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Advances in Brief

Identification of the Interferon-inducible Double-Stranded RNA-dependent Protein Kinase as a Regulator of Cellular Response to Bulky Adducts[1]

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Abstract

The double-stranded RNA-dependent protein kinase PKR plays a central role in IFN-mediated antiviral response. The ability of PKR mutants to transform rodent fibroblasts led to the hypothesis that PKR acts as a tumor suppressor. Recent studies have identified an expanding network of PKR signaling partners, including signal transducers and activators of transcription 1 (STAT1), p53, and IκB-kinase. Here we demonstrate that PKR is involved in the cellular response to genotoxic stress. PKR-deficient mouse-embryonic fibroblasts ($PKR^{-/-}$) are hypersensitive to bulky adduct DNA damage caused by cisplatin, melphalan, and UV radiation but not to other DNA-damaging agents such as Adriamycin. PKR-deficient cells are highly susceptible to cisplatin-induced apoptosis. They demonstrate retarded cisplatin adduct removal kinetics. Most strikingly, PKR localizes to the nucleus rapidly upon cisplatin treatment. Restoration of PKR in $PKR^{-/-}$ cells results in resistance to cisplatin and enhanced cell capacity to remove cisplatin DNA adducts. We conclude that PKR has a function in the regulation of cellular response to bulky adduct-inducing agents, possibly by modulating DNA repair mechanisms.

Introduction

The double-stranded RNA-dependent protein kinase PKR[4] is a central regulator of the IFN-mediated antiviral pathway. The transformation of NIH-3T3 fibroblasts by dominant-negative, catalytically inactive PKR led to the hypothesis that PKR also acts as a tumor suppressor (1–3). The role of PKR in tumorigenesis remains enigmatic, however, because two lines of mouse knockouts have been generated and characterized, neither demonstrating an increase in cancer incidence (4, 5). Furthermore, although a decrease in PKR expression and activity has been associated with a subset of human leukemias (6) and a rearrangement of the murine homologue, TIK, has been found in a murine lymphocytic leukemia (7), the role of this kinase in malignancy and its status as a tumor suppressor remain ambiguous.

PKR is involved in a number of signaling pathways and is emerging as a mediator of several stress response, antiproliferative, and apoptotic programs (reviewed in Ref. 8). Although its classical function in virally-induced apoptosis is to inhibit protein translation by phosphorylation of the eIF-2α, PKR has also been found to associate with STAT1 (9) and to phosphorylate p53 (10), as well as mediate signaling through nuclear factor-κB (NF-κB), likely via interaction with an IκB-kinase (11). An up-regulation of the FAS receptor coinciding with apoptosis is also observed in response to dsRNA in PKR-overexpressing cells (12). Interestingly, although originally identified as the factor responsible for arresting cell-free translation in reticulocyte lysates in response to dsRNA (13, 14), it has subsequently been demonstrated that PKR can be activated in the absence of dsRNA, notably by polyanions and protein activators (15). PKR expression is induced 5–10-fold by type I interferons but is present at significant basal levels in their absence. PKR activity is tightly controlled not only at the transcriptional level but also by interaction with protein inhibitors and activators, such as p58 and PACT, respectively (15, 16).

In this study, we provide novel evidence that human and mouse PKR are translocated to the nucleus after DNA damage, facilitate cisplatin adduct removal, and oppose apoptosis in response to genotoxic stress. Previously characterized PKR-knockout MEFs show hypersensitivity to bulky adducts and increased susceptibility to apoptosis after cisplatin treatment.

Materials and Methods

Cell Lines and Cell Culture. The cell lines used in this study consisted of a human breast carcinoma cell line MDA-MB231 (American Type Culture Collection) and isogenic MEFs with $PKR^{+/+}$ and $PKR^{-/-}$ genotype (4, 5). Cells were cultured as monolayers in αMEM supplemented with 10% heat-inactivated calf serum (MDA-MB231) or in DMEM supplemented with 10% heat-inactivated fetal bovine serum (MEFs) and 100 units/ml penicillin G and 100 units/ml streptomycin. Cells were maintained in 5% CO_2/95% air at 37°C in a humidified incubator.

