have also been identified in different human cancer tissues (2). There are seven known classes of human GSTs. One, GSTπ, is highly expressed in many cancers and appears to be involved in drug resistance (4-6). However, there are very few studies on the role of RFK expression in cancer. Cisplatin is widely used at the initial anticancer agent because of its activity against many human solid tumors (7,8). Its anticancer effect is obtained by several mechanisms, including formation of DNA adducts and production of reactive oxygen species (9,10). Multiple mechanisms for cisplatin resistance have been proposed such as reduced drug accumulation and

transport (11-13) and enhanced DNA repair (14,15). Another mechanism for cisplatin resistance is the up-regulation of the detoxification system via glutathione biosynthesis and conjugation (1,3,16,17). In fact, once inside the cell, cisplatin has to be activated through aquation reactions before being able to interact with DNA. The formation of aquated cisplatin is rate limited by its interaction with many endogenous detoxification molecules, among which glutathione is the most important and effective (5,18). On the other hand, it is unknown whether the glutathione recycling system is involved in cisplatin resistance.

The present studies were designed to determine whether RFK is involved in the glutathione-dependent antioxidant system. These initial studies indicate that RFK expression is involved in the cellular sensitivity against cisplatin or oxidative stress and malignant progression of cancer.

Materials and methods

Cell culture. Human epidermoid cancer HeLa cells and prostate cancer PC3 cells were cultured in Eagle's minimal essential medium. Human lung cancer A549 and H1299 cells, liver cancer HepG2 cells and colon cancer DLD1 and CaCo-2 cells were cultured in RPMI-1640 medium. Human pancreatic cancer MIA PaCa and Panc2 cells were cultured in Dulbecco's modified Eagle's medium. Media were purchased from Nissui Seiyaku (Tokyo, Japan) and contained 10% fetal bovine serum. The cisplatin-resistant P/CDP6 cells were derived from PC3 cells as described previously (19,20). All cell lines were maintained in a 5% CO_2 atmosphere at 37˚C.

Antibodies and drugs. To generate the polyclonal anti-RFK antibody, the synthetic peptide CNFFQVSKSKIMNGH was used as an immunogen and the antisera were obtained from New Zealand white rabbits after multiple immunization. Anti-GSTπ antibody (610719) was purchased from BD Bioscience (San Jose, CA, USA). Antibodies against γ-GCSc (sc-22755) and glutathione reductase (sc-32886) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-caspase-3 (no. 9662) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin (AC-15) and anti-Flag (M2) (F3165) antibodies were purchased from Sigma (St. Louis, MO, USA). Cisplatin, paclitaxel, 5-fluorouracil (5-FU) and etoposide (VP16) were purchased from Sigma. Adriamycin was a kind gift from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Oxaliplatin and SN-38 were a kind gift from Yakult Co., Ltd. (Tokyo, Japan). H_2O_2 was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Diamide was purchased from MP Bio Japan K.K. (Tokyo, Japan). Thapsigargin was purchased from Calbiochem (Darmstadt, Germany). FAD was purchased from ICN Biomedical, Inc. (Aurora, OH, USA). FMN, RF and EDTA ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) were obtained from Kanto Chemicals (Tokyo, Japan). Galactoflavin (GF) was kindly gifted from Merck & Co., Inc. (Rahway, NJ, USA).

Plasmid construction. To obtain full-length complementary DNA (cDNA) sequences for human RFK, PCR was carried out with a SuperScript cDNA library (Invitrogen, San Diego, CA, USA) using the following primer pairs (single underlining indicates the start codon and stop codon): 5'-ATGCCCCGAGCG GACTGCATTATG-3' and 5'-TCAGTGGCCATTCATTATTT TGCTTTTAG-3'. This PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). To construct a plasmid expressing Flag-tagged RFK, N-terminal Flag-tagged RFK cDNA were ligated into pcDNA3 vector (Invitrogen).

Cloning of stable transfectants. The cloning of stable transfectant has been described (21). Briefly, PC3 cells were transfected with pcDNA3-Flag RFK with Superfect reagent and cultured with 500 μg/ml neomycin for 15-20 days. The resulting colonies were isolated and the cellular expression level of Flag-RFK in each clone was investigated by Western blot analysis with anti-Flag (M2) antibody.

Western blot analysis. Whole-cell lysates were prepared as previously described (22,23). The indicated amounts of whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) microporous membranes (Millipore, Billerica, MA, USA) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in 10 mM Tris, 150 mM NaCl and 0.2% (v/v) Tween-20, and incubated for 1 h at room temperature with the primary antibody. The following antibodies and dilutions were used: 1:1,000 dilution of anti-RFK, 1:1,000 dilution of anti-GSTπ, 1:1,000 dilution of anti-γ-GCSc, 1:1,000 dilution of anti-glutathione reductase, 1:1,000 dilution of anti-caspase 3, 1:10,000 dilution of anti-Flag, and 1:5,000 dilution of anti-β-actin. The membranes were then incubated for 45 min at room temperature with a peroxidase-conjugated secondary antibody and were developed using chemiluminescence (Amersham, Piscataway, NJ, USA). The images were obtained by image analyzer (LAS-4000 mini, Fujifilm, Tokyo, Japan). To induce cleaved caspase 3 expression, PC3 cells were incubated with 1 μM VP16 for 24 h.

Knockdown analysis using siRNAs. The following double-stranded RNA 25-bp oligonucleotides were commercially generated (Invitrogen): 5'-AAGUAAGGCAGGUGCCUCA UAAUGC-3' (sense) and 5'-GCAUUAUGAGGCACCUGCCU UACUU-3' (antisense); RFK siRNA #1, 5'-AAGAUUAUCUAC CACUUGCUCAGGA-3' (sense) and 5'-UCCUGAGCAAGUG GUAGAUAAUCUU-3' (antisense); RFK siRNA no. 2, 5'-UUC GUAUUCUUGUAAUAUGGGUUCC-3' (sense) and 5'-GGAA CCCAUAUUACAAGAAUACGAA-3' (antisense); RFK siRNA no. 3. Transfection of siRNA is described previously (22,23). Briefly, 250 pmol of siRNA and 5 μl of lipofectamine mixture were combined with aliquots of $3.0x10^5$ PC3 cells in 500 μl of culture medium, and incubated for 20 min at room temperature. All cells were seeded into 35-mm dishes containing 2 ml culture medium and were harvested after culture for 72 h for Western blot analysis by using anti-RFK antibody, or for 120 h by using anti-caspase-3 antibody, as described above. PC3 cells ($1.0x10^3$) were used in the WST-8 assay, $5.0x10^3$ cells were used in the cell proliferation assay, or all cells were used in the flow cytometry, as described below.

