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ARTICLE

A Cellular Screening Assay to Test the Ability of PKR to Induce Cell Death in Mammalian Cells

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Long double-stranded RNA (>30 bp), usually expressed in cells infected with RNA viruses, triggers antiviral responses that induce apoptosis of the infected cells. PKR can be selectively activated in glioblastoma cells by *in situ* generation of dsRNA following introduction of antisense RNA complementary to an RNA expressed specifically in these cells. Harnessing PKR for the selective killing of cancer cells is potentially a powerful strategy for treating cancer, but we were unable to induce apoptosis by this approach in a T cell lymphoma. We therefore established a cellular screening assay to test the ability of PKR to induce death in cell lines, especially those originating from human cancers. This ''PKR killing screen'' is based on the infection of cells with an adenoviral vector encoding GyrB-PKR, followed by coumermycin treatment. Cancers represented by cell lines in which PKR activation leads to cell death are good candidates for the dsRNA killing approach, using antisense to RNA molecules specifically expressed in these cells. The PKR killing screen may also serve as a tool for exploring PKR signaling and other related pathways, by identifying new cases in which PKR signaling is inhibited or impaired.

Key Words: PKR, GyrB-PKR, eIF-2α, prostate cancer, breast cancer

INTRODUCTION

Double-stranded RNA longer than 30 nucleotides, expressed in virally infected cells, activates multiple antiviral cellular mechanisms. These include the dsRNA-dependent protein kinase PKR and 2′–5′ oligo A synthetase, both of which turn off protein synthesis and proapoptotic signals induced by p38, JNK, IRF3-DRAF1, NFκB, and others [1–3]. The dsRNA-induced mechanisms efficiently kill the infected cells and induce expression of the antiproliferative cytokines interferons, thereby preventing spread of the virus [2]. The death-promoting action of dsRNA can be harnessed to kill cancer cells selectively by the activation of PKR in the diseased cell. PKR is a Ser/Thr protein kinase that phosphorylates the α subunit of the protein synthesis initiation factor eIF-2α, resulting in sequestration of the GDP/GTP exchange factor eIF-2B and strong inhibition of translation initiation [4]. PKR is expressed constitutively in a wide range of cell types but is activated by viruses, dsRNA, and interferons [5]. PKR is responsible for a significant fraction of the antiviral and antiproliferative effects of these interferons [6]. The complete cessation of translation by PKR has toxic effects on cells [7] and even stable expression of PKR in yeast, mammalian, and insect cells results in reduced growth rates. On the other hand, transfection with a transdominant negative mutant of PKR causes malignant transformation of mouse cells [8]. Transient overexpression of PKR in mouse cells is sufficient to induce apoptosis [7]. A functional PKR pathway is required to induce apoptosis in response to dsRNA, LPS, serum deprivation, or TNFα treatment (reviewed in [9]). Activation of PKR by dsRNA induces the expression of proapoptotic genes of the tumor necrosis factor receptor family, including Fas and proapoptotic Bax [10]. 3T3/L1 cells overexpressing PKR become extremely sensitive to dsRNA and TNFα-induced apoptosis, while cells expressing catalytically inactive PKRΔ6 are completely resistant [10]. Thus, PKR is a strong antiproliferative protein and selective activation of this enzyme in tumors could become a powerful strategy to treat cancer.

Activation of PKR involves two molecules binding in tandem to dsRNA and then phosphorylating each other in an intermolecular event (Fig. 1A) [11]. PKR exhibits different affinities for dsRNAs of various sizes, and molecules shorter than 30 bp fail to bind stably and do