Retroviral Expression of PKR. A bicistronic retroviral vector (17) based on the Moloney murine leukemia virus was used for the expression of PKR. The empty vector or vector containing the coding sequence of wild-type PKR were transfected by Lipofectamine into the Ψ2 producer cell line. Stable producer cells were selected 1 week after daily exposure to 1 μg/ml of puromycin as described earlier (17). Retroviral infection of $PKR^{-/-}$ cells was performed by filtering the producer cell culture supernatant through a 0.45 μm filter and by adding Polybrene to a final concentration of 80 μl/10 ml 48 h after infection; cells were selected until drug-resistant colonies were visible.

Cytotoxicity Assay. For drug cytotoxicity assays, exponentially growing cells ($1–3 \times 10^3$ cells/well) were seeded in 96-well plates. The next day, cells were exposed continuously to cisplatin (Oncology Pharmacy at the Jewish General Hospital), Adriamycin, or melphalan (Sigma Chemical Co., St. Louis, MO) for 96 h. Cell survival was evaluated by the MTT assay using MTT (Sigma). The absorbance was measured at 570 nm with a microplate reader (Bio-Rad). For UVC, 5000 cells/3 ml were seeded in six-well plates. After 16 h, medium was removed, and cells were covered with 0.5 ml of PBS and

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[4] The abbreviations used are: PKR, double-stranded RNA-dependent protein kinase; STAT1, signal transducers and activators of transcription 1; dsRNA, double-stranded RNA; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; UVC, UV light C; MEF, mouse embryonic fibroblast; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; NER, nucleotide excision repair; IRF, interferon regulatory factor; TCR, transcription coupled repair; GGR, global genome repair; TFII, transcription factor II; XP, xeroderma pigmentosum.

irradiated using a UV source. PBS was then removed, and cells were collected and cultured in 96-well plates as described above.

Apoptosis Assay. Cells were seeded at 1×10^6 cells/T75 cm^2 plate and continuously exposed to cisplatin for 72 h. Briefly, cells were collected by trypsinization and washed twice with PBS and then diluted to 1×10^6 cells/100 μl of PBS and placed in a 96-well plate. After fixation, cells were permeabilized with 1% Triton X-100 in 0.1% sodium citrate and labeled in 50 μl/well TUNEL reaction mixture (Boehringer Mannheim *In Situ* Cell Death Detection kit; Laval, Quebec, Canada) at 37°C in the dark for 1 h. Cells were then washed three times with 1% BSA in PBS and resuspended in 500 μl of PBS for analysis by flow cytometry.

Cisplatin Accumulation, DNA Adduct Formation, and Removal. Cells (5×10^5) were seeded in T75 cm^2 flasks. When cells reached 80–90% confluence, they were treated with 25 μM cisplatin for 3 h in serum-free medium. Treatment was stopped by washing cells three times with a solution of cold PBS. Cells were collected immediately to determine the total intracellular accumulation of cisplatin, using total cell extract or genomic DNA extract to measure the initial amount of DNA adducts formed. Cells from duplicate flasks were maintained in culture in drug-free complete medium to allow DNA repair. At the indicated times, total DNA was isolated using DNAzol solution (Life Technologies, Inc.) added directly to the flasks. Platinum-DNA was determined by injecting a volume of 20 μl of sample into a pyrocoated graphite cuvette using a Hitachi polarized Zeeman Model z-8100 flameless atomic absorption spectrophotometer. A calibration curve was established using standard platinum solutions. Total cisplatin accumulation was expressed per μg protein, whereas the amount of DNA adducts was expressed per μg of DNA. Comparisons between DNA adducts in PKR-deficient and PKR-proficient cells were done by the Student's *t* test.