Cytotoxicity analysis with WST-8. For the water-soluble tetrazolium salt (WST-8) assay, $1.0x10^3$ PC3/mock, PC3/RFK cl 1,

cl 2 cells or PC3 cells transfected with RFK siRNA were seeded into 96-well plates. The following day, the indicated concentrations of the drugs were applied. After 48 h, surviving cells were stained with TetraColor ONE (Seikagaku Corp., Tokyo, Japan) for 1-2 h at 37˚C. The absorbance was then measured at 450 nm.

Cell viability analysis with Adam-MC. PC3 and P/CDP6 cells ($4.0x10^4$ cells) transfected with siRNA, as described above, were cultured in 6-well plates. The next day, these cells were treated with indicated concentration of cisplatin. After 72 h, these cells were harvested with trypsin and were collected. Living cells were counted by Adam-MC (NanoEnTek Inc., Seoul, Korea) with instruction manual. Briefly, cells in each condition were suspended with 100 μl PBS and each 40 μl was mixed with 40 μl solution T containing propidium iodide (PI) and detergent or 40 μl solution N containing PI without detergent. Living cell number was calculated by (T number - N number)/T number. Living cell number without cisplatin was set to 100%.

Cell proliferation assay. PC3 cells ($5.0x10^3$ cells) transfected with siRNA, as described above, were seeded onto 12-well plates. The cells were harvested with trypsin and counted every two days with a Coulter-type cell size analyzer (CDA-500; Sysmex, Hyogo, Japan).

Flow cytometry. PC3 cells ($3.0x10^5$) transfected with siRNA, as described above, were seeded onto 100-mm dishes and cultured for 96 h. The cells were harvested, washed twice with ice-cold PBS, and fixed in 70% ethanol. After washing twice with ice-cold PBS, the cells were resuspended in PBS, incubated with RNase (Sigma), and stained with propidium iodide (Sigma). The cells were analyzed using an EpicsXL-MCL flow cytometer (Beckman Coulter, Brea, CA, USA).

Measurement of intracellular FAD, FMN and RF by HPLC. PC3/mock ($2.0x10^6$), PC3/RFK cl 1, PC3/RFK cl 2, PC3 or P/CDP6 cells harvested after treatment with 0.02% EDTA and 0.1% trypsin, were washed twice with ice-cold phosphate-buffered saline (PBS). The washed sample was centrifuged at 300 g for 5 min at 4˚C and removed the supernatant. The residue was thoroughly mixed with 100 μl ice-cold water containing 100 mM EDTA and 0.1% Triton-X for 1 min at 4˚C. The mixture was centrifuged at 10,000 g for 5 min at 4˚C. Supernatant (60 μl) was mixed with 180 μl ice-cold methanol, and centrifuged at 10,000 g for 5 min at 4˚C again. Then, the obtained supernatant was filtered using a 0.45-μm membrane filter. Ice-cold water (100 μl) containing 20 ng/ml GF, an internal standard, was added to 100 μl filtrate. The mixture was used as final sample for HPLC (high-performance liquid chromatography) measurement and stored in an ice-bath until use. These samples were analyzed by internal standard method using GF for HPLC with a fluorescence detector, as previously reported (24). The HPLC system consists of pump (LC-10ADvp), fluorescence detector (RT-10AxL), column oven (CTO-10ACvp) (Whole parts; Shimadzu, Kyoto, Japan) and C18 reversed-phase column (250x4 mm, 5 μm) (Lichro-CART250-4 RP18). Mobile phase was 0.01 mol/l KH_2PO_4 (pH 5.0):methanol = 75:25 and the flow rate was 0.8 ml/min. The column oven was set at 40˚C. The spectro-fluorometer was set at 440 nm and 560 nm for excitation wavelength and emission wavelength, respectively. All operations were carried out under low-intensity light.

Quantitative analysis of intracellular glutathione. The intracellular glutathione concentration was measured using the Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Kumamoto, Japan) (22).

Preparation of human tissue samples. Human tissue samples were obtained from autopsy cases. For human neoplastic samples, surgically resected tissues were examined in the Department of Pathology and Cell Biology at The University of Occupational and Environmental Health in Kitakyushu, Japan. These cases were classified according to the World Health Organization Histological Typing of each tissue. The diagnosis was re-evaluated and confirmed by at least three board-certified surgical pathologists who had examined formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin (H&E) or other appropriate immuno-histochemical stains. This study protocol was approved by the committee of University of Occupational and Environmental Health (25). H&E-stained specimens were classified according to the method of Gleason.

Immunohistochemistry. The specimens were fixed in 20% formaldehyde and embedded in paraffin. Four micrometer-thick tissue sections were deparaffinized, dehydrated with graded xylene and alcohol and incubated in 3% hydrogen peroxide for 5 min at room temperature to eliminate endogenous peroxidase activity. Antigen retrieval for the anti-RFK antibody was performed by microwaving and then autoclaving the samples, for 15 min each, in 0.1 mol/l citrate buffer (pH 6.0). The sections were incubated with anti-RFK antibody (1:200). Staining was visualized using the Envision plus system (Dako, Glostrup, Denmark) followed by counterstaining with hematoxylin. According to the intensity of the positive reaction for RFK in the cancer nests, the tumors were divided into four groups: Group 1, tumors that tested negative or faintly positive (i.e., the same as that in normal prostatic epithelium); Group 2, tumors with weak intensity; Group 3, tumors with a moderately stronger intensity to stain than that of normal prostatic epithelium; and Group 4, tumors with a markedly stronger intensity to stain than that of normal prostatic epithelium.

