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Regulation of Tumor Cell Sensitivity to TRAIL-Induced Apoptosis by the Metastatic Suppressor Raf Kinase Inhibitor Protein via Yin Yang 1 Inhibition and Death Receptor 5 Up-Regulation

Stavra Baritaki,* Alina Katsman,* Devisah Chatterjee,† Kam C. Yeung, Demetrios A. Spandidos,§ and Benjamin Bonavida1*

Raf-1 kinase inhibitor protein (RKIP) has been implicated in the regulation of cell survival pathways and metastases, and is poorly expressed in tumors. We have reported that the NF-κB pathway regulates tumor resistance to apoptosis by the TNF-α family via inactivation of the transcription repressor Yin Yang 1 (YY1). We hypothesized that RKIP overexpression may regulate tumor sensitivity to death ligands via inhibition of YY1 and up-regulation of death receptors (DRs). The TRAIL-resistant prostate carcinoma PC-3 and melanoma M202 cell lines were examined. Transfection with CMV-RKIP, but not with control CMV-EV, sensitized the cells to TRAIL-mediated apoptosis. Treatment with RKIP small interfering RNA (siRNA) inhibited TRAIL-induced apoptosis. RKIP overexpression was paralleled with up-regulation of DRs, transcription and expression; no change in DR4, decay receptor 1, and decoy receptor 2 expression; and inhibition of YY1 transcription and expression. Inhibition of YY1 by YY1 siRNA sensitized the cells to TRAIL apoptosis concomitantly with DR5 up-regulation. RKIP overexpression inhibited several antiapoptotic gene products such as X-linked inhibitor of apoptosis (XIAP), c-FLIP long, and Bcl-xL that were accompanied with mitochondrial membrane depolarization. RKIP overexpression in combination with TRAIL resulted in the potentiation of the above effects and activation of caspases 8, 9, and 3, resulting in apoptosis. These findings demonstrate that RKIP overexpression regulates tumor cell sensitivity to TRAIL via inhibition of YY1, up-regulation of DRs, and modulation of apoptotic pathways. We suggest that RKIP may serve as an immune surveillance cancer gene, and its low expression or absence in tumors allows the tumor to escape host immune cytotoxic effector cells. The Journal of Immunology, 2007, 179: 5441–5453.
TRAIL in combination with subtoxic concentrations of several chemotherapeutic drugs has been shown to sensitize TRAIL-resistant cells to TRAIL-mediated apoptosis (14, 15). We and others have demonstrated that one of the mechanisms responsible for tumor cell sensitization to TRAIL-mediated apoptosis after drug pretreatment is via up-regulation of DR5/TRAIL-R2 (14–17). TRAIL binds to four distinct receptors, as follows: DR4/TRAIL-R1, DR5/TRAIL-R2, DcR1/TRAIL-R3, and DcR2/TRAIL-R4 (18, 19); however, only DR4 and DR5 contain functional death domains and are able to induce apoptosis. The apoptotic signaling pathways induced by ligation of the TRAIL receptors are not fully characterized. It has been suggested that the limited availability of functional TRAIL receptors, as a function of upstream or downstream negative regulators, as well as the constitutive activation of signaling pathways such as MAP/ERK, might have a dominant protective effect over apoptotic signaling from the TRAIL DRs (11, 20). Recent studies have proposed the crucial role of the NF-κB components, RelA (p65) and c-Rel, in the DR5 regulation and TRAIL signaling (21, 22). Evidence of the direct NF-κB involvement in DR5 transcriptional regulation is based primarily on the identification of a putative NF-κB DNA binding site in the first intronic region of the DR5 promoter region, which when it is mutated reduces the promoter activity (23).

We have recently shown that cancer cells can be sensitized to drug-induced apoptosis by expression of Raf-1 kinase inhibitor protein (RKIP) (24). RKIP, a member of the phosphatidy ethanolamine-binding protein family, had initially been characterized to be involved in many different physiologic activities, including reproduction and neurophysiology (25–27). Recent findings, however, have identified RKIP as a modulator of apoptosis and metastasis through regulation of important signaling cascades, i.e., the Raf-MEK-ERK kinase cascade, G protein-coupled receptors, and the NF-κB pathway (28–34). RKIP blocks Raf-induced phosphorylation of MEK, via direct interaction with Raf-1 kinase, and consequently the activation of ERK (35–37). RKIP has weak binding affinity to MEK-1 and ERK-2 (38), but when coexpressed with downstream phosphorylation events. In addition to its modulation of Raf sig- naling, RKIP inhibits NF-κB activity by interfering with upstream NF-κB activators such as the NF-κB-inducing kinase (NIK) and TGF-β-activated kinase 1 (TAK1) (29). Because an impact of the above pathways in the protection of cancer cells against cell death is well known, the inhibitory effects of RKIP on these cascades grant the ability to play a role in apoptosis induction.

We have reported in several types of tumors (ovarian, prostate, melanoma) that Fas as well as DR activators such as the NF-κB-inducing kinase (NIK) and TAK1 are able to induce apoptosis. Our findings corroborate the above hypothesis and support the direct role of RKIP in the regulation of tumor cell sensitivity to TRAIL-mediated apoptosis.

Materials and Methods

Reagents

Soluble human TRAIL was purchased from PeproTech. The cis-diammine-dichloroplatinum (II) (CDDP) was obtained from Sigma-Aldrich, and Taxol (Paclitaxel) was purchased from Bristol Myers Squibb. The NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) was provided by Dr. K. Umezawa (Keio University, Yokohama, Japan). Stock solutions of CDDP, Taxol, and DHMEQ were prepared in DMSO. The Bcl-2 inhibitor androst-5-ene-3,7-dione (A2/2MMAM-43) was purchased from BIOMOL. The PE-labeled anti-DR4 and anti-DR5 Abs and FITC-labeled anti-active FLIP, and anti-NIK mAbs, and polyclonal anti-Bax, anti-DR4, anti-pNIK, and HRP-labeled anti-mouse IgG Abs were obtained from Santa Cruz Bio-techology. Polyclonal anti-caspase 8, anti-caspase 9, anti-X-linked inhibitor of apoptosis (XIAP), anti-BID, and HRP-conjugated anti-rabbit IgG Abs were purchased from Cell Signaling Technology. Small interfering (si)RNAs for RKIP and YY1 and siRNA transfection reagent were purchased from Santa Cruz Biotechnology.