Immunoblotting. Total proteins from $PKR^{+/+}$, $PKR^{-/-}$, and $PKR^{-/-}$ transduced with empty or wild-type PKR were collected from subconfluent cells, using a lysis buffer [0.5% NP40, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, and 1 mM orthovanadate] containing protease inhibitors. Proteins (50 μg) were resolved by 10% SDS-PAGE and transferred into nitrocellulose (Bio-Rad) in transfer buffer [25 mM Tris-HCl (pH 7.5), 190 mM glycine, and 20% v/v methanol]. Transfer of proteins was confirmed by staining with Ponceau S (0.02% w/v). The membrane was then blocked with 10% (w/v) skim milk in 1× PBS and then incubated with the mouse monoclonal antibody against mouse PKR (B-10; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed three times with 1× PBS and incubated with goat antimouse (Bio-Rad) immunoglobulin. Immunocomplexes were revealed using enhanced chemiluminescence detection reagent (Amersham).

Immunofluorescence Microscopy. Cells were plated on coverslips 2 days before each experiment at a concentration of 30,000–50,000 cells/35-mm dish. Cells were treated with 10–20 μM of cisplatin for 60 min, fixed by the addition of precooled (−80°C) methanol:acetone (80%:20% v/v) directly to the coverslips, and then placed at −20°C for 15 min. After fixation, the cells were rinsed extensively with PBS (pH 7.4) and then incubated for 1 h with PBS containing 2% BSA, 2% normal goat serum, and 0.2% gelatin at room temperature to reduce nonspecific binding. The cells were then incubated with primary antibodies (rabbit anti-PKR K-17, Santa Cruz Biotechnology; mouse anti-α-tubulin, ICN, Mississauga, Ontario, Canada) for 1 h at room temperature. After washing, cells were incubated with secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min at room temperature with Texas Red goat antirabbit to reveal anti-PKR and incubated with Cy2 goat antimouse to reveal anti-α-tubulin. After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals, Inc., Allentown, PA) and viewed with a Zeiss Axiophot fluorescent microscope equipped with 63× Plan Apochromat objectives and selective filters. Confocal analyses were performed with a Zeiss LSM 410 inverted confocal microscope (Institut Universitaire de Geriatrie de Montreal, Montreal, Quebec, Canada).

Results

The impact of PKR on sensitivity to chemotherapeutics was measured by the MTT cytotoxicity assay. As shown in Fig. 1, $PKR^{-/-}$ cells showed approximately 3–4-fold sensitization to cisplatin, 2–3-fold sensitivity to melphalan, and approximately 5-fold sensitization to UV radiation. Interestingly, we observed no difference in sensitivities of these cell lines to Adriamycin. To determine whether the observed differences in cytotoxicity correlate with induction of apoptosis, we exposed both lines to 5 or 10 μM cisplatin for 48 h and then examined DNA fragmentation by a fluorescence-activated cell sorter TUNEL assay. Fig. 2 demonstrates significantly higher levels of cisplatin-induced apoptosis in the $PKR^{-/-}$ cells.