RT-PCR. The cDNA libraries constructed using various human tissues were purchased (Invitrogen). The primers used for semi-quantitative PCR are shown in Table I in addition to PCR conditions and the size of PCR products. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis. The protein expression levels, as determined by Western blotting and PCR products were assessed numerically using the Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan). Pearson's correlation was used for statistical analysis, and significance was set at the 5% level.

Table I. Primers, PCR conditions and expected PCR product lengths.

Gene name	Protein name	Sequence	Cycle	Product size (bp)
RFK	Riboflavin kinase (RFK)	5'-ATGCCCCGAGCGGACTGCATTATG-3' 5'-TCAGTGGCCATTCATTATTTTGCTTTTAG-3'	28	489
GSR	Glutathione reductase (GR)	5'-CCCCCTATTGGGACAGTGGGACTCACGG-3' 5'-GTCTGCCTTCGTTGCTCCCATCTTCACTGC-3'	28	255
FLAD1	FAD synthase (FADS)	5'-CGCAGCATCTCCCCTTTCCCTGAGCTGG-3' 5'-GATAGGCTGGACGGTATGTGGGGTGTCC-3'	28	415
GSTP1	Glutathione S-transferase P (GSTπ)	5'-AGACGTGGCAGGAGGGCTCACTCAAAGC-3' 5'-CCTCATGGATCAGCAGCAAGTCCAGCAGG-3'	20	384
ACTB	Actin, cytoplasmic 1 (β-actin)	5'-GCGGGAAATCGTGCGTGACATT-3' 5'-GATGGAGTTGAAGGTAGTTTCGTG-3'	20	232

Results

Establishment of RFK overexpressing cell lines. We first generated a RFK-specific polyclonal antibody. The specific binding activity of the antibody was validated by knockdown of RFK expression in human prostate cancer PC3 cells using siRNA transfection (Fig. 1A). To verify the specificity of the antibody, we used three siRNAs targeted for RFK. The original antibody could recognize a ~20-kDa protein, which is close to the expected size (18.3 kDa) based on RFK gene information. The expression of this protein was significantly decreased by transfection with three different siRNAs, indicating that this antibody can recognize human RFK protein (Fig. 1A). To investigate whether RFK plays a role in cisplatin resistance, we established two RFK overexpressing cell lines, PC3/RFK cl 1 and PC3/RFK cl 2, using human prostate cancer PC3 cells. Western blotting of RFK using the original antibody showed that the expression of RFK was about 4-fold higher in both cell lines than in the PC3/mock transfectant (Fig. 1B). We found two bands when the original antibody was employed in Western blotting. The upper band corresponds to the Flag-tagged RFK derived from expression plasmid and the lower band to the endogenous RFK. The upper band was also detected when anti-Flag antibody was employed (data not shown).

We next examined the expression of enzymes involved in the glutathione-dependent antioxidant system. Interestingly, the expression of GSTπ was increased by ~2.7-fold and that of GR was increased by ~2.5-fold in the RFK-overexpressing cell lines as compared with the PC3/mock cells, but the expression of the γ-GCS catalytic subunit was not (Fig. 1B). Next, we measured the cellular concentration of both B_2-vitamers and total glutathione in the RFK overexpressing cells (Table II). Both cellular FAD and FMN level were significantly increased by ~1.3- and 1.2-fold, respectively (Fig. 1C). Although the expression of γ-GCS was not increased in these cells, total glutathione level was slightly but significantly increased by ~1.2-fold (Fig. 1D).

RFK expression and cellular sensitivity against oxidative stress. To elucidate the association between increased RFK expression and oxidative stress, we performed a WST-8 assay using RFK overexpressing cell lines. As shown in Fig. 2A, both cell lines overexpressing RFK were resistant to cisplatin, which induce oxidative stress, but not thapsigargin, which induces endoplasmic reticulum (ER) stress, as compared with PC3/mock transfectant. Moreover, RFK overexpressing cells were resistant to H_2O_2 and diamide. The half-maximal inhibitory concentration (IC_{50}) values for cisplatin, H_2O_2 and diamide were significantly increased in RFK overexpressing cells, when compared with those in control cells (Fig. 2B). However, RFK overexpressing cells had similar sensitivity to other anticancer agents such as 5-FU and oxaliplatin (Fig. 2A) in addition to adriamycin, paclitaxel and SN-38 (7-ethyl-10-hydroxycamptothecin) (data not shown). We also investigated the cellular sensitivity against H_2O_2 or cisplatin using RFK specific siRNAs using PC3/mock cells. As shown in Fig. 2C, down-regulation of RFK sensitized with PC3 and P/CDP6 cells to cisplatin.

Association of RFK expression and cell growth. We performed cell cycle and cell growth analysis. RFK overexpressing cells and control PC3 cells had similar cell cycle profiles and doubling time (data not shown). Furthermore, whether the cells were cultured in riboflavin-abundant medium by adding riboflavin or in normal culture medium did not affect the proliferation rates or drug resistance (data not shown). Therefore, it appears that the normal culture medium contains sufficient levels of riboflavin. Then, we silenced RFK expression and performed the analysis of cell growth and cell cycle profiles. As shown in Fig. 3A, down-regulation of RFK significantly abolished the cell growth of PC3 cells. An increase of subG1 population of cells and a decrease of G2/M phase cells were observed (Fig. 3B). Consistent with these results, immunoblot analysis showed that knockdown of RFK expression significantly activated and cleaved caspase-3, indicating that RFK knockdown induces apoptosis in PC3 cells (Fig. 3C).

