Discoidin Domain Receptor 1 Receptor Tyrosine Kinase Induces Cyclooxygenase-2 and Promotes Chemoresistance through Nuclear Factor-κB Pathway Activation

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Abstract

Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase activated by various types of collagens and is known to play a role in cell attachment, migration, survival, and proliferation. However, little is known about the molecular mechanism(s) underlying the role of DDR1 in cancer. We report here that DDR1 induces cyclooxygenase-2 (Cox-2) expression resulting in enhanced chemoresistance. Depletion of DDR1-mediated Cox-2 induction using short hairpin RNA (shRNA) results in increased chemosensitivity. We also show that DDR1 activates the nuclear factor-κB (NF-κB) pathway and blocking this activation by an IκB superrepressor mutant results in the ablation of DDR1-induced Cox-2, leading to enhanced chemosensitivity, indicating that DDR1-mediated Cox-2 induction is NF-κB dependent. We identify the upstream activating kinases of the NF-κB pathway, IκKβ and IκKY, as essential for DDR1-mediated NF-κB activation, whereas IκKα seems to be dispensable. Finally, shRNA-mediated inhibition of DDR1 expression significantly enhanced chemosensitivity to genotoxic drugs in breast cancer cells. Thus, DDR1 signaling provides a novel target for therapeutic intervention with the prosurvival/antiapoptotic machinery of tumor cells. (Cancer Res 2006; 66(16): 8123-30)

Introduction

Discoidin domain receptor 1 (DDR1) belongs to a small family of receptor tyrosine kinases that contain an extracellular domain of 160 amino acids exhibiting strong homology to the Dictyostelium discoideum protein discoidin 1 (1). DDR1 was isolated as a novel member of the receptor tyrosine kinase (RTK) family (2) and was also identified as a gene overexpressed in breast cancer cells (3). Most RTKs are activated by soluble proteins present in the blood or other body fluids. The DDR1 RTK is unusual in that it is activated by the extracellular matrix protein collagen (4, 5), but independent of the major cellular collagen receptor integrins (6). Various types of collagen have been identified as ligands capable of activating DDR1 (4, 5). The activation process is unique in that it is slow but is sustained over long periods with no down-regulation by endocytosis or receptor degradation (4, 5). DDR1 is widely expressed in human and mouse epithelial cells (7). It has been found to be involved in cell interactions with the extracellular matrix and in controlling adhesion and cell motility (8). DDR1 signaling is essential for cerebellar granule differentiation (9), the development of adaptive immune responses (10), arterial wound repair (11), and mammary gland development (12). A number of studies have reported overexpression of DDR1 in lung, esophageal, breast, ovary, and pediatric brain cancers, suggesting a role for DDR1 in tumor progression (2, 7, 13–17). Moreover, the elevated expression of DDR1 in a number of fast-growing invasive tumors has suggested that this matrix-activated RTK may be involved in the proliferation and stromal invasion of tumors.

Accumulating evidence indicates that cyclooxygenase-2 (Cox-2) plays an important role in the promotion of various proliferative diseases, including cancer. Cox-2 catalyzes the synthesis of prostaglandins (18), and Cox-2 up-regulation as well as increased levels of prostanoids have been reported in a high percentage of tumors (19). Prostanoids affect multiple mechanisms that have been implicated in carcinogenesis (19–21). For example, prostaglandin E2 can stimulate cell proliferation and angiogenesis while inhibiting apoptosis and immune surveillance (21). Furthermore, overexpression of Cox-2 is sufficient to induce tumorigenesis in transgenic mice (22). Epidemiologic studies have also shown that the use of nonsteroidal anti-inflammatory drugs, prototypic inhibitors of cyclooxygenases, is associated with a reduced risk of several malignancies (23).