Cell culture

The prostate carcinoma cell line PC-3 (metastatic bone-derived human androgen-independent human prostatic adenocarcinoma) was obtained from American Type Culture Collection. The metastatic melanoma cell line M202 was provided by Dr. A. Ribas (University of California, Los Angeles, CA). Both cell lines were maintained in a humidified 5% CO2 environment in RPMI 1640 supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 1% nonessential amino acids, 2 mM l-glutamine, 1% sodium pyruvate, and 10% FBS (Invitrogen Life Technologies).

Transient transfections

For overexpression of RKIP, exponentially growing PC-3 and M202 cells cultured in six-well plates were transiently transfected with RKIP expression plasmid (CMV-RKIP) or the relevant empty vector (CMV-EV) as control. The expression plasmid contained the full-length cDNA of RKIP under control of a CMV promoter, as described previously (24). Transfection solutions consisted of 0.5 ml/well reduced serum medium Opti-MeM (Invitrogen Life Technologies) supplemented with 2 μl of Lipofectamine 2000 (Invitrogen Life Technologies) and 2 μg of plasmid DNA (for PC-3), or 4 μl of Lipofectamine and 4 μg of plasmid DNA (for M202) were prepared according to manufacturer’s instructions. After transfection, cells were incubated in antibiotic-free medium for 5 h, followed by replacement with fresh normal growth medium for a further incubation of 48 h before determination of the RKIP levels.

Cotransfection experiments were similarly performed in six-well plates containing exponentially grown PC-3 cells. Combinations of CMV-RKIP or CMV-EV vectors were used with each one of the reporter plasmids.
listed below: pYY1-Luc, pNF-xB-Luc, pDR5 wild type, pDR5−/−605, and pDR5/YY1 mutant. The DR5 luciferase constructs pDR5 wild type, pDR5−/−605 with the 5′-deletion (−605) that excludes the YY1 binding site, and the pDR5/YY1 mutant missing active YY1 binding sequence have been previously characterized (14, 23) (S. Huerta-Yepez, M. Vega, S. Escoto-Chavez, B. Murdock, T. Sakai, and B. Bonavida, submitted for publication). The pYY1-Luc reporter carrying the full length of the relevant wild-type YY1 promoter sequence was synthesized, as previously described (23) (S. Huerta-Yepez, M. Vega, S. Escoto-Chavez, B. Murdock, T. Sakai, and B. Bonavida, submitted for publication), whereas the pNF-xB-Luc plasmid was purchased from Invitrogen LifeTechnologies. Transfection solution was the solution used for RKIP overexpression in PC-3 cells supplemented with 2 μg of the pDR5-Luc reporters or 2 μg of pNF-xB-Luc or 1 μg of pYY1-Luc plasmids. Four hours after the addition of the solution to the cells, the transfection medium was removed and fresh medium containing 10% FBS was added to allow the recovery of cells for 44 h. Luciferase activity in protein extracts was measured in an analytical luminance counter according to the manufacturer’s protocol (BD Biosciences). Data were normalized to protein concentration levels using the Scintillation counter according to the manufacturer’s protocol (BD Biosciences). Application of siRNAs A total of 3 × 10⁵ PC-3 or M202 cells/well was plated in a six-well plate, 24 h before transfection in an antibiotic-free growth medium. A total of 10 μg (1 μg) of YY1 siRNA or siRKIP or a relevant amount of a control siRNA solution was mixed with 6 μl of transfection reagent LipofectAM. Transfection was performed for 72 h, according to the manufacturer’s protocol (Santa Cruz Biotechnology). For determination of cell sensitization to TRAIL-mediated apoptosis, 48 h posttransfection, untransfected or siRNA-transfected cells were treated with CDDP (3 μg/ml) or TRAIL (10 ng/ml), or the combination for 24 h. Cells transfected with siYY1 for 48 h were treated only with different concentrations of TRAIL (10, 25, and 50 ng/ml) for 24 h. Apoptosis was determined by staining in a processus suspensory flow cytometry, as described below. Flow cytometric evaluation of the surface DR5 protein levels was also performed in siTRAIL-transfected cells 72 h posttransfection.

Determination of apoptosis PC-3 and M202 cells transfected for 24 h with the CMV-RKIP or the CMV-EV plasmid were treated for additional 24 h with various concentrations of TRAIL. Apoptosis and apoptosis were determined by cleavage of caspase 3 using flow cytometry as previously described (14). Similarly, apoptosis was assessed in 3 × 10⁵ cells treated for 6 h with the NF-xB inhibitor DMEQ (5 or 10 μM) or the Bel-xL inhibitor 2MAM-A3 (15 μg/ml), followed by 18-h treatment with various concentrations of TRAIL. Determination of apoptosis in PC-3 cells transfected with siRKIP was performed by annexin-V/pi iodide (PI) staining method. Seventy-two hours posttransfection, cells were resuspended in 100 ml of staining solution (containing annexin V fluorescein and PI in a HEPES buffer; annexin-V-FLUOS staining kit; Boehringer Mannheim). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Apoptotic cells were defined as annexin-V positive and annexin-V/PI positive because annexin-V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. All samples were analyzed on an Epics XL flow cytometer. Data were processed using the incorporated System I and II software and presented as percentage of apoptotic cells within the gated population.

Flow cytometry for evaluation of DR and YY1 expression The surface expression of DR5, DR4, DcR1, and DcR2 as well as the YY1 expression were assessed by flow cytometry in PC-3 or M202 cells transfected with the CMV-RKIP or CMV-EV constructs or after various treatments, as previously described (14). All samples were analyzed on an Epics XL flow cytometer. Data were processed using the incorporated System II Software, and the mean fluorescence intensity (MFI) was recorded.