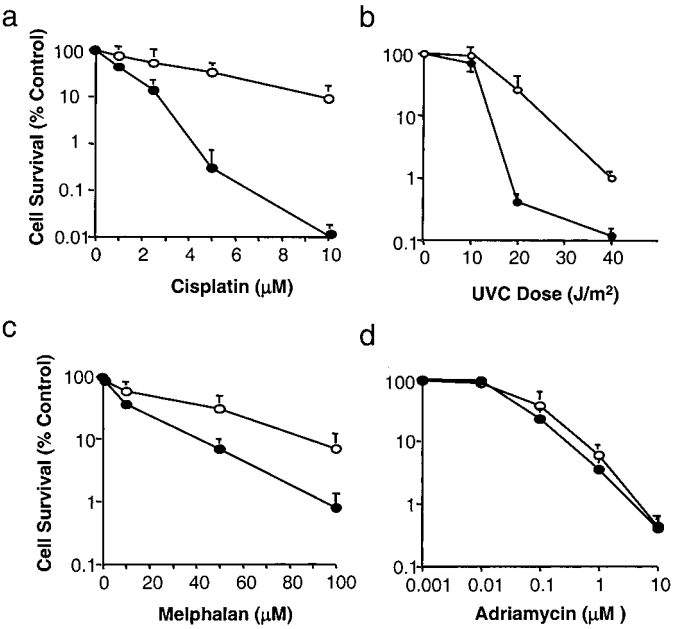

Fig. 1. Cytotoxic effect of DNA-damaging agents on $PKR^{+/+}$ and $PKR^{-/-}$ cells. $PKR^{+/+}$ (○) and $PKR^{-/-}$ (●) cells were exposed to cisplatin, melphalan, or Adriamycin for 96 h or to UVC, and then cytotoxicity was assayed by MTT. $PKR^{-/-}$ cells were significantly more sensitive to bulky adduct-inducing agents (*a–c*) than $PKR^{-/-}$ cells but showed no increase in sensitivity to Adriamycin (which induces double strand breaks; *d*). Bars, SEM.

The specific sensitivity of $PKR^{-/-}$ cells to bulky adducts induced by cisplatin and UVC, both preferred substrates of nucleotide excision repair, led us to question whether the observed difference in apoptosis might be attributable to a defect in this repair pathway. To assay for repair capacity, we measured the accumulation and removal of cisplatin adducts in $PKR^{+/+}$ and $PKR^{-/-}$ cells by atomic absorption spectrometry. Cells were incubated with cisplatin for 3 h and then maintained in culture for various periods of time to allow for repair. Fig. 3*A* demonstrates that there were no significant differences in either cisplatin uptake (147 ± 27 and 135 ± 17 pg/μg total protein for $PKR^{-/-}$ and $PKR^{+/+}$, respectively) or adduct formation (29.9 ± 2.8 and 28.8 ± 3.7 pg/μg DNA for $PKR^{-/-}$ and $PKR^{+/+}$, respectively) between the two cell lines. Strikingly, however, the kinetics of adduct removal are significantly impeded in $PKR^{-/-}$ cells. After a 4-h incubation in drug-free medium, $PKR^{+/+}$ cells had removed 1.5-fold more adducts than their $PKR^{-/-}$ *counterparts (90% versus* 60% of adducts removed, respectively). By 8 h of incubation in drug-free medium, the $PKR^{+/+}$ cells had removed virtually all adducts, whereas the $PKR^{-/-}$ cells had removed only 66% of adducts (Fig. 3*B*). The specificity of these effects is confirmed by restoration experiments in which $PKR^{-/-}$ cells were stably transfected with either wild-type PKR ($PKR^{-/- + WT}$) or with empty vector ($PKR^{-/- + empty\ vector}$), using a retroviral expression system. PKR is expressed to a lower level than the endogenous protein of $PKR^{+/+}$ *cells (Fig. 4a*) and was also found to be phosphorylated (data not shown). Restoration of PKR confers resistance to cisplatin and UVC (Fig. 4, *b* and *c*). However, this resistance is not restored to the same level as that observed in $PKR^{+/+}$ cells, possibly because of the lower expression level of wild-type PKR in $PKR^{-/- + WT}$ cells. Although neither of these transfections alters cisplatin uptake or adduct formation, by 8 h