There is as yet relatively little information concerning DDR1 downstream signaling pathways or functions. It was shown that the DDR1 is a direct p53 transcriptional target and that it can function as a survival effector in wild-type (wt) p53-containing cells exposed to genotoxic stress (24), suggesting that inhibition of DDR1 function may provide a novel approach to selectively enhance therapy of such tumors. We undertook our present studies in an effort to identify downstream signaling targets of DDR1, in particular those that may contribute to the DDR1-induced prosurvival pathways. Our results identify a signaling pathway mediated by DDR1 involving activation of nuclear factor-κB (NF-κB) and its downstream effectors, including Cox-2 and XIAP. These findings establish the molecular mechanisms underlying DDR1 protection against genotoxic stress.

Materials and Methods

Cell lines and culture conditions. Human breast cancer cell lines MDAMB435 and T47D were cultured in DMEM containing fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C. All mouse embryonic fibroblasts (MEF), including Cox-2−/− MEF, IκKα−/− MEF, IκKβ−/− MEF, and IκKY−/− MEF (25–27) were also maintained in DMEM containing 10% FBS. For drug treatment, cells were grown to ~50% confluency before exposure to the DNA-damaging agents, mitomycin C at a concentrations of 2.5 to
pRL-TK, which expresses Renilla luciferase. In transfection efficiency across samples was normalized by cotransfecting the Luciferase Reporter assay system (Promega, Madison, WI). The difference 24 hours posttransfection and luciferase activity was determined using Dual Luciferase Reporter assay system (Promega, Madison, WI). The difference in transfection efficiency across samples was normalized by cotransfecting pBL-TK, which expresses Renilla luciferase.

**Apoptosis assay.** Apoptosis was measured using the Cell Death Detection ELISA kit (Roche, Indianapolis, IN). Briefly, the cells were lysed and the amount of nucleosomes in the cytoplasmatic fraction of the cell lysates was measured using antihistone antibody and anti-DNA antibody linked to peroxidase in a sandwich ELISA-based protocol. For calculating relative nucleosome content, the absorbance of all the samples was normalized with respect to that of untreated/untransfected cells.

**Short hairpin RNAs.** Vectors expressing short hairpin RNA (shRNA) against Cox-2 (5'-GCTCAACACCGGAAATTTC-3'), luciferase (5'-GTGTGGGCGCGTATTTC-3'), DDR1 (5'-AAATCCTCATTCTCTGAAT-3'), and GFP (5'-GCAGCTAGCTCAGCTCT-3') were generated using the pBabe-U6-shRNA plasmid. Cells were transfected with LipofectAMINE 2000 (Invitrogen) according to the protocol of the manufacturer in the presence of shRNA.

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assays (EMSA) were done as described previously (28, 29). Briefly, after the indicated drug treatment, nuclear extracts were made using a Nuclear Extraction Kit (Panomics, Redwood City, CA). Ten micrograms of nuclear extract were incubated with 32P-labeled NF-κB consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) in gel shift binding buffer (Promega). The DNA-protein complexes were then resolved by electrophoresis through Novex 6% DNA retardation gel (Invitrogen). Control oligos representing the consensus oligonucleotide unlabeled or unlabeled NF-κB mutant oligonucleotide (Santa Cruz Biotechnology) were added in 500-fold excess.

**Western blot analysis.** Western blots were done as described previously (30). The following antibodies were used: DDR1 (Santa Cruz Biotechnology), β-actin (Sigma), Bcl-2 (BD Transduction Laboratories, San Diego, CA), XIAP (Cell Signaling Technology, Danvers, MA), Cox-2 (BD Transduction Laboratories), and b-B (Santa Cruz Biotechnology).