Measurement of mitochondrial membrane depolarization The mitochondria-specific dye 3,3'-dihexyloxocarbocyanine (DiOC6; Molecular Probes) was used to measure the mitochondrial membrane potential. PC-3 cells transfected with the CMV-RKIP plasmid or the relevant control vector were treated or left untreated 24 h posttransfection with TRAIL (10 ng/ml) for a further 24 h. Mitochondrial membrane depolarization was analyzed at 48 h posttransfection by flow cytometry, as previously reported (14). Data were collected as MFI of DiOC₆.

RT-PCR Total RNA was extracted and purified from 1 × 10⁶ PC-3 cells for each experimental condition using TRIzol reagent (Invitrogen Life Technologies). Total RNA (1 μg) was reverse transcribed to first strand cDNA for 1 h at 42°C with 200 U of AMV reverse transcriptase and 20 μM random hexamer primers according to the manufacturer’s instructions (Promega). Amplification of 1/20 of these cDNA by PCR was performed using the following gene-specific primers: YY1 forward (5'-GGGACTACCTTGCCAT TGCAG-3') and YY1 reverse (5'-TGTTTGTGACCTTGTG-3') (127-bp expected product); DR5 forward (5'-ATCTTAGACACA ATTGGAATAGCTG-3') and DR5 reverse (5'-AAATTAATGACAG TACACAAGG-3') (502-bp expected product). Internal control for equal RNA loading in each reaction was assessed using the gene-specific primers: GAPDH forward (5'-GGGACATTGGAAGTAG CCAGC-3') and GAPDH reverse (5'-GTCATGGTATGCGAAAC CATAT-3') (100-bp expected product). CR amplifications of each specific RNA sequence were conducted using Taq polymerase (Denville Scientific), followed by three deep thermal cycling incubations (94°C/30 s; 54°C/35 s; 72°C/30 s) for 30 cycles, and a final extension at 72°C for 10 min. The amplification cycle was established based on preliminary titration of the optimal amount of amplified product for each gene representing maximum linear phase of the amplification process. The amplified products were resolved in 1× agarose gel electrophoresis, and their relative concentrations were assessed by densitometric analysis of the digitized gel images obtained from the image analyzer.

Western blot analysis Whole-cell lysates from PC-3 or M202 cells transfected for 48 h with the CMV-RKIP or CMV-EV plasmids with or without treatment with TRAIL (10 ng/ml) were prepared in radioimmunoprecipitation assay lysis buffer, as previously described (14). Lysates from untreated and untransfected cells were served as control. Protein lysates were also extracted from PC-3 cells treated with CDDP (3 μg/ml) or DMEQ (10 μg/ml) or M202 cells treated with Taxol (30 μM) for 24 h. A total of 15 μg of cell extracts was subjected to Western blot analysis (14) for determination of RKIP protein levels after transfection by using as primary Ab a 1/10000 dilution of a mouse anti-RKIP mAb and 1/5000 dilution of a secondary goat anti-mouse IgG-HRP Ab. Western analyses for YY1, DR4, DR5, c-FLIP, procasps 9, caspase 8, Bax, Bel-xL, Bid, XIAP, and β-actin were performed using specific mAbs or polyclonal Abs according to protocols reported previously (14, 41). The expression of β-actin was used as an internal control.

Statistical analysis Significant differences between values obtained in a population of PC-3 or M202 cells treated with different experimental conditions were determined by Mann-Whitney U test. Kruskal-Wallis H test was also applied to the results obtained as function of drug or TRAIL concentration used. Probability (p) was set significant at the level of 0.05. Statistical analyses were performed using the software SPSS.

Results RKIP overexpression sensitizes TRAIL-resistant tumors to TRAIL-mediated apoptosis via DR5 up-regulation The PC-3 human prostate carcinoma cell line is resistant to TRAIL-mediated apoptosis. We have previously shown that this resistance can be reversed by the combination of TRAIL and several chemotherapeutic drugs (e.g., CDDP, adriamycin, etoposide, vinblastine) via drug-induced DR5 up-regulation due to inhibition of NF-xB and YY1 (14). We hypothesized that gene products that are able to inhibit NF-xB and YY1 might mimic the chemotherapeutic drugs and be considered as suppressors of TRAIL resistance. Taking into account the inhibitory effect of RKIP on NF-xB
signaling and the role of RKIP in tumor cell sensitization to drug-induced apoptosis (24, 29), we examined the sensitivity of PC-3 cells to TRAIL-mediated apoptosis. The prostate cancer cell line PC-3 (A) and the melanoma cell line M202 (B) were transfected with 2 or 4 μg of CMV-RKIP vector, respectively, in the presence of the relevant empty vector plasmid (CMV-EV) for 48 h, as described in Materials and Methods. Twenty-four hours posttransfection, the cells were treated with increasing concentrations of TRAIL for an additional 24 h, and apoptosis was determined by detection of active caspase 3 by flow cytometry. The data represent the mean values ± SEM from three independent experiments. *, p < 0.03. Untransfected cells treated with TRAIL vs RKIP-transfected cells treated with TRAIL (Mann-Whitney U test); **, p < 0.02, TRAIL concentration-dependent increase in apoptosis in RKIP-overexpressing cells (Kruskal-Wallis H test). RKIP overexpression after cell transfection was confirmed by Western blot analysis 48 h posttransfection in cell lysates, as described in Materials and Methods. Actin expression was used as internal control.

