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Seminars in Cancer Biology 13 (2003) 309–314

seminars in CANCER BIOLOGY

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Tumor specific activation of PKR as a non-toxic modality of cancer treatment

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Abstract

Over the past decade progress has been made in the development of therapies against cancer. Small molecules, mainly tyrosine kinase inhibitors (tyrphostins) like Gleevec, Iressa targeting CML and EGFR overexpressing tumors have entered the clinic, where a large number of other tyrphostins are at various stages of clinical development. In parallel a few antibodies like Herceptin targeting breast cancer overexpressing Her-2 and Rituxan targeting B cell malignancies are utilized in the clinic. In all these cases success is moderate and restricted to a narrow population of patients, except for Gleevec which is effective for a long duration for chronic CML. The cancer community agrees that this is actually a unique exception that proves the rule. Over the past few years a few modalities of cancer gene therapies have emerged. In this short review we shall summarize our efforts to develop methods to activate PKR selectively in cancer cells.

Keywords: dsRNA; Glioblastoma; PKR

1. Regulation of translation by PKR

PKR was discovered over 25 years ago as a protein kinase that inhibits protein synthesis upon addition of double-stranded RNA (dsRNA) to the reticulocyte lysate cell-free translation system [1–5]. Interest in this enzyme increased dramatically when it was demonstrated that it was not a peculiarity of immature red cells but was also inducible in a wide range of cell types by treatment with interferons [6–9] and likely to be responsible at least partly for the antiviral and antiproliferative effects of these cytokines [10–13].

PKR is a member of a small family of protein kinases that phosphorylate the alpha subunit of the protein synthesis initiation factor eIF-2 and therefore play an important role in the translational regulation of gene expression [14]. The initiation factor eIF-2 is responsible for binding the initiating methionyl-tRNA (Met-tRNA$_f$), together with a molecule of GTP, and placing Met-tRNA$_f$ on native 40S ribosomal subunits [15]. During the course of this process GTP is hydrolyzed to GDP, and when eIF-2 leaves the ribosome later in initiation (at the 60S subunit joining stage), it does so as an inactive [eIF-2.GDP] complex (Fig. 1). Regeneration of active eIF-2α requires the exchange of the GDP for a new molecule of GTP, catalyzed by the guanine nucleotide exchange factor eIF-2B [15]. When eIF-2 becomes phosphorylated by PKR the initiation factor acquires an increased affinity for eIF-2B, sequestering the latter within an inactive complex (Fig. 1). As a result the rate of guanine nucleotide exchange on both phosphorylated and unphosphorylated eIF-2 falls, as eIF-2B present is cells in less than stoichiometric levels with respect to eIF-2. This sequestration leads to the cessation of polypeptide chain initiation. This has effects on the global rate of protein synthesis, but it may also selectively inhibit the translation of specific mRNAs that for various reasons have a greater than average requirement for active eIF-2 in the cell.

2. PKR, cell growth, apoptosis and cancer

PKR can strongly inhibit cell growth through phosphorylation of eIF-2α. Inducible expression of PKR in *Saccharomyces cerevisae* resulted in inhibition of growth, whereas a transdominant negative PKR mutant exhibited no phenotype. Growth inhibition was reversed by the co-expression of a mutant yeast eIF-2α, which cannot be phosphorylated by PKR, supporting the notion that the mechanism of cell growth control was through phosphorylation of eIF-2α [16]. Stable expression of PKR in yeast, mammalian and insect

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doi:10.1016/S1044-579X(03)00045-2

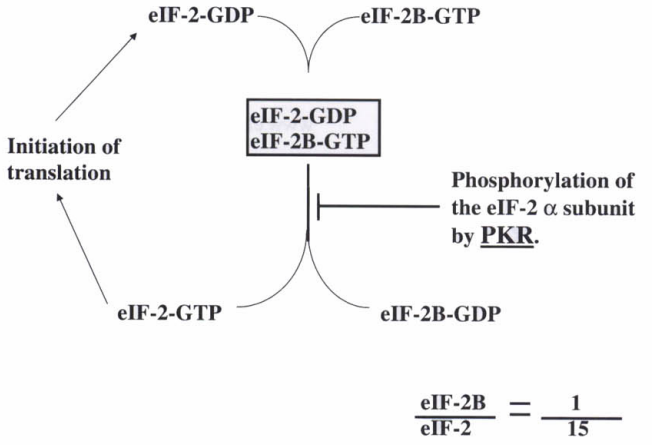

Fig. 1. Mechanism of translation inhibition by PKR.

cells results in reduced growth rates. On the other hand transfection with a transdominant negative mutant of PKR causes malignant transformation of mouse cells [17].

PKR has been implicated in antiviral activities, cell growth regulation and tumorigenesis processes that rely on apoptosis as control mechanisms in vivo. Transient overexpression of PKR in mouse cells is sufficient to induce apoptosis [18]. A functional PKR pathway is required to induce apoptosis in response to LPS, dsRNA, serum deprivation or TNF-α treatment (reviewed in Ref. [19]).

In studies with 3T3/L1 cell lines which allow tetracycline controlled expression of either wild type PKR or the catalytically inactive PKRΔ6, cells overexpressing PKR became extremely sensitive to dsRNA and TNFα-induced apoptosis, whereas cells expressing PKRΔ6 were completely resistant [20]. These studies also demonstrated that activation of PKR induces the expression of members of the tumor necrosis factor receptor (TNFR) family, including Fas (CD95/Apo-1) and of the pro-apoptotic protein Bax. In contrast, transcripts representing Fas, TNFR-1, Fas-associated death domain (FADD), FLICE, Bad and Bax were ablated in cells expressing PKRΔ6 where levels of Bcl-2 mRNA increased significantly. The involvement of death receptors in PKR-induced apoptosis was underscored by demonstrating that murine fibroblasts lacking FADD were almost completely resistant to dsRNA-mediated cell death. Thus, PKR, a key cellular target for viral repression, is a receptor/inducer for the induction of pro-apoptotic genes by dsRNA and probably functions in interferon-mediated host defense to trigger cell death in response to virus infection and perhaps tumorigenesis [20].