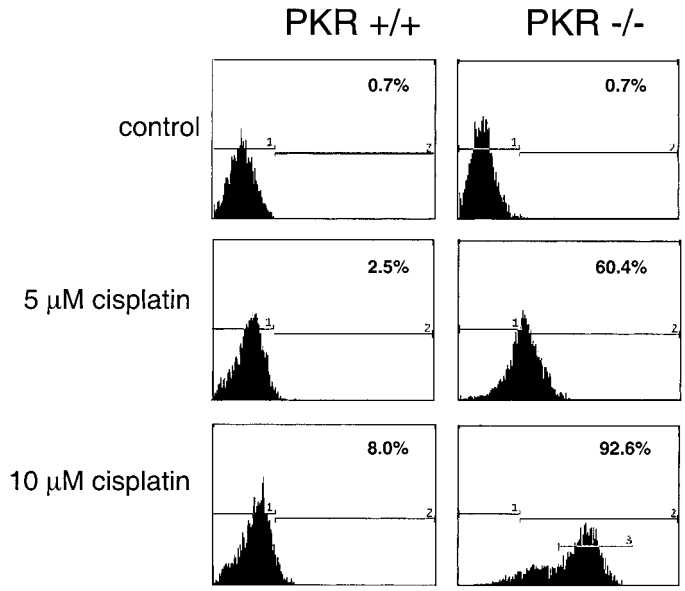

Fig. 2. Cisplatin-induced apoptosis in $PKR^{+/+}$ and $PKR^{-/-}$ cells. $PKR^{-/-}$ cells are susceptible to cisplatin-induced apoptosis. Cells were harvested after 48 h of exposure to 5 or 10 μM cisplatin and then analyzed by fluorescence-activated cell sorter after terminal DNA labeling (TUNEL). The percentage of apoptotic cells is indicated.

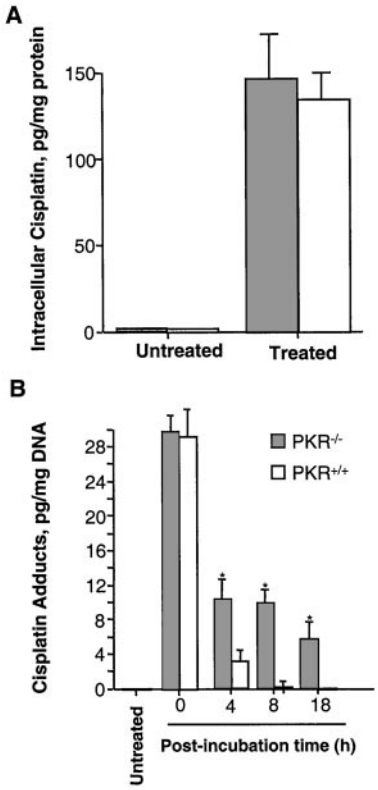

Fig. 3. Cisplatin uptake and DNA adduct formation and removal in $PKR^{+/+}$ and $PKR^{-/-}$ cells. Cells were treated with 25 μM cisplatin for 3 h and postincubated with drug-free medium for the indicated times. Cisplatin uptake (*a*) and adduct formation and removal (*b*) were measured by atomic spectrometry as described in "Materials and Methods." *Bars*, SEM for two independent experiments, each performed in duplicate. *, significantly different from $PKR^{+/+}$ at $P < 0.001$.

$PKR^{-/- + WT}$ cells show ~50% increase in repair capacity relative to $PKR^{-/- + empty\ vector}$ (Fig. 4, *d* and *e*). Therefore, the amount of PKR expressed in $PKR^{-/-}$ cells is capable of partially restoring the capacity of $PKR^{-/- + WT}$ cells to remove cisplatin adducts.

PKR is believed to modulate signaling to the nucleus via interactions with STAT1 and IκB-kinase. It also interacts with the nuclear proteins p53 and DRBP76. To gain insight into whether the observed differences in repair capacity were the result of a PKR-mediated signaling cascade or direct PKR action in the nucleus, we used immunofluorescence to observe the localization of PKR in response to cisplatin. As shown in Fig. 5, untreated $PKR^{+/+}$ MEFs (*A*) and MDA-MB231 (a human breast adenocarcinoma cell line; *B*) show diffuse cytoplasmic labeling and nuclear exclusion of PKR. Upon treatment with cisplatin, however, PKR rapidly localizes to the nucleus (Fig. 5). This translocation was not observed in $PKR^{-/-}$ cells.