C, Reversal of CDDP-induced sensitization of PC-3 cells to TRAIL-mediated apoptosis by RKIPsiRNA. PC-3 cells were transfected with 1 μg of RKIPsiRNA or equivalent amount of control siRNA for 72 h. Forty-eight hours posttransfection, the cells were treated with 3 μg/ml CDDP for 6 h, followed by TRAIL treatment (10 ng/ml) for an additional 18 h. Apoptosis was determined at 72 h by PI/annexin-V assays. Total whole-cell lysates were prepared at the same time from identical cell treatments and examined for RKIP expression by Western blot analysis. Actin was used as internal control. Apoptosis was significantly reduced in RKIP siRNA-transfected cells treated with the combination of CDDP and TRAIL compared with that observed in similarly treated cells without transfection (p < 0.005, Mann-Whitney U test). The data shown represent the mean values ± SEM from two independent experiments.

FIGURE 1. Overexpression of RKIP results in increased sensitivity of TRAIL-resistant cells to TRAIL-mediated apoptosis. A and B, RKIP overexpression sensitizes tumor cells to TRAIL-mediated apoptosis. The prostate cancer cell line PC-3 (A) and the melanoma cell line M202 (B) were transfected with 2 or 4 μg of CMV-RKIP vector, respectively, in the presence of the relevant empty vector plasmid (CMV-EV) for 48 h, as described in Materials and Methods. Twenty-four hours posttransfection, the cells were treated with increasing concentrations of TRAIL for an additional 24 h, and apoptosis was determined by detection of active caspase 3 by flow cytometry. The data represent the mean values ± SEM from three independent experiments. *, p < 0.03. Untransfected cells treated with TRAIL vs RKIP-transfected cells treated with TRAIL (Mann-Whitney U test); **, p < 0.02, TRAIL concentration-dependent increase in apoptosis in RKIP-overexpressing cells (Kruskal-Wallis H test). RKIP overexpression after cell transfection was confirmed by Western blot analysis 48 h posttransfection in cell lysates, as described in Materials and Methods. Actin expression was used as internal control. C, Reversal of CDDP-induced sensitization of PC-3 cells to TRAIL-mediated apoptosis by RKIPsiRNA. PC-3 cells were transfected with 1 μg of RKIPsiRNA or equivalent amount of control siRNA for 72 h. Forty-eight hours posttransfection, the cells were treated with 3 μg/ml CDDP for 6 h, followed by TRAIL treatment (10 ng/ml) for an additional 18 h. Apoptosis was determined at 72 h by PI/annexin-V assays. Total whole-cell lysates were prepared at the same time from identical cell treatments and examined for RKIP expression by Western blot analysis. Actin was used as internal control. Apoptosis was significantly reduced in RKIP siRNA-transfected cells treated with the combination of CDDP and TRAIL compared with that observed in similarly treated cells without transfection (p < 0.005, Mann-Whitney U test). The data shown represent the mean values ± SEM from two independent experiments.

The human melanoma cell line M202. The baseline level of RKIP protein expression in M202 cells was found to be minimal, as assessed by Western blot analysis (Fig. 1B, top panel). As with PC-3 cells, RKIP overexpression in M202 cells also significantly sensitized the cells to TRAIL-mediated apoptosis, and the extent of apoptosis was dependent on the TRAIL concentration used (Fig. 1B).

To show that the observed induction of apoptosis was the consequence of RKIP overexpression, we examined whether the sensitization of PC-3 cells to TRAIL-mediated apoptosis by CDDP (14) could be reversed by inhibiting RKIP expression. PC-3 cells were transfected with RKIP siRNA, followed by treatment with CDDP for 48 h (3 μg/ml) and TRAIL (10 ng/ml) treatment for an additional 24 h. Apoptosis was determined at 72 h posttransfection by the PI/annexin-V assay. The combination of CDDP and TRAIL sensitized cells to apoptosis, whereas RKIP siRNA...
significantly reversed this sensitization (Fig. 1C). Cells transfected with control siRNA retained their sensitization pattern, indicating that RKIP has a specific biological role in TRAIL-mediated apoptosis. It is important to note that cell treatment with CDDP for 24 or 48 h resulted in RKIP overexpression compared with untreated control (Fig. 1C, top panel with Western blots). Similar observations were reported in the DU145 prostate cell treated with camptothecin, CDDP, or etoposide (24), suggesting that chemotherapeutic drugs may mediate their apoptotic function via RKIP induction.

DR5 is one of the main DRs of TRAIL, and it has been shown to be up-regulated in tumor models after drug treatment (14, 16). Therefore, we addressed whether the increased sensitivity of tumor cells to TRAIL by RKIP overexpression could be mediated by DR5 up-regulation. To examine at which level RKIP may interfere with DR5 regulation, we performed cotransfection experiments using the CMV-RKIP construct and a wild-type DR5 reporter construct as well as analysis of RKIP protein and mRNA levels before and after RKIP overexpression for 48 h. DR5 promoter activity was consistently up-regulated in PC-3 cells that overexpress RKIP, as indicated by increased luciferase activity (Fig. 2A). This finding was corroborated by the use of DHMEQ, an NF-κB inhibitor, which has been previously shown to up-regulate DR5 promoter activity (11). In contrast, no difference was detected in cells transfected with the control vector. We have previously shown that DR5 is negatively regulated by the transcription repressor YY1. To clearly show that YY1 is directly involved in DR5 up-regulation by RKIP, we examined, in addition to the pDR5 wild-type reporter, two additional DR5 constructs carrying deletions or mutations in the YY1 binding sequence, namely pDR5/Δ505 and pDR5/YY1 mutant, respectively. In both of these reporters, the promoter activity was significantly elevated compared with the wild-type construct. However, RKIP overexpression did not alter the promoter activity of these two constructs because no significant difference was observed between cells transfected with the overexpression and the control vectors (Fig. 2A). The decreased reporter activities of pDR5/Δ505 and pDR5/YY1 mutant after cell transfection with the RKIP or control vectors were attributed to diminished cell viability, followed by cotransfection, as assessed by trypan blue dye exclusion assay (data not shown). These findings show that RKIP regulation of DR5 is via inhibition of YY1. The activation of the DR5 promoter in the presence of high RKIP levels was correlated with increased DR5 mRNA and total protein levels, as detected by RT-PCR and Western blot analysis accompanied by up-regulation of DR5 surface expression, as assessed by flow cytometry (Fig. 2B). The increase in DR5 mRNA and total protein was corroborated by the use of CDDP, an agent that has been previously shown to induce DR5 expression in PC-3 cells (14). In contrast, no change was detected in DcR2 surface level after RKIP overexpression, whereas the DcR1 expression remained unmodified and almost undetected (Fig. 2C). As with the PC-3 cells, elevated surface and total expression of DR5 were observed in M202 cells after RKIP overexpression (Fig. 2D). In contrast to DR5, assessment of DR4 surface levels in M202 cells before and after transfection with the CMV-RKIP vector did not reveal any significant changes (Fig. 2D, right panel). As a positive control for DR5 and DR4 protein expression, we used cells treated with 30 nM Taxol, as previously described (42). The above findings demonstrate that the increased sensitivity of cells to TRAIL following overexpression of RKIP correlates with the enhancement of the DR5 promoter activity and increased DR5 mRNA and protein expression.