**Results**

**DDR1 induces Cox-2 expression.** We have previously shown that both DDR1 and Cox-2 promote cell survival upon p53-dependent genotoxic stress (24, 30). To identify the downstream signaling targets of DDR1, in particular those that may contribute to the DDR1-induced prosurvival pathway, we initially investigated the effects of DDR1 overexpression on Cox-2 expression. For this purpose, we infected wt and Cox-2−/− MEF with recombinant Ad-DDR1 or control Ad-GFP and measured levels of Cox-2 expression. As shown in Fig. 1A, ectopic DDR1 expression in wt-MEF resulted in induction of Cox-2 expression in the presence of its ligand collagen, whereas the control virus, Ad-GFP, had no effect. We also generated tetracycline-regulated (tet-off) DDR1 expression system in MDAMB435 cells (MDAMB435-DDR1) and tested whether DDR1 also induced Cox-2 in a different cell type. The results showed that following tet withdrawal, Cox-2 expression increased concomitantly with increasing DDR1 expression and only in the presence of collagen IV (Fig. 1A, middle), suggesting that DDR1-mediated Cox-2 induction was specific for the activated DDR1. In fact, activated induced DDR1 as measured by tyrosine phosphorylation, upon tet-removal, was detected only in the presence of collagen (Fig. 1A, right). These results support the conclusion that activation of the overexpressed DDR1 is required for its induction of Cox-2 expression.

**DDR1-mediated chemoresistance is Cox-2 dependent.** The DDR1 kinase is activated/phosphorylated in response to genotoxic stress in wt-p53-containing cells and confers increased resistance to apoptosis (24). Cox-2 is also up-regulated in wt-p53-containing cells and is protective against genotoxic stress (30). Thus, we next tested whether DDR1-induced Cox-2 played an important role in DDR1-mediated cell survival upon genotoxic stress. For this purpose, we infected wt- and Cox-2−/− MEFs with Ad-DDR1 or control virus Ad-GFP at a MOI of 20, and measured the extent of cell death at different time points following exposure to different chemotherapeutic agents. DDR1 overexpression resulted in a marked decrease in apoptosis in the presence of etoposide at two different concentrations (40 and 80 μmol/L) in wt-MEFs, but had no significant effects in Cox-2-null MEFs under the same conditions (Fig. 1B). These results were observed with another therapeutic agent mitomycin C. These findings supported the concept that DDR1-mediated survival or chemoresistance is Cox-2 dependent. To test the role of DDR1 in chemoresistance in human cancer cells, we induced DDR1 expression in tet-off–regulated MDAMB435-DDR1 and measured the extent of apoptosis upon the addition of genotoxic drugs, etoposide or mitomycin C. As shown in Fig. 1C, DDR1 induction by tet removal or Ad-DDR1 infection resulted in ~2- to 3-fold reduction in the extent of apoptosis, upon the addition of etoposide or mitomycin C, compared with similarly treated control cells grown in the presence of tet or infected with Ad-GFP (Fig. 1C). To further validate the effect of Cox-2 induction on DDR1-mediated survival, we used shRNA to suppress the expression of endogenous Cox-2 in human breast cancer cells. Transfection of Cox-2 shRNA resulted in the suppression of Cox-2 expression in Ad-DDR1–infected MDAMB435 cells, compared with cells transfected with control shRNA vector (pBabe-U6-shRNA-luciferase; Fig. 2A, left). Next, we examined the effects of Cox-2 depletion on DDR1-mediated chemoresistance in DDR1-overexpressing MDAMB435 cells. As shown in Fig. 2A (right), the Ad-DDR1–infected cells were more resistant to etoposide treatment compared with control Ad-GFP–infected cells. However, depletion of Cox-2 induction via Cox-2 shRNA transfection resulted in a dramatic increase of genotoxic stress–induced apoptosis in Ad-DDR1–infected cells (compare columns 3 and 6). The antiapoptotic protein, Bcl-2, was also increased in response to ectopic expression of DDR1, but Bcl-2 induction was drastically reduced by Cox-2 depletion (Fig. 2A), consistent with several previous reports that Bcl-2 expression is regulated through a Cox-2–dependent mechanism (22, 31). To further validate these results, we examined the effect of Cox-2 inhibition in a human breast cancer cell line, T47D, containing high basal expression of both DDR1 and Cox-2 proteins (Fig. 2B, left). Endogenous Cox-2 expression was significantly suppressed by transfection with Cox-2 shRNA but not by luciferase control shRNA (Fig. 2B). Moreover, depletion of Cox-2 resulted in a reproducible decrease in Bel-2 expression in T47D cells and also caused a marked increase in apoptosis in the presence of etoposide, compared with results with shRNA-transfected or nontransfected T47D cells (Fig. 2B, right). Thus, the high basal expression level of DDR1 in T47D cells seems likely to be a causative factor in the chemoresistant phenotype of these tumors.
cells. Taken together, these results show that DDR1, through a Cox-2–dependent mechanism, can play an important role in the chemoresistant phenotype of tumor cells.