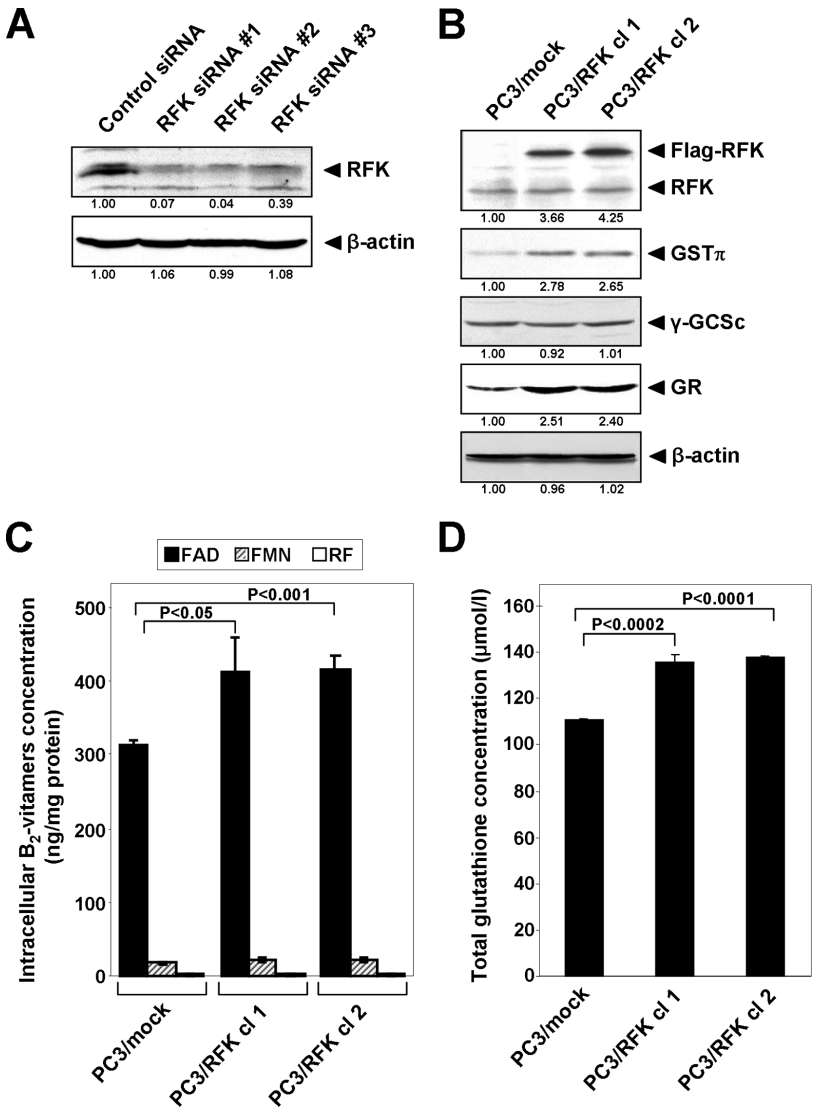

Figure 1. Specificity of the original anti-RFK antibody and establishment of RFK overexpressing cell lines. (A) PC3 cells were transfected with control or RFK siRNAs. After 72 h, whole-cell lysates (100 μg) were subjected to SDS-PAGE and Western blotting was performed using the original anti-RFK antibody. Bands were visualized at 20 kDa, similar to the molecular weight of RFK protein (18.3 kDa). Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. (B) Whole-cell lysates (100 μg) of RFK overexpressing cell lines (PC3/RFK cl 1 and cl 2) or a control cell line (PC3/mock) were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. (C) The intracellular FAD, FMN and RF concentration was measured in PC3/mock, PC3/RFK cl 1 or cl 2 cells. The average (± SD) B_2-vitamer concentration represents the B_2-vitamer (ng)/total mg protein. All values are means of at least three independent experiments. Bars, ± SD. (D) The intracellular total glutathione concentration was measured in PC3/mock, PC3/RFK cl 1 or cl 2 cells. The average (± SD) glutathione concentration represents the glutathione concentration/1 ml/5.0x10^5 cells. All values are means of at least three independent experiments. Bars, ± SD.

RFK expression of cisplatin-resistant prostate cancer cells. We investigated RFK expression in cisplatin-resistant cells. The cisplatin-resistant P/CDP6 cells were derived from PC3 cells and were found to be 63-fold more resistant to cisplatin than their parental cells (19,20). RFK expression was increased in P/CDP6 cells, in addition to γ-GCS, GSTπ and GR (Fig. 4A). Then, we investigated whether RFK expression coordinately up-regulates the expression of genes which are up-regulated in P/CDP6 cells. Interestingly, down-regulation of RFK reduced the expression of GR, GSTπ and γ-GCS (Fig. 4B). The cellular FAD level was ~1.5-fold higher in P/CDP6 cells than in the parental PC3 cells (Fig. 4C and Table II). The cellular glutathione level was 3-fold higher in P/CDP6 cells than in the parental PC3 cells (Table II).

Coordinate expression of RFK with GR, FADS and GSTπ. As shown in both Figs. 1B and 4, RFK expression correlates at least with both GR and GSTπ expression. To confirm these results, the expression levels of RFK, GR, FADS and GSTπ were measured by semiquantitative PCR using cDNA libraries derived from normal tissues (Table I). Among these tissues, β-actin was almost equally expressed. On the other hand, RFK expression was found to vary in these tissues (Fig. 5A). Interestingly, the expression levels of GR and GSTπ strongly correlated and the expression levels of FADS weakly correlated with those of RFK (Fig. 5B).