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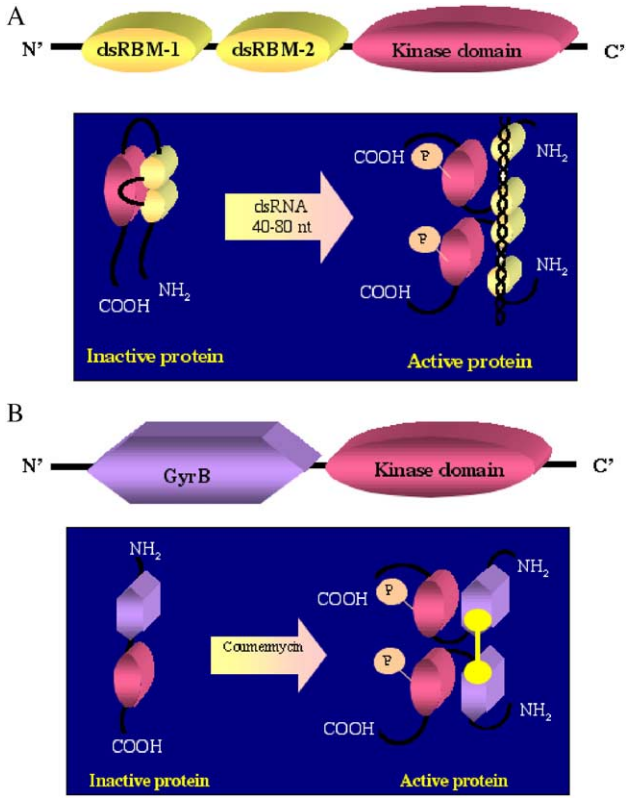

FIG. 1. Schematic presentation of wt PKR, GyrB-PKR, and their activation. (A) Wt PKR consists of two dsRNA-binding motifs (dsRBM) and a kinase domain. dsRNA binding induces dimerization of two PKR molecules and subsequent activation. (B) In GyrB-PKR the regulatory domain of gyrase B replaces the dsRNA binding domains. GyrB-PKR activation is induced by the drug coumermycin, which binds to two molecules of GyrB.

not activate the enzyme. Molecules longer than 30 bp bind and activate the enzyme with an efficiency that increases with increasing chain length, reaching a maximum at about 85 bp.

Based on the fact that in many cancers, chromosomal rearrangements and truncations lead to the production of unique mRNA species, it was shown recently that PKR activation could be harnessed for the selective killing of cancer cells [12]. U87MGΔEGFR, a glioblastoma cell line that harbors a truncated epidermal growth factor receptor (EGFR), was infected with a viral vector encoding a 39-nucleotide-long antisense (AS) RNA molecule, complementary to the unique junction of ΔEGFR mRNA. AS expression caused selective death of U87MGΔEGFR both *in vitro* and *in vivo* but did not affect cells expressing wild-type EGFR [12]. In U87MGΔEGFR cells, the AS RNA hybridized with the unique mRNA sequences flanking the truncation, resulting in dsRNA generation, leading to PKR activation. In cells expressing only the wild-type EGFR, PKR was not activated since the flanking sequences were far apart, and only short dsRNA species could be formed.

PKR and its signaling pathway are not restricted to a given cell line; therefore, in principle, this dsRNA killing approach can be applied to any cancer that expresses unique mRNA sequences. PKR signaling is inhibited, however, in various cancers. We therefore decided to develop a screening assay to establish whether a particular cancer, represented by an established cell line, is amenable to dsRNA-induced killing. Such an assay precludes the lengthy procedure of making the appropriate viral vector harboring the AS RNA, except in cases that prove suitable. In this communication we present a cellular screening assay that tests the ability of PKR to induce cell death in various cancerous cell lines that express unique mRNA sequences due to chromosomal aberrations.

The "PKR killing assay" is based on the expression of GyrB-PKR, a PKR derivative in which the regulatory domain of gyrase B replaces the dsRNA binding domains (Fig. 1B) [13]. This form of PKR can be activated by coumermycin instead of by dsRNA. Coumermycin binds GyrB with a stoichiometry of 1:2 [14] and therefore induces PKR dimerization and its subsequent activation. In our cellular screening assay, GyrB-PKR is delivered into cells by adenoviral infection, followed by coumermycin treatment. We chose adenoviral infection because many cell lines are difficult to transfect. Cell viability was determined by methylene blue assay. Cancers in which PKR activation leads to cell death are suitable candidates for the dsRNA killing approach, using antisense specific to the chromosomal aberration.