**DDR1 activates the NF-κB prosurvival pathway.** It is well established that Cox-2 is a downstream target of the NF-κB pathway (32, 33). To explore whether DDR1 activates NF-κB signaling, we first examined its effects on NF-κB activity by using an NF-κB promoter–luciferase reporter construct. As shown in Fig. 3A, Ad-DDR1 infection activated NF-κB activity, whereas Ad-GFP infection of parental MDAMB435 cells had no effect on the NF-κB promoter. Next, we did EMSA to examine the effects of DDR1 on NF-κB DNA-binding activity. The DNA-binding activity of NF-κB was significantly increased in cells infected with Ad-DDR1 to a level comparable with that observed with tumor necrosis factor-α, activator of this pathway, whereas there was no NF-κB binding activity in Ad-GFP–infected control cells (Fig. 3B). The increased level of NF-κB DNA-protein complexes observed in response to DDR1 overexpression was abrogated when an excess of cold wt-NF-κB binding oligonucleotide was added to compete its binding, whereas the addition of mutant oligo had no effect on NF-κB DNA-binding activity (Fig. 3B). To further determine the specific role of DDR1 in NF-κB–mediated Cox-2

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**Figure 1.** DDR1 induces Cox2 expression, and DDR-mediated chemoresistance is abolished in Cox-2 null cells. A, wt and Cox-2+/− MEFs were infected with Ad-DDR1 or the control Ad-GFP at 20 MOI in the presence of collagen IV (10 μg/mL). Forty-eight hours postinfection, cell extracts were made and Western blotting was carried out for the indicated proteins (left). Right, tetracycline-regulated DDR1 expression (MDAMB435-DRR1 tet-off) induces Cox-2 expression after tet removal for indicated times only in the presence of collagen IV, and cell extracts were made and Western blotting was carried out for DDR1, Cox-2, and β-actin. At the indicated time after tet removal, cells were lysed and DDR1 proteins were immunoprecipitated. The levels of tyrosine phosphorylation were determined by Western blot (WB) analysis using PY20 phosphotyrosine antibody. The quantity of DDR1 proteins in the immunoprecipitate (IP) was determined by Western blotting using DDR1 antibody. B, DDR1 expression renders chemoresistance in a Cox-2–dependent manner. Wt- and Cox-2+/− MEFs were infected with Ad-DDR1 or the control Ad-GFP at 20 MOI, and 24 hours later, cells were treated with the indicated concentrations of the chemotherapeutic drugs, etoposide and mitomycin C, for 24 and 48 hours. At the same time, collagen IV was also added to the cells. At the indicated time points, cell death detection ELISA was carried out to determine the apoptosis rate as described in Materials and Methods. DMSO was treated as a choice of solvent. Columns, mean of three independent experiments with each sample in triplicate; bars, SD. C, DDR1 expression increases cell survival in response to genotoxic stress in MDAMB435 cells. MDAMB435 cells were infected with Ad-DDR1 or the control virus Ad-GFP at 10 MOI in the presence of collagen IV. Twenty-four hours later, indicated concentrations of etoposide and mitomycin C were added. Twenty-four hours after drug treatment, cell death detection ELISA was carried out. A similar experiment was also carried out with MDAMB435-DDR1 tet-off cells, which were treated with etoposide in the presence of collagen IV with or without tet.
induction, we tested whether the IκBα superrepressor mutant abolishes Cox-2 expression induced by DDR1. MDAMB435 cells were transfected with the superrepressor expressing plasmid in the presence of Ad-DRD1 or Ad-GFP. Western blot analysis, as shown in Fig. 3C, shows that the superrepressor IκB markedly inhibited Cox-2 expression induced by DDR1 (Ad-DRD1 infection), whereas transfection of the vector alone (pCDNA3) failed to do so. Taken together, these results show that DDR1 increases NF-κB activity and that DDR1-mediated Cox-2 induction is NF-κB dependent.