RFK expression in prostate cancer cells. We investigated the expression of RFK in human cancer cell lines, and found that

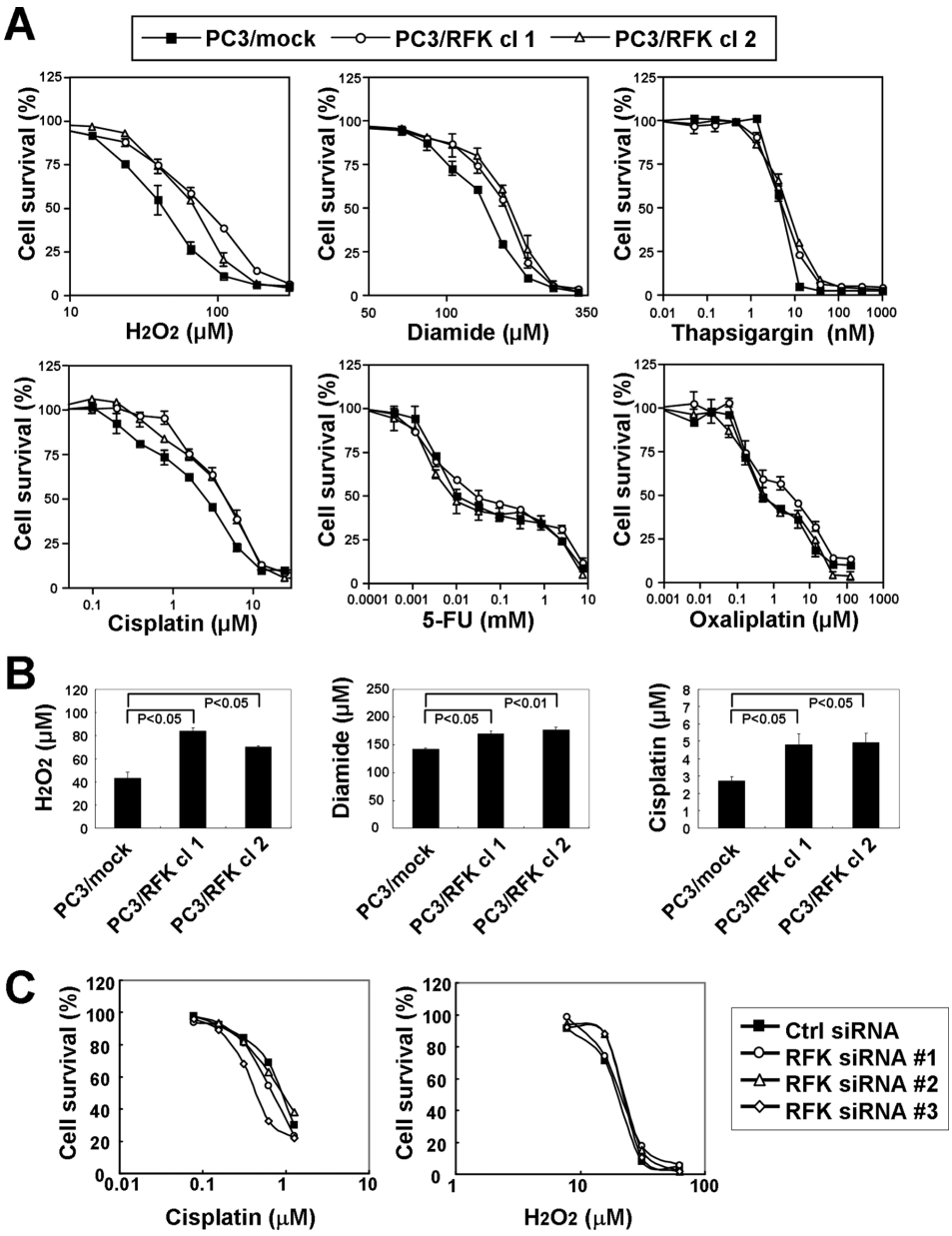

Figure 2. RFK expression correlates with oxidative stress defense. (A) $1.0x10^3$ PC3/mock, PC3/RFK cl 1 or cl 2 cells were seeded into 96-well plates. The next day, the indicated concentrations of the drugs were applied. After 48 h, cell survival was analyzed with a WST-8 assay. Cell survival in the absence of drugs corresponded to 100%. All values are means of at least three independent experiments. Bars, ± SD. (B) The IC_{50} (half-maximal inhibitory concentration) values in (A) were measured to compare the cellular sensitivity for cisplatin, H_2O_2 and diamide. (C) PC3 cells, transfected with 250 pmol of control (Ctrl) siRNA, or RFK siRNA #1, #2 or #3, were seeded onto 96-well plates. The next day, the indicated concentrations of the drugs were applied. After 72 h, cell survival was analyzed with a WST-8 assay. Cell survival in the absence of drugs corresponded to 100%.

the expression level of RFK varied, but was ubiquitously observed in human cancer cell lines (Fig. 6A). The specificity of this anti-RFK polyclonal antibody was again tested by immunohistochemistry of a prostate cancer specimen. Immunoreactivity of an anti-RFK antibody was observed diffusely in the cytoplasm of prostate cancer cells (Fig, 6B, left panel). After incubation of this antibody with excess of synthetic peptides, the positive immunostaining was abolished (Fig. 6B, right panel). Then, we investigated RFK expression of 30 prostatic cancer specimens in relation with pathological classification. The representative immunohistochemical analyses are shown in Fig. 6C. The level of RFK expression significantly correlates with Gleason score (coefficient of correlation, 0.763; $p<0.001$) as shown in Table III.

Discussion

To further investigate the possible functions of RFK in oxidative stress, we established RFK overexpressing cell lines using human prostate cancer PC3 cells. Our results show, for the first time, that RFK overexpression up-regulated the cellular level of both flavin cofactors and glutathione and activity of the antioxidant system, leading to cellular protection from oxidative stress (Fig. 1).