RKIP up-regulates DR5 via both inhibition of NF-κB and YY1 activities

Based on our recent findings on the antiapoptotic effect of YY1 as a transcriptional repressor of DR5 (14), we hypothesized that RKIP overexpression might lead to reduced YY1 expression and consequently to DR5 up-regulation. In agreement with this hypothesis, transfection of PC-3 cells with the CMV-RKIP vector resulted in significant inhibition of the YY1 promoter activity (Fig. 3A), decreased YY1 mRNA levels (Fig. 3B), and reduced YY1 protein expression, as assessed by Western blot (Fig. 3C) and flow cytometry analyses (Fig. 3D). In contrast, no differences were observed in the YY1 promoter activity or YY1 expression in cells transfected with the control vector. DHMEQ and CDDP, previously shown to inhibit YY1 promoter activity and protein expression (14), were used as positive controls to corroborate the inhibition of YY1 by RKIP. Consistent with the findings in PC-3 cells, inhibition of YY1 protein levels was also observed in M202 cells overexpressing RKIP (Fig. 3D).

To examine the direct role of YY1 in the regulation of DR5 expression and PC-3 sensitivity to TRAIL-mediated apoptosis, the cells were initially transfected with a predetermined optimal concentration of YY1 siRNA and then incubated with TRAIL for 18 h. Apoptosis and DR5 surface levels were determined by flow cytometry (Fig. 3, E and F, respectively). The findings demonstrate a statistically significant augmentation of apoptosis after treatment with a combination of TRAIL and YY1 siRNA compared with treatment with either TRAIL or YY1 siRNA treatment alone (Fig. 3E). The increased sensitivity of cells to TRAIL-mediated apoptosis was correlated with significant up-regulation of DR5 surface expression (Fig. 3F). These findings support the direct inhibitory role of YY1 in TRAIL-induced apoptosis.

NF-κB has been shown to play a significant role in cell sensitivity to TRAIL-mediated apoptosis (41, 43, 44). PC-3 cells express constitutively active NF-κB, which acts as regulator of YY1 and protects cells from apoptosis (14, 44, 45). We confirmed that inhibition of NF-κB by its chemical inhibitor DHMEQ can sensitize cells to TRAIL-mediated apoptosis as a function of the DHMEQ concentration used (Fig. 4A, upper panel), resulting in YY1 inhibition and DR5 overexpression as assessed by flow cytometry and Western blot analyses, respectively (Fig. 4A, lower panel). DR5 up-regulation and YY1 inhibition were observed after cell treatment for 24 h with DHMEQ; however, prolonged incubation (48 h) resulted in a higher sharper modification. The above findings confirm the suppressive effect of RKIP on YY1 expression via NF-κB inhibition, followed by DR5 up-regulation and cell sensitization to TRAIL-mediated apoptosis. We have reported that one of the biological roles of RKIP is to exert a repressive effect on the NF-κB pathway via direct interaction and modulation of the active phosphorylation of upstream activators such as NIK and TAK (29). To assess whether NF-κB is inhibited by RKIP overexpression, we examined the NF-κB promoter activity before and after cell transfection with the CMV-RKIP or CMV-EV vectors as well as the phosphorylated levels of NIK. NF-κB activity was found to be significantly reduced, as indicated by decreased expression of the NF-κB reporter in RKIP-overexpressing cells (Fig. 4B, upper panel). The inhibition of NF-κB promoter activity by RKIP was corroborated by the use of DHMEQ. To rule out the possibility that RKIP affects additional sites than NF-κB binding sequences in target promoters, we examined a Bcl-xL reporter system that is known to be under the regulation of NF-κB (Fig. 4B, lower panel). PC-3 cells transfected with a wild-type Bcl-xL reporter showed significantly elevated promoter activity compared with activity observed in cells transfected with a Bcl-xL construct.
lacking active NF-κB binding sequence. Cotransfection with the RKIP expression vector inhibited almost completely the wild-type promoter activity, whereas no significant change was observed in the activity of the Bcl-xL NF-κB-mutated promoter. Cotransfection with the control plasmid did not reveal any alterations in the initial activities of both constructs. These results indicate that RKIP mainly targets NF-κB inhibition, and therefore, it induces down-regulation of NF-κB-regulated promoters. NIK phosphorylation was also inhibited in the presence of ectopic RKIP expression, whereas no differences were observed in the inactive NIK.
form (Fig. 4C). Overall, these findings suggest that RKIP up-regulates DR5 and sensitizes tumor cells to TRAIL-mediated apoptosis via a mechanism involving the inhibition of NF-κB and YY1.