RESULTS

Activation of GyrB-PKR Leads to Glioma U87MG-wtEGFR Cell Death

We examined the ability of GyrB-PKR to induce cell death first in glioma U87MG-wtEGFR cells, which die upon PKR activation by pIC (data not shown). We seeded cells and infected them with GyrB-PKR-encoding adenoviruses and treated them with coumermycin as described under Materials and Methods. Fig. 2A shows the effect of GyrB-PKR activation on U87MG-wtEGFR growth. U87MG-wtEGFR cells infected with GyrB-PKR-encoding adenoviruses died upon coumermycin treatment. Viral infection without coumermycin treatment had a much milder effect on the cells. Infection with adenoviruses encoding green fluorescence protein (GFP) had no effect on cell growth (Fig. 2B), although the infection efficiency was high (data not shown). This pilot experiment showed that the PKR killing screen was feasible.

GyrB-PKR Activation Leads to eIF-2α Phosphorylation in U87MG-wtEGFR Cells

To examine whether GyrB-PKR activation indeed leads to eIF-2α phosphorylation, we used a PKR mutant with inactive kinase (K296H). We infected U87MG-wtEGFR

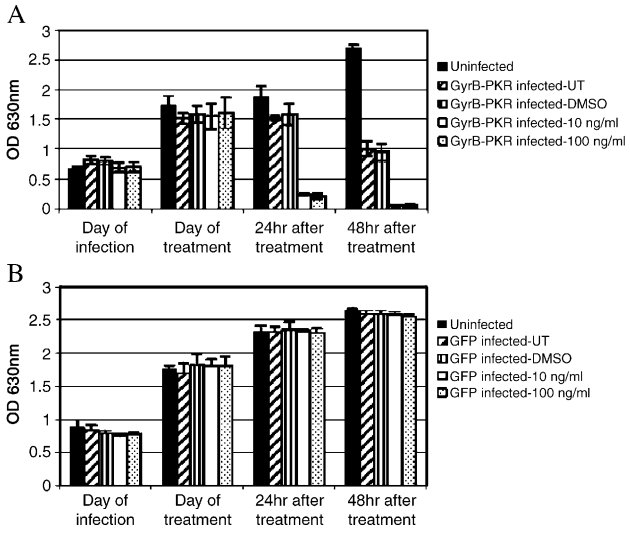

FIG. 2. GyrB-PKR activation leads to U87MG-wtEGFR cell death. U87MG-wtEGFR cells were seeded in 96-well plates, infected with adenoviruses encoding (A) GyrB-PKR or (B) GFP, and treated as described under Materials and Methods. The graphs depict cell growth as determined by methylene blue assay. Infection with GyrB-PKR-encoding viruses followed by coumermycin treatment caused the death of U87MG-wtEGFR cells.

cells with either GyrB-PKR wt- or GyrB-PKR K296H-encoding adenoviruses. We treated the cells with coumermycin and harvested them 24 h later. At this time, we observed cell death in cells infected with GyrB-PKR wt and treated with coumermycin. We determined the levels of PKR and eIF-2α phosphorylation by Western analysis, as described under Materials and Methods. Antibody anti-PKR K-17 detected both endogenous PKR and GyrB-PKR in lysates derived from U87MG-wtEGFR cells. As can be seen in Fig. 3, eIF-2α was highly phosphorylated upon infection with GyrB-PKR wt-encoding adenoviruses (lane 1), compared to its phosphorylation state in cells that were not infected (lane 9). Phosphorylation was further increased upon coumermycin treatment (lanes 3 and 4). In cells that were infected with GyrB-PKR K296H, however, no change in the phosphorylation state of eIF2α was observed (lanes 5–8). Note that GyrB-PKR wt levels decreased upon activation with coumermycin, probably due to degradation (unpublished data).

A Human Prostate Adenocarcinoma Cell Line, LNCaP, Dies Upon GyrB-PKR Activation

We seeded the LNCaP cell line, infected it with GyrB-PKR-encoding adenoviruses, and treated it with coumermycin as described under Materials and Methods. Fig. 4A shows the effect of GyrB-PKR activation on LNCaP growth. Infection with GyrB-PKR-encoding adenoviruses led to growth inhibition, but infection followed by coumermycin treatment killed the cells. While infection of LNCaP cells with adenoviruses encoding GFP also led to cell death (Fig. 4B), treatment of GyrB-PKR-infected cells with coumermycin had a stronger inhibitory effect than infection without coumermycin treatment.