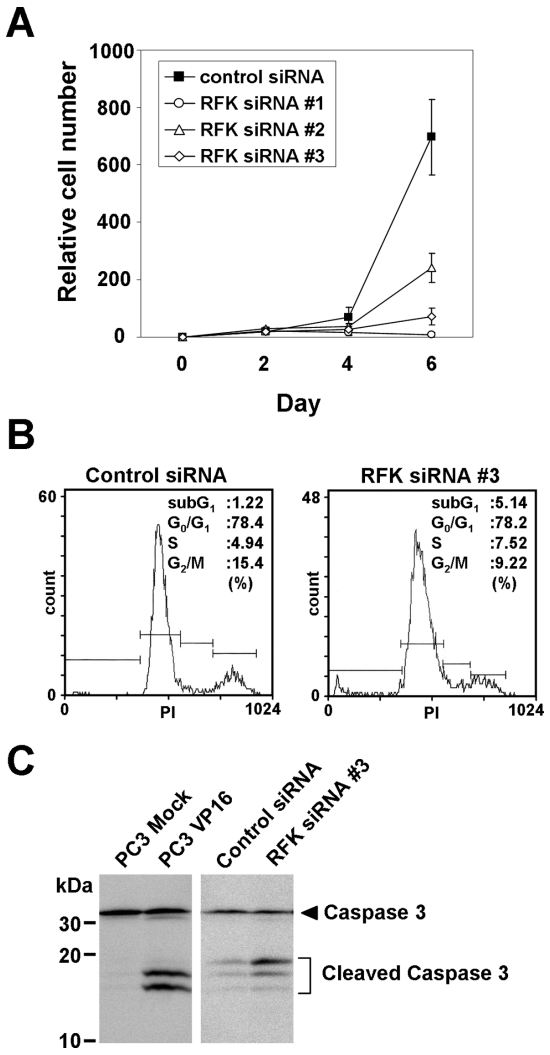

Figure 3. RFK expression is indispensable for cancer growth. (A) PC3 cells ($5.0x10^3$ cells), transfected with 250 pmol of control siRNA (siCtrl), or RFK siRNA (siRFK) nos. 1, 2 or 3, were seeded onto 24-well plates and counted every two days. The cell number on day 0 corresponded to 1. All values are means of at least three independent experiments. Bars, ± SD. (B) PC3 cells were transfected as in (A). After 96 h, the cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle fraction is shown at the top right of each graph. (C) PC3 cells were treated with VP-16 (1 μM) for 24 h and whole-cell lysates were used for positive control. PC3 cells were transfected as in (A). After 120 h, whole-cell lysates (100 μg) were subjected to SDS-PAGE, and Western blotting was performed using anti-caspase 3 antibody.

Glutathione synthesis is primarily regulated by γ-GCS, but γ-GCS expression was not up-regulated in the RFK overexpressing cells (Fig. 1B). Other regulators of glutathione metabolism, such as catalytic reaction by glutathione synthetase, intracellular levels of available amino acid precursors (Cys, Glu and Gly), or glutathione consumption within the cell and loss through efflux, might increase total glutathione levels (3).

The cellular sensitivity against oxidation agents such as cisplatin, H_2O_2 and diamide decreases in RFK overexpressing cells not only due to elevated levels of total glutathione but also due to the reduced redox conditions. FMN and FAD are both required by important cellular oxidation/reduction systems as coenzymes. Riboflavin is first phosphorylated to form FMN, which is catalyzed by RFK. FMN is then converted to FAD by adenylation. FAD, a coenzyme, promotes the regeneration of reduced glutathione by GR. As expected, cellular concentration of FAD and GR expression was increased in both RFK overexpressing cells and cisplatin-resistant cells (Figs. 1 and 4). Furthermore, forced expression of RFK up-regulated the expression of GSTπ (Fig. 1). GSTπ catalyses the conjugation of glutathione with various substrates including ROS and cisplatin. This result is consistent with previous reports showing that GSTπ plays an important role in the development of resistance to oxidative stress or cisplatin (4-6).

We also investigated the cellular sensitivity against cisplatin using RFK-specific siRNA. The down-regulation of RFK expression significantly sensitized cells to cisplatin (Fig. 4C). On the other hand, RFK down-regulation did not reduce the total glutathione level (data not shown). Transient down-regulation of RFK for 3 days might not be enough to decrease cellular glutathione levels. As shown in Fig. 3, down-regulation of RFK reduced cell growth and induced apoptosis. On the other hand, riboflavin deficiency induced G1 cell cycle arrest in HepG2 cells (26). Recently, it has been reported that RFK interacts with TNFR1 (tumour necrosis factor receptor 1) and is involved in TNF (tumour necrosis factor)-dependent signal transduction (27). These observations suggest that RFK may involve diverse cellular functions.

Moreover, RFK expression was up-regulated in cisplatin-resistant P/CDP6 cells in addition to FAD, total glutathione level, GR, GSTπ and γ-GCS (Fig. 4). These results suggest that RFK might play an important role to protect cells from

Table II. Cellular concentration of both B_2-vitamers and total glutathione in cell lines used.

Cell line	FAD (ng/mg protein)	FMN (ng/mg protein)	RF (ng/mg protein)	Total glutathione (μmol/l)
PC3/mock	313.63 (±4.75)	18.66 (±0.99)	3.03 (±0.96)	110.8 (±0.8)
PC3/RFK cl 1	410.91 (±48.9)[a]	22.56 (±1.83)[a]	2.67 (±0.60)	135.6 (±3.4)[a]
PC3/RFK cl 2	416.33 (±17.8)[a]	22.70 (±1.76)[a]	3.00 (±1.11)	137.7 (±0.7)[a]
PC3	300.62 (±16.2)	20.34 (±0.93)	3.01 (±0.34)	109.8 (±6.3)
P/CDP6	460.51 (±30.4)[a]	12.36 (±1.75)	1.93 (±0.24)	299.8 (±20.4)[a]

Each value represents the average (± SD) of three samples. [a]Statistically significant increase, $p<0.05$ (PC3/RFK cl 1 or 2 vs. PC3/mock, P/CDP6 vs. PC3).