Abrogation of NF-κB activity abolishes DDR1-mediated chemoresistance. Given the ability of DDR1 to enhance NF-κB activity, we investigated whether DDR1 actually exerts its activity in DDR1-overexpressing breast cancer cells. Ectopic DDR1 expression in MDAMB435 cells with little basal DDR1 expression significantly reduced the apoptotic response to etoposide, compared with that of cells with ectopic GFP expression (Fig. 44). However, transfection of the superrepressor IκB resulted in a significant reduction of DDR1-induced chemoresistance (Fig. 4A, columns 3 and 6). We also examined the effect of NF-κB inhibition in T47D cells, which have a high basal level of DDR1 expression. As shown in Fig. 4B, transfection of the IκB superrepressor resulted in a drastic increase in etoposide-induced apoptosis in a dose-dependent manner. In contrast, transfection of vector itself along with increasing doses of etoposide induced only a marginal increase in apoptosis. These results indicate that DDR1 increases NF-κB activity and its activation plays an important role in DDR1-mediated chemoresistance in some breast cancer cells.

IκKβ and IκKγ are required for DDR1-mediated NF-κB activation and chemoresistance. The IκK complex (IκB kinase) is the upstream activating kinase of the NF-κB pathway. It is composed of two catalytic subunits, IKKα and IκKβ, which can directly phosphorylate IκB, whereas IκKγ acts as the regulatory subunit. Activation of the canonical NF-κB pathway based on IκB degradation is mostly dependent on IκKβ. To investigate whether these NF-κB upstream regulators affect DDR1-mediated NF-κB activation, we examined the ability of DDR1 to activate NF-κB and its targets in IKK knockout MEFs (i.e., IKKα−/−, IKKβ−/−, and IκKγ−/− MEFs). Each MEF was transfected with an NF-κB luciferase reporter construct (NF-κB-Luc) or control luciferase construct (pGL3) along with Ad-DDR1 or Ad-GFP. DDR1 enhanced NF-κB activity in wt-MEF and IKKα−/− MEF (Fig. 5a), but the activation of NF-κB by DDR1 was significantly diminished in both IKKβ−/− and IκKγ−/− MEFs (Fig. 5a). Ectopic DDR1 induced increased expression of NF-κB targets, Cox-2 and XIAP, in wt-MEFs, and transfection of the superrepressor IκB mutant into wt-MEFs significantly inhibited the DDR1-mediated increase of Cox-2 and XIAP expression (Fig. 5b, left). Moreover, the DDR1-mediated activation of NF-κB targets, Cox-2 and XIAP, was abolished in IKKβ−/− and IκKγ−/− MEFs (Fig. 5b, right). Taken together, these data showed that IκKβ and IκKγ are essential for DDR1-mediated NF-κB activation, but that IκKα is dispensable. To determine whether DDR1-mediated survival or chemoresistance also requires the IKK subunits, we examined the antiapoptotic role of DDR1 in response to etoposide in IKK knockout MEFs. As seen in other cell types, DDR1 overexpression suppressed etoposide-induced apoptosis in wt-MEFs and IKKα−/− MEFs (Fig. 5c and D). Moreover, abrogation of NF-κB pathway via the superrepressor IκB mutant further enhanced the apoptotic response to etoposide in wt-MEFs. Of note, the DDR1-mediated antiapoptotic effect was not observed in IKKβ−/− or IκKγ−/− MEFs (Fig. 5d). These results strongly imply that DDR1 promotes cell survival via the activation of NF-κB and its target genes and that IκKβ and IκKγ subunits of the IKK complex are required for mediating the prosurvival signal of DDR1 upon genotoxic stress.