To examine the phosphorylation state of eIF-2α in the LNCaP cell line, we seeded cells, infected them with GyrB-PKR-encoding adenoviruses, and treated them as described under Materials and Methods. We lysed the cells 12 and 36 h after coumermycin treatment and observed the levels of GyrB-PKR and eIF-2α phosphorylation (Fig. 5). We observed neither GyrB-PKR nor phosphorylation of eIF-2α in lysate derived from uninfected cells (lane 1). GyrB-PKR was expressed in infected cells (lanes 2–8). Phosphorylation of eIF-2α was induced by coumermycin treatment as early as 12 h after treatment (lanes 4 and 5). EIF-2α phosphorylation was even stronger 36 h after treatment (lanes 7 and 8). As in U87MG wt-EGFR cells, in LNCaP cells GyrB-PKR levels decreased upon coumermycin treatment.

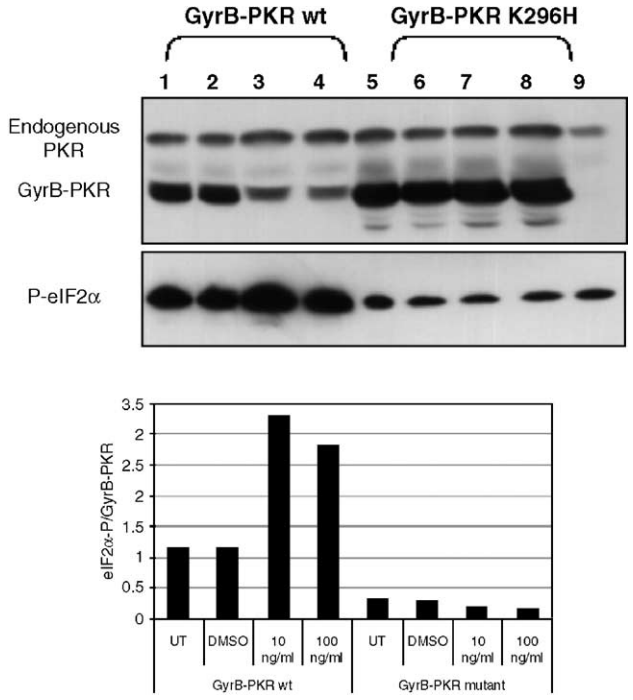

FIG. 3. GyrB-PKR activation leads to eIF-2α phosphorylation in U87MG-wtEGFR cells. U87MG-wtEGFR cells were seeded in six-well plates and infected with adenoviruses encoding GyrB-PKR wt (lanes 1–4) or GyrB-PKR K296H (lanes 5–8). Samples 1 and 5 were not treated, samples 2 and 6 were treated with DMSO, and samples 3, 7, 4, and 8 were treated with coumermycin (10 ng/ml (samples 3 and 7) or 100 ng/ml (4 and 8)). Sample 9 was from uninfected cells. 24 h after treatment, the cells were lysed and the various samples were electrophoresed and blotted. The graph shows normalization of eIF-2α levels to GyrB-PKR levels. eIF-2α phosphorylation was specifically induced in cells infected with GyrB-PKR wt and not with GyrB-PKR K296H. Furthermore, this phosphorylation was elevated by coumermycin treatment.

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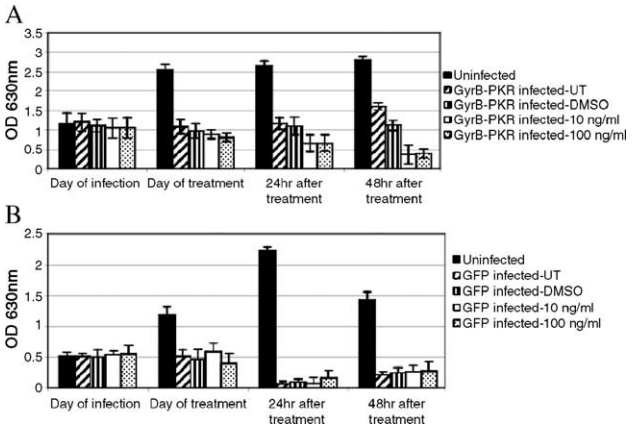

FIG. 4. GyrB-PKR activation leads to LNCaP cell death. LNCaP cells were seeded in 96-well plates, infected with adenoviruses encoding GyrB-PKR or GFP, and treated as described under Materials and Methods. The graphs depict their growth as determined by methylene blue assay. (A) Infection with GyrB-PKR-encoding viruses followed by treatment with coumermycin caused death of LNCaP cells. (B) Infection with adenoviruses coding only for GFP also inhibited growth, but no further effect of coumermycin was observed.