Discussion

In addition to the established role of PKR as a mediator of antiviral signaling, PKR is now recognized as a central molecule in several stress-related signaling pathways. Within the cytoplasm, PKR functions in response to infection by halting translation via phosphorylation of eIF-2α (18). It has also been reported to interact with STAT1,

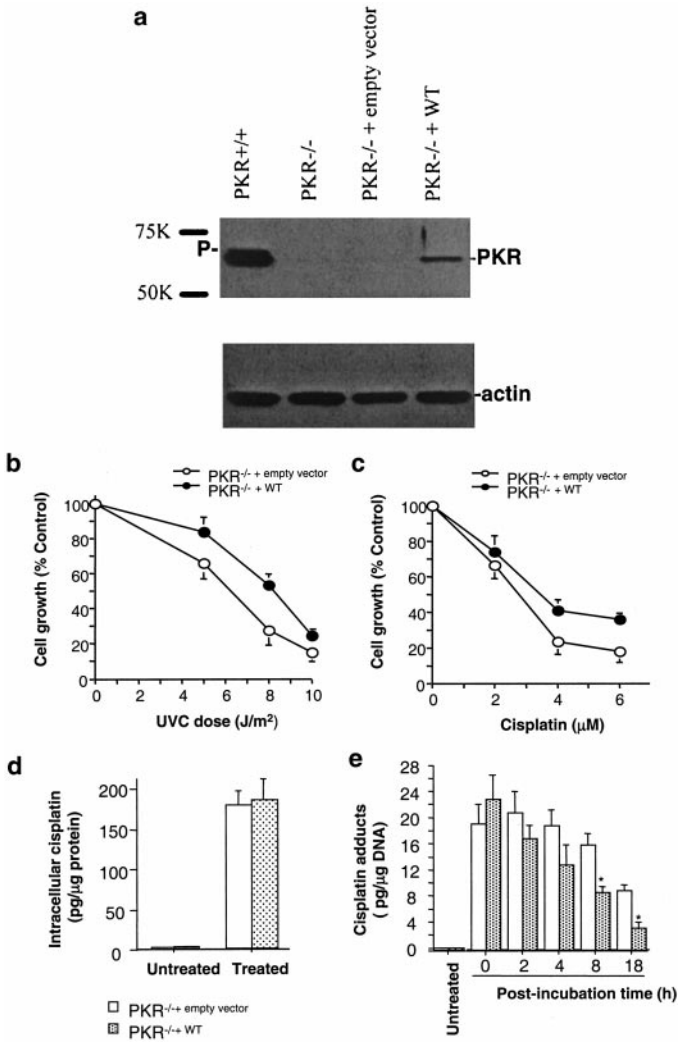

Fig. 4. Effect of PKR expression on drug resistance and cisplatin adduct removal. *a*, $PKR^{-/-}$ cells transduced with wild-type murine PKR ($PKR^{-/- + WT}$) showed lower expression when compared with the endogenous protein level of mouse ($PKR^{+/+}$) cells. *Upper band*, hyperphosphorylated form of PKR. The blot was stripped and reprobed with anti-actin (Boehringer Mannheim). Restoration of WT PKR confers resistance to cisplatin (*b*) and UV (*c*) as measured by live cell counting. $PKR^{-/- + WT}$ and $PKR^{-/- + empty\ vector}$ do not differ in cisplatin uptake or adduct formation (*d*), but $PKR^{-/- + WT}$ have demonstrated enhanced adduct removal kinetics relative to $PKR^{-/- + empty\ vector}$ (*e*). *Bars: b–e*, SEM.