Chemosensitization of breast cancer cells by depletion of DDR1 expression. Given that DDR1 is involved in NF-κB prosurvival signaling and chemoresistance, we sought to determine whether DDR1 inhibition might be sufficient to promote chemosensitization in human breast cancer cells exhibiting high basal levels of DDR1. Inhibition of DDR1 expression by DDR1 shRNA in T47D cells in which DDR1 and Cox-2 are expressed at high levels significantly suppressed the levels of endogenous DDR1 and Cox-2 as measured by immunoblotting (Fig. 6a). Moreover, depletion of

Figure 2. DDR1-mediated chemoresistance is Cox-2 dependent. A, depletion of Cox-2 induction represses Bcl-2 expression and reduces DDR1-mediated chemoresistance. MDAMB435 cells were transfected with Cox-2 shRNA or luciferase shRNA constructs, then the cells were infected with Ad-GFP or Ad-DDR1 at 10 MOI. Twenty-four hours later, 40 μmol/L etoposide was added in the presence of collagen IV. Twenty-four hours after drug addition, cell extracts were made and Western blotting were carried out for the indicated proteins or subjected to cell death detection ELISA. Left, Western blot analysis using lysates prepared from MDAMB435 cells transfected with Cox-2 shRNA or control shRNA. Right, the pattern of apoptosis analysis by cell death ELISA. B, knockdown of basal Cox-2 expression suppresses Bcl-2 expression and enhances apoptosis in response to etoposide treatment. T47D cells were transfected with Cox-2 shRNA or luciferase shRNA constructs. Twenty-four hours later, 40 μmol/L etoposide was added in the presence of collagen IV. Twenty-four hours after drug treatment, cell extracts were made and Western blots were carried out for the indicated proteins or subjected to cell death detection ELISA.
DDR1 modulated total IκB protein level (Fig. 6A). Although transfection of control shRNA (luciferase shRNA) as well as vector alone (pBabe-U6-shRNA) had no effect on the levels of Cox-2, Bcl-2, and IκB, cotransfection of the superrepressor IκB mutant with either luciferase-shRNA or vector alone inhibited both Cox-2 and Bcl-2 expression (Fig. 6A). These data further argue that DDR1 is an upstream regulator of the NF-κB pathway. To directly assess the involvement of DDR1 in survival or chemoresistance of T47D tumor cells, we compared the levels of etoposide-induced apoptosis observed in these breast cancer cells transfected with a control shRNA construct (pBabe-luciferase-shRNA), vector alone (pBabe), or DDR1 shRNA (pBabe-DDR1-shRNA). As shown in Fig. 6B, depletion of DDR1 expression resulted in a significant increase in apoptosis with increasing etoposide concentration, whereas there was only a marginal increase in apoptosis with empty vector or luciferase shRNA-transfected cells. These findings establish that the reduced levels of endogenous DDR1 contributed directly to etoposide-induced sensitization of these tumor cells to apoptosis. Finally, we examined whether DDR1-mediated chemoresistance in T47D cells was NF-κB dependent. Suppression of Cox-2 expression or NF-κB activity by the superrepressor IκB mutant significantly reduced apoptosis induced by etoposide in control shRNA or vector-transfected cells. Of note, DDR1 depletion by DDR1 shRNA failed to further increase the level of etoposide-induced apoptosis in the presence of the superrepressor (Fig. 6C). These results indicate that DDR1-mediated chemoresistance in breast cancer cells, which exhibit high basal levels of this receptor, is dependent upon activation of NF-κB pathway and consequent Cox-2 induction.