A Human Colon Adenocarcinoma Cell Line, HT-29, Is Resistant to GyrB-PKR Activation

We seeded the HT-29 cell line, infected it with GyrB-PKR-encoding adenoviruses, and treated it as described under Materials and Methods. The effect of GyrB-PKR activation on HT-29 growth is depicted by the growth curves in Fig. 6A. Activation of GyrB-PKR had no effect at all on the growth of the HT-29 cell line, which was also not affected by the infection itself. We obtained similar results when infection was performed with adenoviruses encoding GFP. While infection efficiency was high (Fig. 6B), no toxic effect of the adenoviral infection was observed (Fig. 6C).

The Effect of GyrB-PKR Activation on Human Breast Adenocarcinoma Cell Lines

We seeded the MDA-MB-468 and MCF-7 cell lines, infected them with GyrB-PKR-encoding adenoviruses,

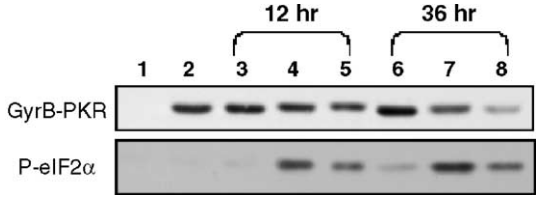

FIG. 5. GyrB-PKR activation leads to eIF-2α phosphorylation in LNCaP cells. LNCaP cells were seeded in six-well plates and infected with adenoviruses encoding GyrB-PKR wt (lanes 2–8). Sample 1 was from uninfected cells. The various lysates derived from infected cells were treated as follows: sample 2, untreated; samples 3 and 6, treated with DMSO; samples 4, 7, 5, and 8, treated with coumermycin (10 ng/ml (samples 4 and 7) or 100 ng/ml (5 and 8)). EIF-2α phosphorylation was induced by activation of GyrB-PKR.

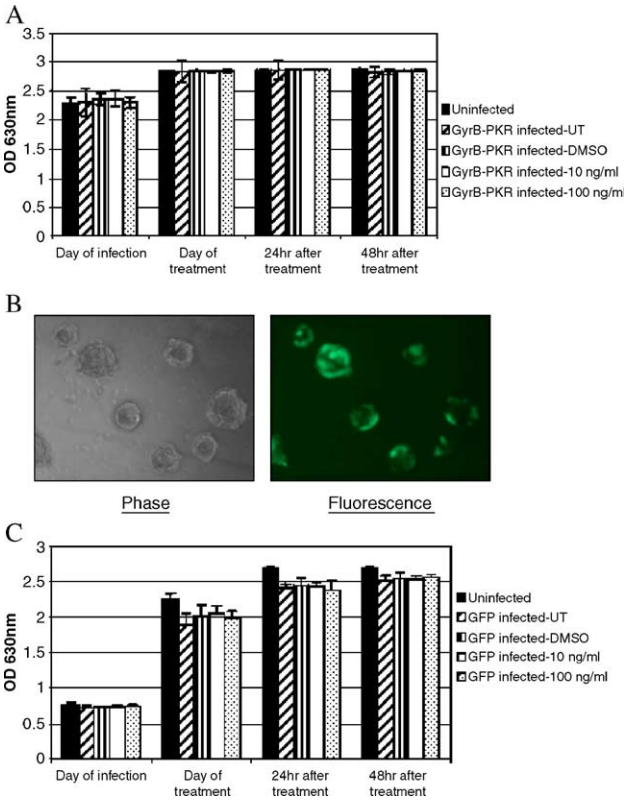

FIG. 6. GyrB-PKR activation did not inhibit HT-29 cell growth. HT-29 cells were seeded in 96-well plates, infected with GyrB-PKR- or GFP-encoding adenoviruses, and treated with coumermycin as described under Materials and Methods. The growth curves of GyrB-PKR-infected cells and GFP-infected cells, as determined by methylene blue assay, are shown in (A) and (C), respectively. Neither viral infection nor GyrB-PKR activation had any detectable effect on HT-29 cell growth. (B) GFP fluorescence confirmed that adenovirus infection of HT-29 cells was efficient.

and treated them as described under Materials and Methods. MDA-MB-468 cells were extremely sensitive to viral infection and therefore the effect of GyrB-PKR on their growth, if any, could not be detected (Fig. 7A). We obtained a similar result when MDA-MB-468 cells were infected with adenoviruses encoding only GFP (Fig. 7B). MCF-7 cells were somewhat sensitive to infection, but activation of GyrB-PKR caused additional growth inhibition (Fig. 7C). We observed growth inhibition when MCF-7 cells were infected with adenoviruses encoding only GFP, but coumermycin treatment had no further effect (Fig. 7D).