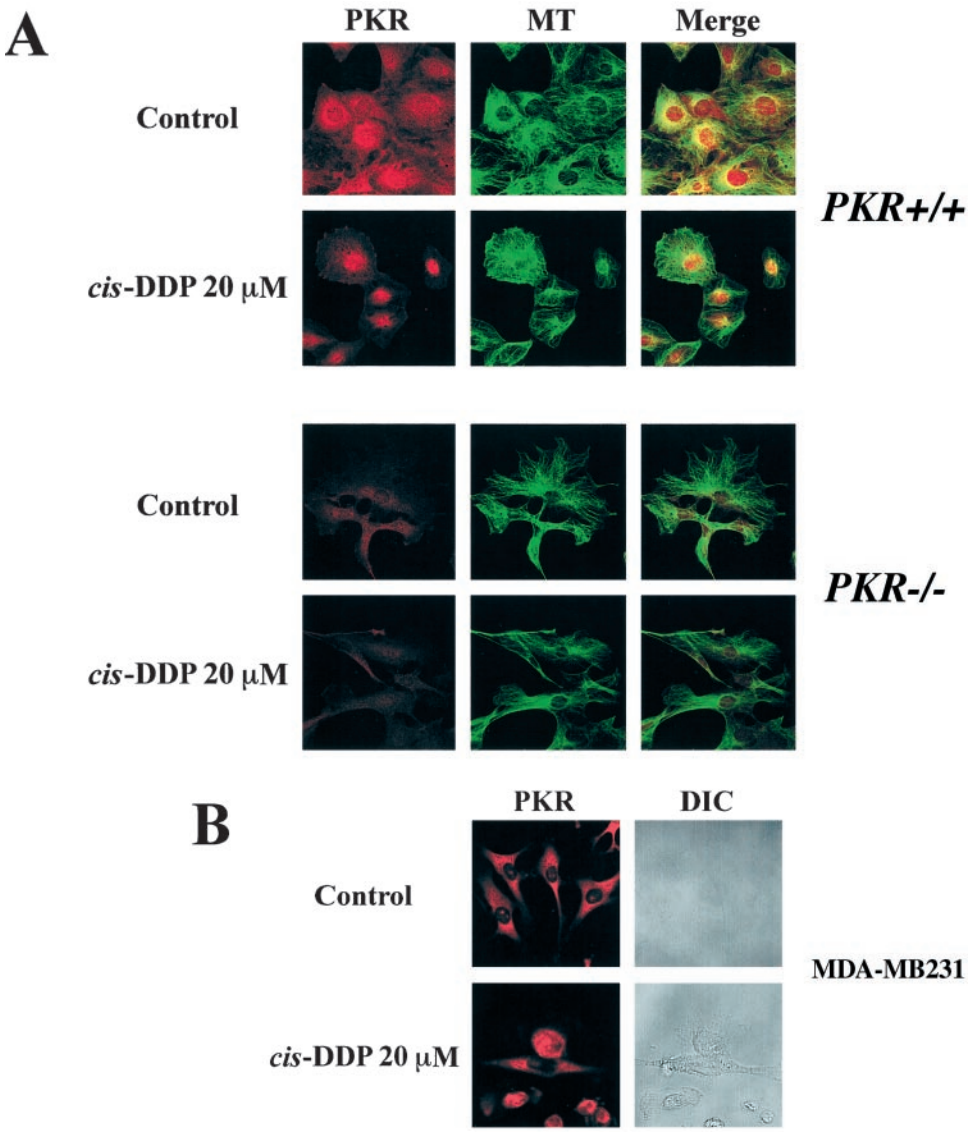

Fig. 5. Cellular localization of PKR after exposure to cisplatin. MEFs (*A*) and MDA-MB231 (*B*) were treated with 20 μM cisplatin for 60 min, fixed for immunocytochemistry, and double-labeled for PKR (*red*) and microtubules (*green*). Untreated cells (*Control*) show diffuse cytoplasmic localization of PKR in both $PKR^{+/+}$ and MB-MDA231. Upon treatment with cisplatin (*cis-DDP*), PKR becomes predominantly localized to the nucleus. In contrast, $PKR^{-/-}$ cells show very weak and nonspecific labeling with the anti-PKR antibody. *MT*, microtubule; *DIC*, differential interference contrast.

perhaps limiting its DNA binding and transactivation in the absence of PKR activation by IFN (10). Furthermore, PKR interacts with p53, and PKR-deficient cells show a reduction in p53 phosphorylation and induction of p53-mediated transcription upon DNA damage. Interestingly, this reduction in phosphorylation was seen after treatment with Adriamycin or γ-radiation but not UV (19).