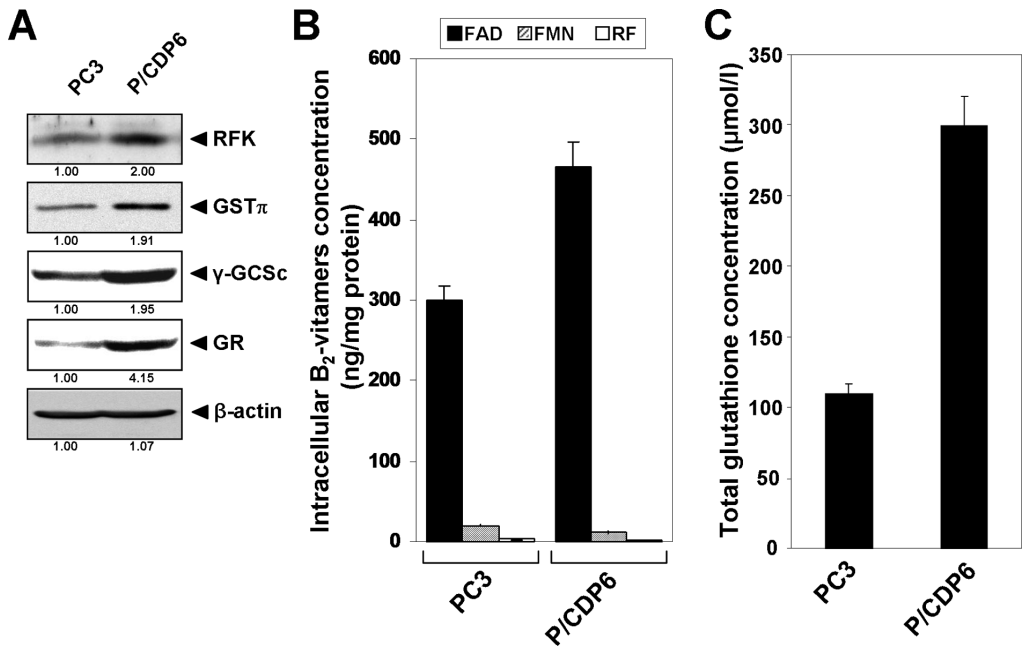

Figure 4. RFK expression in cisplatin-resistant cell lines. (A) Whole-cell lysates (100 μg) of cisplatin-resistant cell lines (P/CDP6) or parent PC3 cell lines were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. (B) The intracellular FAD, FMN and RF concentration was measured in PC3 or P/CDP6 cells. The average ± SD B_2-vitamer concentration represents the B_2-vitamer (ng)/total mg protein. All values are the means of at least three independent experiments. Bars, ± SD. (C) The intracellular total glutathione concentration was measured in PC3 or P/CDP6 cells. The average ± SD glutathione concentration represents the glutathione concentration/1 ml/5x10^5 cells. All values are the means of at least three independent experiments. Bars, ± SD.

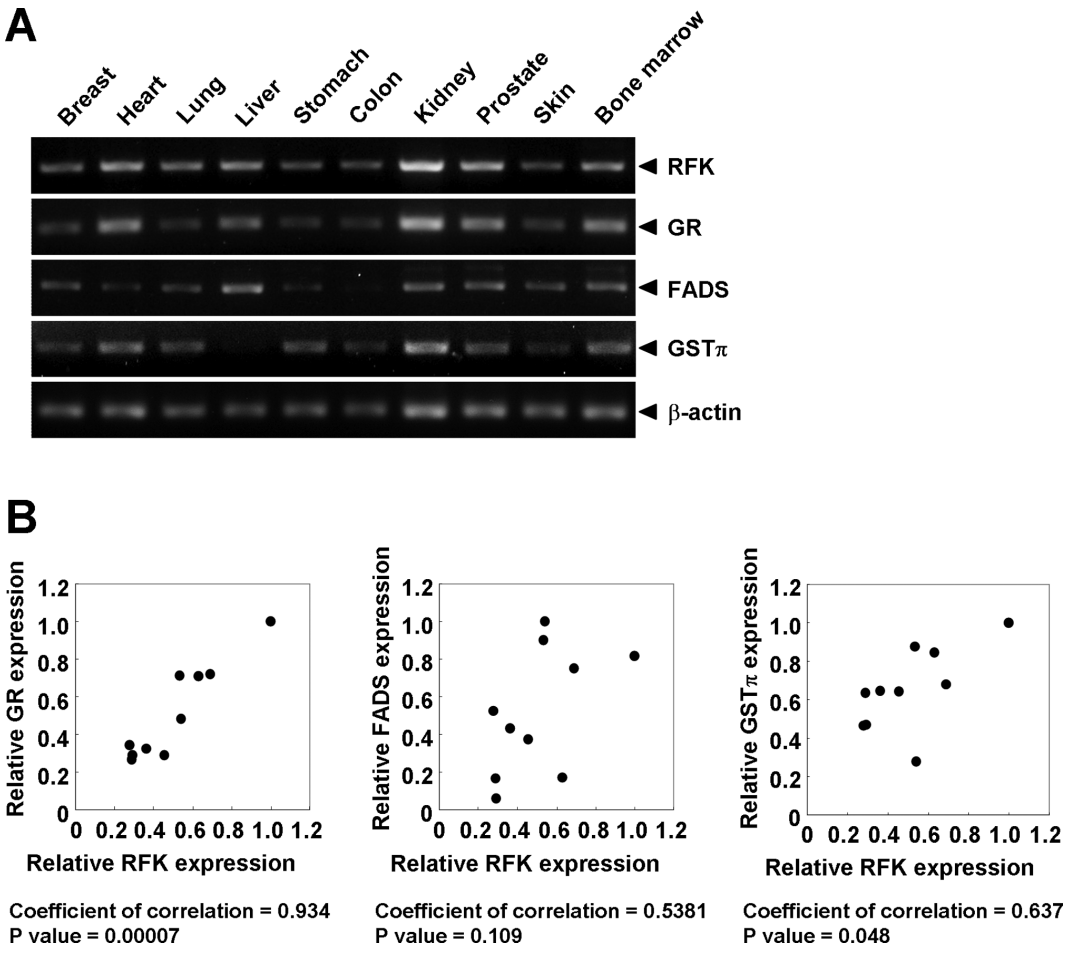

Figure 5. Coordinate expression of GR, FADS and GSTπ with RFK. (A) Semi-quantitative PCR of the cDNA library from normal human tissues was performed by using specific primer pairs for the indicated genes (Table I). (B). Expression levels of RFK, GR, FADS and GSTπ were determined by the Multi Gauge Version 3.0 using (A), and were normalized by each β-actin band. The maximum expression levels of RFK, GR, FADS or GSTπ were set to 1.