DISCUSSION

In this study, we have screened human cancer cell lines for death upon PKR activation using the GyrB-PKR assay. We have demonstrated the utility of the screen using a glioblastoma cell line (U87MG-EGFRwt), two breast adenocarcinoma cell lines (MCF-7 and MDA-MB-468), a

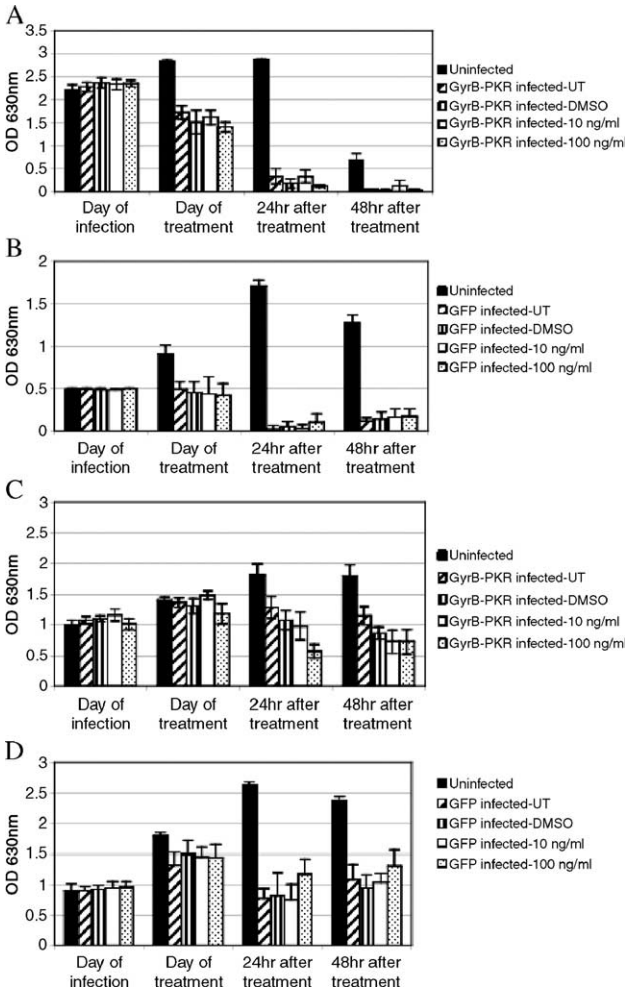

FIG. 7. The effect of GyrB-PKR activation on human breast adenocarcinoma cell lines. MDA-MB-468 and MCF7 cells were seeded, infected with GyrB-PKR-encoding adenoviruses, and treated as described under Materials and Methods. (A and B) The growth of MDA-MB-468 cells infected with GyrB-PKR- or GFP-encoding adenoviruses, respectively, is depicted. (C and D) The growth of MCF-7 cells infected with GyrB-PKR- or GFP-encoding adenoviruses, respectively, is depicted. The growth was determined by methylene blue assay.