**Discussion**

It is well documented that Cox-2 can be induced by growth factors and cytokines, inflammatory stimuli, and tumor promoters (34). Some studies have provided evidence that oxidative and DNA damage stresses can induce Cox-2 expression (35, 36). However, the precise mechanisms of induction of Cox-2 in response to these stresses are unknown. Our previous studies showed that tumor suppressor p53 or DNA-damaging agents acting through p53 can induce Cox-2, which is able to counteract p53-mediated cell death (30). These studies indicated that Cox-2 is a p53 response gene that is induced to alleviate the adverse effects arising from DNA damage stresses.
damage/genotoxic stress. A number of recent reports strongly imply that enhanced Cox-2 expression plays a significant role in resistance to chemotherapy-induced apoptosis, by protecting cancer cells against apoptosis (37). Moreover, increased Cox-2 levels are commonly detected in a variety of premalignant and malignant tissues (19). Expression of Cox-2 in several types of cancer, including breast cancer, correlates with poor prognosis, and Cox-2 enzyme inhibitors have been reported to reduce breast cancer incidence in humans (38, 39). Recent studies have shown that overexpression of Cox-2 in the mammary gland of CD1 transgenic mice results in tumorigenic transformation as well as angiogenic stimulation (22, 40). Cox-2 is expressed by the tumor endothelium in human colon, breast, prostate, lung, and other cancers (41), whereas normal vascular endothelial cells express only Cox-1 (42). In addition, several studies have shown that selective inhibition of Cox-2 activity suppresses angiogenesis in vitro and in vivo (43). Thus, it has been strongly suggested that Cox-2 is a promising molecular target for cancer prognosis and treatment (44).

The wide range of genetic, environmental, and metabolic stimuli that activate p53 clearly distinguishes it from other known tumor suppressor genes. Our previous studies have shown that DDR1 is a direct p53 target gene and can be functionally activated/phosphorylated in a p53-dependent manner (24). We also found that inhibition of DDR1 function resulted in strikingly increased apoptosis of wt-p53-containing cells in response to genotoxic stress (24). Hence, DDR1 seems to play a critical role in determining the cellular outcome in response to genotoxic/therapeutic stress in wt-p53-containing cells. Our present studies show that DDR1 acts as a novel upstream regulator for Cox-2, and that Cox-2 functions as an important mediator for DDR1-mediated survival in the p53-dependent response to genotoxic stress. Moreover, we showed that the mechanism by which DDR1 causes increased Cox-2 expression is mediated through DDR1 activation of the NF-kB pathway, which induces increased levels of its known targets, including Cox-2 and XIAP. DDR1 up-regulation of Cox-2 as well as XIAP was shown to be dependent on functional components of the NF-kB signaling. DDR1 activation led to strong induction of NF-kB-mediated transcription, and MEFs null for either IKKβ or IKKγ failed to induce Cox-2 in response to DDR1 signaling. One well-known characteristic of cancer cytotoxic treatment is the activation of the transcription factor NF-kB, which regulates cell survival (45). NF-kB activation suppresses the apoptotic potential of chemotherapeutic agents and contributes to chemotherapy resistance. It is also known that many human tumors display constitutive NF-kB activation that allows malignant cells escape apoptosis (46). Our present findings show that DDR1 induces NF-kB activation, establishing this RTK as an important upstream regulator for the NF-kB prosurvival pathway.