The increased sensitivity of tumor cells to TRAIL-mediated apoptosis by RKIP overexpression involves both type I and type II apoptotic pathways and Bcl-xL down-regulation

To investigate the apoptotic pathways activated in tumor cells expressing high RKIP levels, in the presence or absence of TRAIL, we monitored in PC-3 cells the mitochondrial transmembrane potential with DiOC6, as well as the expression of members of the Bcl-2 family known to control the relative susceptibility of cells to apoptotic stimuli (46). Remarkably, the mitochondrial membrane was found significantly depolarized in both TRAIL-treated and -untreated cells that overexpress RKIP (Fig. 5A), indicating that RKIP by itself causes mitochondrial membrane disruption. This was confirmed by Western blot analysis in whole lysates derived from CMV-RKIP-transfected cells, where we detected active caspase 9, although the combination of RKIP overexpression with TRAIL treatment resulted in a more pronounced activation of pro-caspase 9 (Fig. 5B, upper panel). In contrast, RKIP overexpression without TRAIL moderately activated procaspase 8. However, treatment of RKIP-transfected cells with TRAIL resulted in significant procaspase 8 activation, as shown by the active intermediate p43/41 protein products (Fig. 5B, upper panel). According to reported data, the c-FLIP proteins play a significant role in TRAIL-induced apoptosis, by preventing the formation of the death-inducing signaling complex (DISC) via interaction with Fas-associated death domain protein and/or caspase 8 (47). To evaluate the correlation between c-FLIP variant expression and TRAIL sensitivity...
before and after RKIP overexpression, we measured c-FLIP long (c-FLIPL) and c-FLIPS expression levels in PC-3 cells by Western blot analysis (Fig. 5B, upper panel). Interestingly, we did not detect any c-FLIPS expression in the treatments tested. In contrast, c-FLIPL showed constitutively high basal levels that were significantly decreased following RKIP overexpression. A more potent c-FLIPL inhibition was observed after cell treatment with TRAIL, indicating that c-FLIPL expression is affected by RKIP and may regulate TRAIL-induced cell death.

Taking into account that active caspase 8 may activate mitochondria and apoptosome formation via Bid cleavage and Bax dimerization, we investigated whether the enhancement of TRAIL-induced apoptosis by RKIP overexpression involves the modulation of Bid, Bax, Bcl-xL, and XIAP expression (Fig. 5B, upper panel). The levels of tBid and Bax in lysates from cells overexpressing RKIP were almost comparable with those detected in control lysates. However, the levels of these proteins remarkably increased after TRAIL treatment, indicating that there may be a cross-talk between type I and type II apoptotic pathways after cotreatment of cells with CMV-RKIP vector and TRAIL. Moreover, Western blot analyses on the same whole-cell extracts indicated that PC-3 cells had higher levels of Bcl-xL and XIAP expression than of Bax expression. RKIP overexpression led to significant down-regulation of Bcl-xL and XIAP levels that would, presumably, lead to decreasing the Bcl-xL/Bax ratio. RKIP overexpression and TRAIL treatment in combination resulted in more efficient down-regulation of Bcl-xL and XIAP expression and induction of Bax, thus, presumably, increasing the Bax/Bcl-xL ratio, which, in turn, resulted in procaspase 9 and 3 activation and potentiation of TRAIL-mediated apoptosis. Down-regulation of XIAP, Bcl-xL,
and c-FLIPL expression was also observed after treatment of PC-3 cells with the NF-κB inhibitor DHMEQ for 24 h. The inhibition of the above antiapoptotic gene products was more potent after longer (48 h) cell incubation with DHMEQ (Fig. 5B, lower panel). These findings suggest that the inhibitory effects of RKIP on the expression of the above antiapoptotic gene products are attributed to inhibition of NF-κB.

It has been reported that both Bcl-xL and XIAP transcription are regulated, in part, by NF-κB (48–50). Because RKIP inhibits NF-κB, it is possible that sensitization of PC-3 by RKIP to TRAIL-induced apoptosis might have resulted from down-regulation of Bcl-xL expression via NF-κB inhibition. Accordingly, inhibition of Bcl-xL should sensitize PC-3 cells that overexpress RKIP to TRAIL-mediated apoptosis. Thus, treatment of PC-3 with the Bcl-xL inhibitor 2MAM-A3 resulted in significant cell sensitivity to TRAIL-mediated apoptosis, and the extent was a function of the TRAIL concentration used (Fig. 5C). Moreover, treatment of cells with RKIP siRNA restored Bcl-xL levels, as assessed by Western blot analysis (lower panel).

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The above findings demonstrate that type I and type II apoptotic pathways are activated in the presence of high RKIP levels in tumor cells, which after costimulation with TRAIL facilitate the induction of apoptosis.

Discussion

RKIP expression has been reported to be diminished in many tumors and completely absent in metastases (51–53). Although the molecular mechanisms of how RKIP inhibits Raf and NF-κB pro-survival pathways have been partially delineated (28), little is known about the role of RKIP in the pathogenesis of tumor cell resistance to immune host defense mechanisms. Consistent with the inhibitory effects of RKIP on NF-κB and Raf/MEK/ERK signaling, we have recently reported that RKIP can reverse cancer cell resistance to chemotherapy in prostate and breast carcinoma cell lines (24). These findings imply that RKIP is part of a cell fail-safe mechanism against apoptosis. The effect of RKIP on apoptosis signaling appears to be specific to cancer cells because it has not been observed on primary or immortalized untransformed cells (28). Furthermore, the levels of RKIP in tumor cells have been
considered crucial determinants of tumor fate to apoptosis, because down-regulation of RKIP expression is another mechanism that cancer cells use to evade apoptotic stimuli (54).

In the present study, we have investigated the role of RKIP in tumor cell sensitivity to TRAIL-mediated apoptosis and have explored the underlying mechanism. As experimental models, we used a human prostate and a melanoma cell line, namely PC-3 and M202, respectively, which are relatively resistant to TRAIL apoptotic stimulus and express basal levels of RKIP. In this study, we provide evidence for the first time demonstrating that RKIP regulates tumor cell sensitivity to TRAIL, and RKIP induction sensitizes TRAIL-resistant tumor cell lines to TRAIL-mediated apoptosis. The direct effect of RKIP in the induction of TRAIL-mediated apoptosis was confirmed by the ability of RKIP siRNA to reverse the drug-induced sensitization of PC-3 cells to TRAIL-mediated apoptosis. RKIP overexpression inhibited both NF-κB and YY1 activities concomitant with up-regulation of DR5 expression. Furthermore, RKIP overexpression modulated type I and type II apoptotic pathways and in combination with TRAIL triggered TRAIL-induced apoptosis.