human colon adenocarcinoma cell line (HT29), and a human prostate adenocarcinoma cell line (LNCaP). The feasibility of the PKR killing screen was shown using the U87MG-EGFRwt cell line, for which it was already known that PKR, upon activation, leads to cell death [12]. Applying the PKR killing screen to the other human cancer cell lines, we observed that the effect of viral infection itself and of treatment with coumermycin varied between the cell lines. PKR induced the most pronounced cell death for the LNCaP cell line, in which the activation of GyrB-PKR had a dramatic effect beyond that of viral infection alone (Fig. 4). In LNCaP cells, as well as in U87MG-EGFRwt cells, phosphorylation of eIF-2α was induced upon GyrB-PKR activation (Figs. 5 and 3, respectively). The fact that the other cell lines examined were found to be refractory to GyrB-PKR activation correlates with a growing body of evidence showing that in many cancers PKR activity is inhibited or impaired. PKR activation and eIF-2α phosphorylation have been found to be diminished in Ras-transformed cells [15]. PKR inactivation, apparently resulting from the presence of an inhibitor in the cells, has been reported for leukemic cells from B cell chronic lymphocytic leukemia patients [16], as well as for various HTLV-1-transformed and non-transformed human T cells [17]. PKR from MCF-7 cells was reported to be highly expressed but its ability to autophosphorylate, or to phosphorylate eIF-2α, was attenuated [18]. MCF-7 extracts were shown to contain a transdominant inhibitor of PKR, which could be responsible for this inhibition [18]. On the other hand another group, working with the same cell line, reported that PKR activation is not inhibited. It was found that although eIF-2α is highly phosphorylated, translation is not arrested, presumably owing to the high levels of eIF-2B in these cells [19].

The impetus for developing the PKR killing screen was our finding that glioblastoma cells can be targeted specifically for killing by activation of PKR [12]. In principle, PKR can be specifically induced in many cancer cells, by taking advantage of the unique sequences associated with the genomic rearrangements characteristic of cancer cells to generate cancer-specific dsRNA. Except for the MDA-MB-468 cell line, in which the chromosomal abnormalities have yet to be identified, all other cell lines were reported to possess defined chromosomal aberrations leading to the expression of unique mRNA sequences. The MCF-7 cell line harbors several translocations, such as the t(1;20), t(8;11) [20]. The HT-29 cell line harbors the t(6;14) (q23;q13) translocation [21] and the LNCaP cell line harbors the t(6;16) (p21;q22) translocation [22]. Therefore the respective cancers, expressing these unique mRNA sequences, are candidates for the dsRNA killing approach by specific activation of PKR. Nonetheless, given the growing body of evidence that the PKR pathway is repressed in some cancers, it is clear that this approach to cell killing will be effective only for cell lines and tumors that die upon PKR activation. Therefore, it is important to verify that the PKR-directed apoptotic pathways are active, before attempting to apply the dsRNA killing approach for the treatment of a given cancer.

We are currently generating AS for the tpc/hpr fusion mRNA resulting from the t(16;6) (p21;q22) translocation found in the LNCaP human prostate cancer cell line. Expression of this AS RNA should result in formation of dsRNA and hence PKR activation specifically in the LNCaP cell line. The remarkable susceptibility to PKR killing of a cell line established from prostate cancer suggests that the dsRNA killing approach can be extended to the treatment of certain types of prostate cancers in

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which this translocation occurs. Nevertheless, since cancers are heterogeneous, the dsRNA killing approach will have to be combined with other anti-cancer therapies such as drugs or irradiation. Accumulating data indicate that patients develop resistance to anti-cancer drugs after continuous treatment. Treatment with combinations of anti-cancer therapies, targeting a number of signaling pathways in parallel, should prevent or slow the development of resistance. The dsRNA killing approach might be particularly effective as a means of eliminating cells remaining after surgery or irradiation.

The PKR killing screen may also serve as a useful tool for exploring PKR signaling and other related pathways, by identifying new cases in which PKR signaling is inhibited or impaired, thus leading to a better understanding of cell biology in general and cancer cell biology specifically.

MATERIALS AND METHODS

Materials. All cell culture growth media and sera were purchased from Biological Industries (Beit Haemek, Israel). Insulin, DMSO, and coumermycin A1 were bought from Sigma (St. Louis, MO, USA). Penicillin and streptomycin were bought from Teva (Israel). Testosterone was purchased from Fluka Chemicals Ltd. (England).

Plasmids. Plasmids pSG5-GyrB-PKR wt and pSG5-GyrB-PKR K296H, a mutant form that cannot phosphorylate eIF-2α, were a generous gift from Professor Thomas E. Dever [13]. A *Sac*I–*Hin*dIII fragment encoding the GyrB-PKR (wt or mutant) was "filled in" and subcloned into the *Eco*RV site of vector pBluescript II SK(+) (Stratagene, La Jolla, CA, USA). The resulting plasmids were cut with *Xho*I and *Xba*I enzymes and the fragment encoding GyrB-PKR wt or GyrB-PKR mutant was transferred to the adenoviral vector pAdeTrack-CMV [23] cut with the same enzymes, generating the plasmids pAdeTrack-CMV-GyrB-PKR wt and pAdeTrack-CMV-GyrB-PKR K296H.