Our study demonstrates that, relative to $PKR^{+/+}$ MEFs, $PKR^{-/-}$ are hypersensitive to melphalan, UV, and cisplatin but not to Adriamycin. Neither differences in uptake nor adduct formation contribute to the cisplatin-sensitive phenotype of $PKR^{-/-}$ cells, whereas the removal of these adducts is severely impeded. To preclude the possibility that the genetic background of the wild-type and knockout cells was responsible for the observed phenotype, we restored expression of PKR in the $PKR^{-/-}$ cells and found that its expression does indeed confer resistance to cisplatin. The partial restoration of cisplatin resistance compared with that observed with isogenic $PKR^{+/+}$ can be attributed to differences in the level of PKR expression. Although we used a highly efficient retroviral system to express PKR, the amount obtained is significantly lower than the endogenous level expressed within $PKR^{+/+}$ cells. Given that no significant difference in intracellular accumulation of cisplatin was observed between $PKR^{+/+}$ and $PKR^{-/-}$, it is logical to speculate that decreased removal of cisplatin-adducts in $PKR^{-/-}$ may be attributable to impaired DNA repair or associated cell cycle checkpoint mechanisms.

Bulky adducts, such as those induced by UV and cisplatin, are substrates for NER (reviewed in Ref. 20). Recognition of damage results in sequential recruitment of TFIIH, XPF, and XPG. Assembly of the repair complex then leads to 3′ and 5′ incisions around the site of damage and excision of a 28–32-base fragment, leaving a gap that is then filled and ligated. This repair process has two classical branches: TCR and GGR. TCR is mediated by stalling of transcription at the site of bulky lesions. It is possible that such stalling facilitates secondary structure formation in the nascent mRNA, allowing binding of PKR or other double-stranded RNA binding motif proteins. GGR occurs after damage sensing and recognition, likely by a combination of XPC, XPA, and/or RPA, and the subsequent recruitment of the several other repair proteins including TFIIH (reviewed in Ref. 20). The adduct removal assays presented here do not, however, distinguish between GGR and TCR; it is not known whether PKR is involved in one or both branches of NER nor whether RNA is required. To date, no direct interaction has been demonstrated between PKR and any proteins known to be involved in NER, with the exception of p53. Interestingly, PKR localization to the nucleus is unimpaired in p53-null SaOS-2 (data not shown), suggesting that PKR

trafficking in response to bulky adduct damage is p53-independent. It is also interesting to recall that p53 phosphorylation in response to UV is unaltered in PKR knockout MEFs (19).

It is also worth mentioning that another protein that is induced in response to viral infection, IRF-7, has been shown recently to localize to the nucleus after genotoxic stress. The activation and localization of IRF-7 are reported to be mediated by c-Jun NH_2-terminal kinase 1 (JNK1) and mitogen-activated protein kinase kinase-4 (MKK4) (21), which is also notable because c-Jun NH_2-terminal kinase inhibition reportedly sensitizes cells to cisplatin (22). Furthermore, IRF-1, which is required for basal, but not inducible, expression of PKR (6), may modulate p53 stability in response to DNA damage. Interestingly, IRF-1 deficiency resulted in accelerated p53 stabilization after genotoxic insult and reduced DNA repair activity after UV treatment (23). This is consistent with a report that p53-induced apoptosis may occur via modulation of specific helicase activities (24–26). Thus, it seems plausible that, in response to bulky adduct DNA damage, PKR may modulate NER activity via its previously reported interactions with p53 (10, 19), perhaps reducing the p53 interaction with the TFIIH helicases XPB and XPD, thereby promoting repair and opposing apoptosis.

PKR-deficient cells have been reported previously to have a defect in the G_1-S checkpoint induced by Adriamycin treatment (19). Our unpublished observations indicate a similar defect in the G_2-M checkpoint in response to cisplatin; the role of these checkpoint defects in the observed bulky adduct-sensitive phenotype must also be examined. In summary, our results provide compelling evidence that PKR is involved in a novel pathway facilitating repair and opposing apoptosis in response to bulky adduct DNA damage.

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