To uncover the underlying mechanism of the increased tumor sensitivity to TRAIL under high RKIP levels, we explored the involvement of the TRAIL receptor, DR5. Increased RKIP levels were correlated with elevated DR5 mRNA and protein expression as well as increased DR5 promoter activity. We have previously shown that DR5 up-regulation is also involved in drug-induced sensitization of several tumor cells to TRAIL-mediated apoptosis via inhibition of DR5 transcriptional repressor YY1 through NF-κB suppression (14). Considering the inhibitory effect of RKIP on the NF-κB signaling pathway, we monitored the YY1 promoter activity and YY1 expression in our cell lines. When RKIP was overexpressed, both YY1 promoter activity and expression were decreased upon high RKIP levels, suggesting that YY1 could be a target of RKIP function. The direct involvement of YY1 as a suppressor of tumor cell sensitization to TRAIL-induced apoptosis was revealed by the use of YY1 siRNA, which was shown to reverse TRAIL resistance and to reduce apoptosis by TRAIL. The induction of cell sensitization to TRAIL by inhibiting YY1 with YY1 siRNA was correlated with DR5 up-regulation, indicating that modulation of DR5 expression by YY1 suppression is a key component for tumor cell response to TRAIL in our system. The direct involvement of YY1 in DR5 up-regulation by RKIP was consistent with the findings showing that deletions or mutations in the putative YY1 binding sites in the DR5 promoter abrogated the enhancing effect that the RKIP overexpression had on the wild-type DR5 promoter. Thus, we propose RKIP as a regulator of TRAIL-mediated apoptosis via a mechanism that involves both YY1 and DR5. In contrast to DR5 assessment of DR4 surface levels in M202 cells before and after transfection with the CMV-RKIP vector did not reveal any significant changes. Additionally, examination of the DR4 promoter by computer-based transcription search (TESS) analysis revealed the absence of YY1 putative binding sites clustered within the promoter region (~1000 bp from the start codon). Similarly, we did not detect any significant change in the expression of decoy receptors in the surface of PC-3 after RKIP overexpression.

We have shown that chemical inhibitors of NF-κB are able to suppress YY1 expression and promoter activity in vitro in several cancer cell lines (14, 39). Our observations are consistent with a recent report demonstrating a positive regulation of YY1 by NF-κB in myoblasts (45). In this study, we present findings showing that inhibition of NF-κB by DHMEQ or inhibition of YY1 by YY1 siRNA sensitizes cells to TRAIL-mediated apoptosis ensuing by DR5 up-regulation. Thus, we propose that RKIP mimics the chemical inhibitors of NF-κB, resulting in YY1 inhibition by NF-κB down-regulation and potentiation of TRAIL-mediated apoptosis via DR5 up-regulation. We have shown that RKIP acts upstream of the kinase complex that mediates the phosphorylation and inactivation of the inhibitor of NF-κB, IκB, by interacting physically with four kinases of the NF-κB activation pathway, namely, NIK, TAK1, IκB kinase (IKK)α, and IKKβ (29). Hence, modulation of RKIP expression levels was shown to antagonize the signal transduction pathways that mediate the activation of NF-κB in response to TNF-α and IL-1β by antagonizing the activation of IKK elicted by TNF-α (29). Consistent with those findings, we report in this study that overexpression of RKIP in PC-3 cells results in decreased NF-κB promoter activity and limited NIK phosphorylation. Additionally, we show using an NF-κB-target promoter, Bcl-xL, that RKIP affects mainly the NF-κB-binding sites because RKIP expression down-regulated Bcl-xL wild-type promoter activity, but it did not alter the minimal activity of the Bcl-xL promoter with inactive NF-κB binding site. Similar observations have also been shown in our previous reports (29), indicating that RKIP mainly functions as an NF-κB inhibitor, and promoters that are transcriptionally regulated by NF-κB could most likely be inhibited by RKIP.

Overall, we suggest that NF-κB inhibition by RKIP down-regulates YY1 and increases DR5 expression, accounting for more potent TRAIL signaling. However, we cannot rule out the possibility that inhibition of the Raf/MEK/ERK pathway by RKIP might also have an enhancing effect in DR5 signaling after TRAIL ligation. It has been shown that the MAPK/ERK pathway is able to override the apoptotic signaling by DRs, including TRAIL receptors (11, 20). This protecting effect over apoptosis, which was found to be independent of newly synthesized proteins, might act in all cases by suppressing the activation of the caspase effector machinery (11). Although we have reported that modulation of RKIP expression affected NF-κB signaling independently of the MAPK pathway, the inhibitory effects of RKIP in both pathways might account for reinforcement of TRAIL signaling through the cognate receptors. Our proposed model for RKIP-induced DR5 up-regulation and tumor sensitization to TRAIL via YY1 and NF-κB inhibition could be extended to other DRs like Fas. We have shown that Fas expression is also under the negative regulation of YY1, and inhibition of YY1 and NF-κB by agents like NO sensitizes cells to FasL-mediated apoptosis (35). Thus, we can speculate that RKIP-induced down-regulation of YY1 and NF-κB might have similar effects on Fas overexpression, resulting in increased cell sensitivity to FasL-mediated apoptosis.