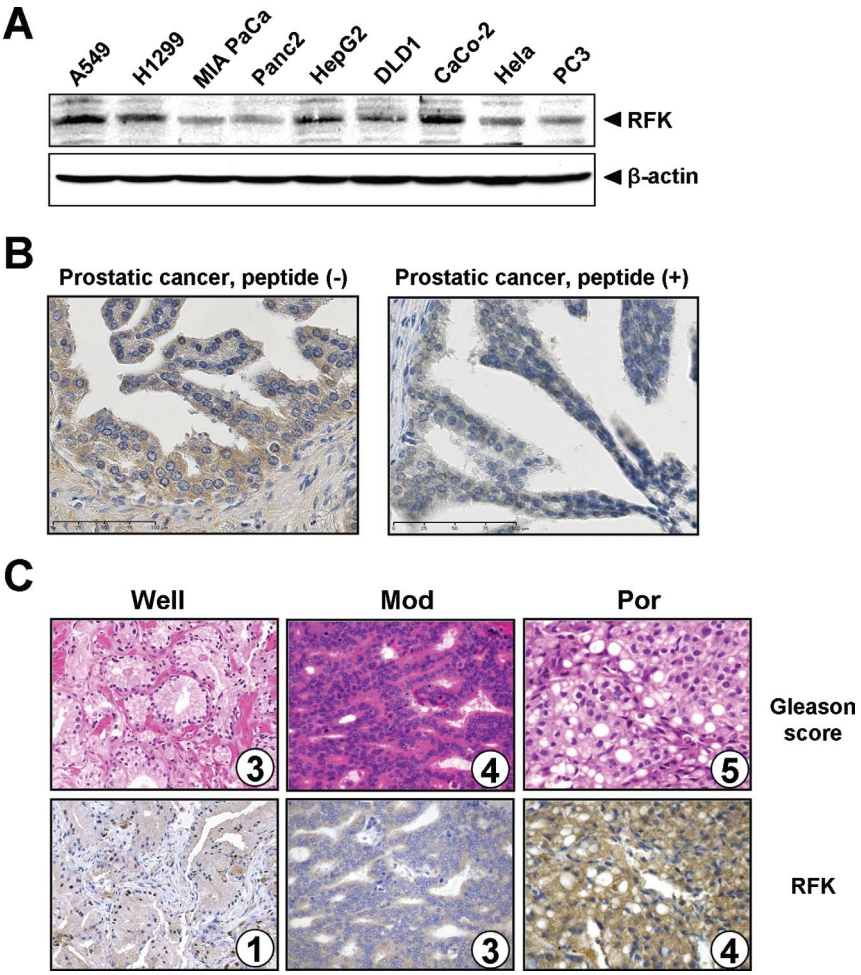

Figure 6. Expression of glutathione-related genes in cancer. Western blot analysis was performed with 100 μg of whole-cell lysates for RFK expression in nine human cancer cell lines. Immunoblotting of β-actin is shown as a loading control. (B) Immunostaining of RFK was performed in prostate cancer by using the original anti-RFK antibody (left panel). Immunostaining of RFK was performed after incubation of the antibody with the synthetic peptide of RFK (right panel). Scale bar, 100 μm. (C) The representative results of H&E staining and immunostaining of RFK are shown. Immunostaining of RFK was performed in prostate cancer specimens. Well, mod and por indicate well differentiated, moderately differentiated and poorly differentiated tumor, respectively. Gleason score and Group number according to the intensity of RFK staining are shown in the lower right corner.

Table III. Immunohistochemical analysis of RFK expression and Gleason grade in prostate cancers.

	Gleason score				
RFK[a]	6	7	8	9	10
Group 1	10[b]	1	0	0	0
Group 2	3	3	4	0	0
Group 3	1	0	1	0	2
Group 4	0	0	2	1	2

[a]RFK expression level is defined by intensity of immunostaining and was divided into four groups as described in Materials and methods. [b]Number of cases is shown (i.e., Gleason score 6 and Group 1 is observed in 10 cases).

oxidative stress. Preliminary data showed that both RFK and Ets expression were also induced by treatment with cisplatin or H_2O_2 (data not shown). It has been recently shown that the expression of Ets is a critical transcription factor of ROS stress (28) and that there is one Ets binding site in the promoter region of the RFK gene. These data suggest that stress-dependent activation of the Ets-related transcriptional factors might be involved in the regulation of RFK.

Coordinated gene regulation of the glutathione-dependent detoxification system is often observed. Expression of MRP (multidrug resistance associated protein) and γ-GCS was shown to be coordinately regulated by heavy metals (29). The cells forced expression of the transcription factor, ATF4, showed elevated expression of γ-GCS, GSTπ and ABC transporters with high levels of cellular glutathione (22). In this study, we also observed coordinated regulation of GR and GSTπ in addition to cellular glutathione in RFK overexpressing cells and cisplatin resistant cells (Figs. 1 and 4). Furthermore, coordinated expression of RFK, GR, FADS and GSTπ were found in normal tissues (Fig. 5). To investigate the common regulatory mechanisms of GR, FADS, GSTπ and γ-GCS genes, we searched the transcription factor binding sites in the promoter region between -3000 bp and transcription start site. Several ATF4 and Ets-binding sites are found in these gene promoters. The information suggests that similar transcriptional regulation may occur via these elements.

One of the major hallmarks of cancer is apoptosis resistance (30). Antioxidant systems were often up-regulated in cancer to evade apoptosis (31,32). As shown in Figs. 4 and 6, RFK expression plays a critical role in apoptosis and malignant progression of prostatic cancers. Both high intracellular glutathione and B_2-vitamers is partly due to high RFK expression and can promote cancer cell growth and survival.

In conclusion, the present study is the first report to show that RFK is involved in protection of cells from oxidative stress. Although further studies are necessary to determine whether RFK can be a target of signal transduction for oxidative stress, our findings provide insights into the biological mechanisms of not only cancer but also other many diseases related to reactive oxygen.

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