Figure 5. DDR1-mediated NF-kB activation requires IKKα and IKKγ. A. IKKα and IKKγ are required for DDR1-mediated NF-kB activation. Wt−, IKKα−/−, IKKγ−/−, and IKKγ−/− MEFs were transfected with pGL3Basic or NF-kB-Luc along with 0.1 μg Ik-Renilla-Luc for normalization. Cells were then infected with Ad-GFP or Ad-DDR1 at 20 MOI. Forty-eight hours postinfection, luciferase activity was measured as described in Materials and Methods. B. DDR1-mediated induction of the NF-kB target genes, Cox-2 and XIAP, is suppressed by the superrepressor IkB mutant (left). Wt-MEFs were infected with Ad-GFP or Ad-DDR1 at 20 MOI. Twenty-four hours later, 40 μM/L etoposide were treated. After additional 24 hours, cell extracts were made and Western blots were carried out for the indicated proteins. DDR1 does not induce Cox-2 and XIAP expression in IKKα-null MEFs (right). IKKα-null MEFs were infected with Ad-GFP or Ad-DDR1 at 20 MOI. Twenty-four hours later, 40 μM/L etoposide were treated. C and D, IKKα and IKKγ are required for DDR1-dependent, NF-kB-mediated prosurvival. Wt-MEFs were transfected with IkB superrepressor, then infected with Ad-GFP or Ad-DDR1 at 20 MOI. Twenty-four hours later, MEFs were treated with 40 μM/L etoposide. Twenty-four hours after drug addition, cell death detection ELISA was carried out. Wt−, IKKα−/−, IKKγ−/−, and IKKγ−/− MEFs were infected with Ad-GFP or Ad-DDR1 at 20 MOI. Twenty-four hours later, cells were treated with 40 μM/L etoposide. Twenty-four hours after drug addition, apoptosis was measured by cell death detection ELISA. Columns (A, C, and D), mean of three independent experiments with each sample in triplicate; bars, SD.
ELISA. Apoptosis was measured by cell death detection ELISA. and control T47D cells (pBabe-shRNA and Luciferase shRNA) were treated with indicated concentrations of etoposide. Twenty-four hours after drug addition, Western blots were carried out for the indicated proteins. DDR1 knockdown or control T47D cells were also transfected with Cox-2 shRNA or GFP-shRNA. Forty-eight hours post-transfection, cell extracts were made and significantly reduced Cox-2 expression along with an increase of total I

Figure 6. Knockdown of endogenous DDR1 expression enhances chemosensitivity. A, modulation of NF-κB pathway proteins, Cox-2, Bcl-2, and IκB. T47D cells were stably transfected with pBabe (vector), luciferase shRNA (pBabe-luciferase shRNA), or DDR1 shRNA construct (pBabe-DDR1 shRNA), and selected for stable clones under puromycin. The resistant clones were pooled and determined for reduction of DDR1 expression. DDR1 knockdown cells (T47-D-DR1 shRNA) as well as control shRNA lines (T47-D-luciferase shRNA and T47-D-pBabe shRNA) were then transiently transfected with IκB mutant superrepressor or vector (pcDNA3), and total cell lysates were isolated for Western blot analysis using anti-DDR1, anti-Cox-2, anti-IκB, anti-Bcl-2, or anti-β-actin antibodies. Note that depletion of DDR1 significantly reduced Cox-2 expression along with an increase of total IκB level and that expression of IκB mutant superrepressor inhibited Cox-2 expression. DDR1 knockdown or control T47D cells were also transfected with Cox-2 shRNA or GFP-shRNA. Forty-eight hours post-transfection, cell extracts were made and Western blots were carried out for the indicated proteins. B, effect of DDR1 depletion on genotoxic agent (etoposide)-mediated apoptosis. DDR1 knockdown T47D cells and control T47D cells (pBabe-shRNA and Luciferase shRNA) were treated with indicated concentrations of etoposide. Twenty-four hours after drug addition, apoptosis was measured by cell death detection ELISA. C, depletion of Cox-2 or IκB superrepressor mutant antagonizes DDR1-mediated prosurvival. The same double-knockdown cells from (A) were also treated with etoposide (40 μmol/L) for 24 hours, then analyzed to determine the patterns of apoptosis by cell death detection ELISA.

Numerous studies have reported overexpression of DDR1 in lung, esophageal, breast, ovary, and pediatric brain cancers (2, 7, 13–17). Moreover, the elevated expression of DDR1 in a number of fast-growing invasive tumors has suggested an important role of this matrix-activated RTK in the proliferation and stromal invasion of tumors (13, 15). In the present studies, we showed that a human breast tumor line, T47D, which overexpressed DDR1, exhibited high constitutive levels of Cox-2. Moreover, inhibition of NF-κB by the IκB superrepressor as well as shRNA inhibition of the expression of Cox-2 or DDR1 itself all dramatically increased the apoptotic response of these tumor cells to genotoxic agents. These findings imply that in DDR1-overexpressing tumors, prosurvival signaling by this newly worked out pathway may offer new tumor-specific therapeutic targets.

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