Because little is known about the mechanism by which RKIP is able to affect apoptotic stimuli and apoptosis-related gene targets, we paid special attention to clarify where the RKIP apoptotic effects may take place in relation to the mitochondrial signaling sequence. We showed that RKIP overexpression by itself causes depolarization of mitochondrial membrane and activation of procaspase 9. In addition, RKIP contributes to down-regulation of antiapoptotic gene products such as XIAP and Bcl-xL by interacting with the mitochondrial apoptotic pathway by RKIP. Because the mitochondrial pathway has been reported to be involved in the TRAIL-induced apoptosis (55), treatment of RKIP-overexpressing cells with TRAIL was shown to enhance the activation of the intrinsic apoptotic pathway by additional effects on Bid cleavage and Bax activation. In addition, the activation of caspase 8 as a consequence of TRAIL and CMV-RKIP cell cotreatment revealed an
The interplay between extrinsic and intrinsic apoptotic cascades under the above conditions. Among the above apoptosis-related gene products shown to be affected by RKIP, we have reported that Bcl-xL has a discrete dominant role in prostate tumor cell resistance to TRAIL-mediated apoptosis (41) because it can be reversed after treatment with Bcl-xL inhibitors, like 3MAM-A3. In this study, we report that Bcl-xL expression, which was significantly reduced under high RKIP levels, can be restored by blocking RKIP with siRNA against RKIP, suggesting that Bcl-xL is a potent target of RKIP and, through its inhibition, RKIP might mediate its proapoptotic function. Similar observations have been derived by treatment of non-Hodgkin's B cell lymphoma with rituximab, which results in cell chemosensitization by up-regulation of RKIP, inhibition of the Raf-MEK1/2-ERK 1/2 signaling pathway, and Bcl-xL down-regulation (56).

The present findings assign a crucial role of RKIP expression in tumor sensitivity to TRAIL and support the notion that RKIP might also regulate host immune surveillance strategies against cancer. Immune surveillance results primary by apoptosis of neoplastic cells induced by host effector cells such as NK and NKT cells, CTL, or IFN-producing killer dendritic cells via TRAIL, FasL, or perforin/granzyme-dependent mechanisms (1, 57). Immuno-selected tumor cells with low expression of the relevant receptors like DR5 or Fas are relatively resistant to killing by the above killer cells. The induction of DR5 in tumors cells by high RKIP expression, as we propose in this study, can be considered as boost for the immune system to fight cancer by restoring normal innate and adaptive cytotoxic functions. Thus, it is possible that RKIP may belong to a new group of immune surveillance cancer gene products that are involved in cancer immune surveillance and may be clinically useful for reversal of tumor resistance by the host.

As a regulator of MAPK signaling, RKIP has also been implicated in cancer progression. RKIP expression is diminished in several tumors, including prostate, melanoma, and breast cancer, and this decrease was found to correlate with the extent of metastatic disease. Restoration of its expression in xenograft mouse models was shown to inhibit metastasis, identifying, therefore, RKIP as a metastasis suppressor gene with prognostic significance (51–53). The role of immune surveillance in preventing metastasis is still not well defined (58). However, because metastasis seeds from primary tumors that have been immunoselected by evading immune surveillance (59), it is reasonable to implicate host immunity in modulating metastatic spread. Taking into account the notion that RKIP regulates immune surveillance and the tumor metastatic potential, we suggest that the levels of RKIP expression in different tumors may be crucial determinants of cancer progression by modulating in part the host immune response against cancer. Thus, the clinical relevance of restoring RKIP expression by treating patients with RKIP-inducible agents might correlate with a favorable clinical outcome in cases of tumors with low or absent RKIP levels. In addition, RKIP overexpression may increase tumor sensitivity to externally administrated immunostimulating agents such as TRAIL or chemotherapeutic molecules via a mechanism that implicates YY1 and NF-κB inhibition. However, the novel identified role of RKIP in the present study suggests that this gene product might prevent cancer establishment and progression via regulation of multiple cascades and checkpoints. Recently, RKIP was identified as a regulator of mitotic progression by altering the integrity of the spindle assembly checkpoint during the cell cycle (60, 61). The mitotic defects elicited by RKIP depletion were shown to be due, at least in part, to elevated MAPK activity via inhibition of Aurora B kinase (60).
In conclusion, the present study provides the first evidence of the involvement of RKIP in the positive regulation of TRAIL-mediated apoptosis, and hence, in the regulation of host immune surveillance strategies against cancer. We propose that one underlying mechanism for this increased sensitivity could be the inhibition of YY1 and DR5 up-regulation via the inhibitory effects of RKIP on the NF-kB pathway. Fig. 6 schematically illustrates RKIP-induced up-regulation of DR5 and tumor cell sensitization to TRAIL, as well as the apoptosis-related gene products shown to be targets for RKIP. We suggest that RKIP mediates its positive effect on TRAIL-mediated apoptosis via activation of both the extrinsic and intrinsic apoptotic pathways. Considering this novel role of RKIP in tumor immunosensitization, we suggest that therapeutic strategies using RKIP overexpression in tumor cells will either improve the host immune surveillance or the efficacy of anti-tumor therapies, especially if they are combined with immunostimulatory or chemotherapeutic regimens. Because RKIP level of expression has been shown to be of prognostic significance in several cancers, our findings in this study suggest that RKIP levels may regulate tumor cell response to host immune surveillance mechanisms, and hence, RKIP may be considered as a novel immune surveillance cancer gene.

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Disclosures

The authors have no financial conflict of interest.

References


Letter of Retraction

A request was received from the Acting Research Integrity Officer of the University of California, Los Angeles (UCLA), to retract this article: “Regulation of Tumor Cell Sensitivity to TRAIL-Induced Apoptosis by the Metastatic Suppressor Raf Kinase Inhibitor Protein via Yin Yang 1 Inhibition and Death Receptor 5 Up-Regulation” by Stavroula Baritaki, Alina Katsman, Devasis Chatterjee, Kam C. Yeung, Demetrios A. Spandidos, and Benjamin Bonavida, The Journal of Immunology, 2007, 179: 5441–5453.

The Editor-in-Chief of The Journal of Immunology was informed that UCLA had conducted a review of the work and concluded that data used in some of the figures in this article could not be supported. In particular, issues involving duplication of images were identified in Figs. 1B, 1C, 2B, 4C, 5B and 5C. The article is therefore retracted.