Cell culture. The two human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-468, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/L), and penicillin (100,000 units/L). The human colon adenocarcinoma cell line, HT29, was grown in McCoy-5A medium supplemented with 10% FCS and antibiotics. The human prostate adenocarcinoma cell line, LNCaP, was grown in RPMI 1640 medium supplemented with 10% FCS, antibiotics, insulin (1 μg/L), and testosterone (1 μg/L). The glioblastoma cell line U87MG-wtEGFR, which overexpresses the EGFR, was grown in DMEM supplemented with 10% FCS and antibiotics, in the presence of 400 μg/ml G418. All cell lines were grown as monolayers on tissue culture plates (Nalge Nunc International, USA).

Adenovirus production. Adenoviruses were produced as described [23]. In brief, recombinant adenoviral plasmids were generated by homologous recombination between the pAdeTrack-CMV (control vector that encodes only GFP), the pAdeTrack-CMV-GyrB-PKR wt or K296H, and the pAdeEasy-1 plasmids in *Escherichia coli* BJ5183. HEK293 cells, grown in DMEM supplemented with 10% FCS and antibiotics, were transfected with the recombinant adenoviral plasmids and virus enrichment cycles were performed. Adenoviruses were easily detected by fluorescence microscopy during the enrichment cycles since the vector coded for the GFP in addition to the GyrB-PKR protein.

Adenoviral infection. On the day of infection, the growth medium in the 96-well plates was replaced with 40 μl/well of fresh growth medium supplemented with only 2% FCS and containing the virus. The plates were returned to the incubator and 2 h later 160 μl of fresh growth medium supplemented with 10% FCS was added to each well. All cell lines were efficiently infected with adenoviruses as monitored by GFP expression 24 h after infection. When infection was done on cells cultured in 6-well plates, the growth medium was replaced with 1 ml/well of fresh growth medium supplemented with only 2% FCS and containing the virus. After 2 h of incubation 2 ml of fresh growth medium supplemented with 10% FCS was added to each well.

PKR killing screen. Cells were seeded in 96-well plates at the following densities: U87MG-wtEGFR, 5000 cells/well; MDA-MB-468, 10,000 cells/well; MCF-7, 6000 cells/well; HT-29, 3000 cells/well; LNCaP, 4000 cells/well. Forty-eight hours after seeding, adenoviral infection was performed as described above. Two days later, the cells were treated with DMSO only or with coumermycin (dissolved in DMSO) at two concentrations, 10 or 100 ng/ml. Measurement of cell viability by methylene blue assay was performed at the following time points: on the day of infection, on the day of treatment, and 24 and 48 h after treatment.

Determination of cell growth. Cell growth was measured by the methylene blue assay [24]. Cells were fixed in glutaraldehyde, 0.05% final concentration, for 10 min at room temperature. After being washed, the plates were stained with 1% methylene blue in 0.1 M borate buffer, pH 8.5, for 60 min at room temperature. The plates were then washed extensively and rigorously in deionized distilled water to remove excess dye and dried. The dye taken up by the cells was eluted in 0.1 N HCl for 60 min at 37°C, and absorbance was monitored at 630 nm. Each point of the growth curve experiments was calculated from five replicate wells.

Determination of eIF-2α phosphorylation upon GyrB-PKR activation. U87MG-wtEGFR cells or LNCaP cells were seeded in six-well plates (150,000 and 450,000 cells/well, respectively). Forty-eight hours after seeding, adenoviral infection was performed as described above. Two days later, the cells were treated with DMSO only or with coumermycin (dissolved in DMSO) at two concentrations, 10 or 100 ng/ml. U87MG-wtEGFR cells were lysed 24 h after treatment. LNCaP cells were lysed 12 and 36 h after treatment. Fifty micrograms of each sample was electrophoresed and blotted. The blots were probed with the antibodies anti-PKR K-17 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5000) and anti-eIF-2α (Research Genetics, USA; 1:10,000) using the ECL procedure.

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