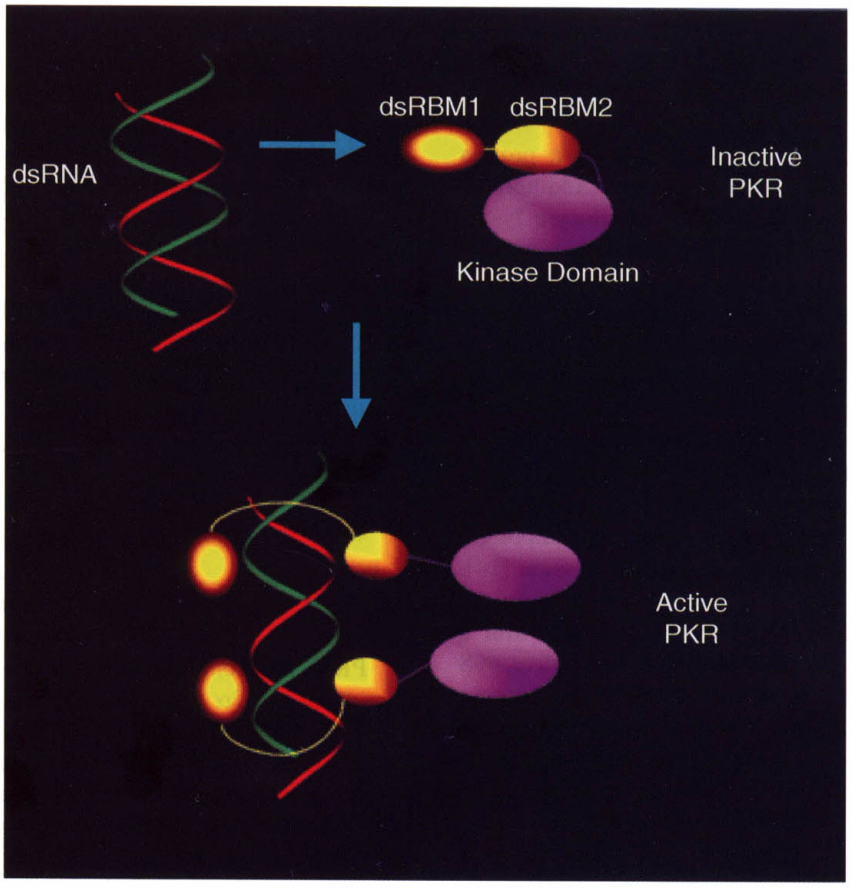

Fig. 2. Mechanism of PKR activation by dsRNA.

3. Mechanism of PKR activation by dsRNA

The only clearly established mechanism for the activation of PKR requires the interaction of dsRNA with the enzyme [21]. Although the exact nature of this interaction is not fully understood, various features of the activation process have been established. A key step in the activation of PKR involves differential binding of dsRNA to its two structurally similar N-terminal dsRNA binding motifs, dsRBM1 and dsRBM2. Recent studies [22] have suggested that dsRBM2 locks the kinase domain in a 'closed' conformation in the latent PKR whereas dsRBM1 is unconstrained by the rest of the protein, fluctuating with millisecond motions. dsRNA is first anchored to the free dsRBM1, inducing cooperative binding to dsRBM2, which then exposes the kinase domain (Fig. 2). This would also relax the major dimerization region that is otherwise restrained due to the interaction between dsRBM2 and the kinase domain. Such concerted conformational change then results in PKR dimerization, autophosphorylation and activation [22].

One of the major properties of PKR is that it is activated by low levels of dsRNA and inhibited by higher levels [2,5]. However, the inhibition only occurs when high concentrations of dsRNA are added simultaneously with the enzyme. In other words, once activated by low concentrations of dsRNA, PKR cannot be inhibited by subsequent addition of high concentrations of dsRNA. It seems that at lower dsRNA concentrations, the flexible dsRBM1 with higher RNA binding affinity first anchors to dsRNA, and facilitates the cooperative dsRNA binding to lower affinity dsRBM2 to expose the kinase domain. Once both dsRBMs are bound to dsRNA, additional dsRNA has no effect because of the slow kinetics of exchange once the stable complex is formed. When high dsRNA concentrations are added at the beginning, the two dsRBMs simultaneously bind to two different dsRNA molecules, which leads to a distorted overall protein conformation, unfavorable for the kinase function.

PKR exhibits differing affinities for dsRNAs of different sizes and it has been shown that molecules shorter than 30 bp fail to bind stably and do not activate the enzyme, where at high concentrations they prevent activation by long dsRNA. 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 [23]. These dsRNAs fail to activate PKR at high concentrations and also prevent activation by long dsRNA.

4. Specific activation of PKR in cancer cells

Recently [44] we demonstrated for the first time, the feasibility of an approach for treating tumors through selective activation of PKR in cancer cells by cancer specific dsRNA molecules generated in situ.

The strategy is based on the following reasoning: in many cancers, chromosomal rearrangements, truncations and alternative splicing of pre-mRNA lead to the production of unique mRNA species. Table 1 summarizes some of the many examples of these aberrations that occur in various malignancies. In principle, a cancer cell can be transfected with a vector encoding antisense RNA (AS-RNA) between 30 and 85 nucleotide length, that complements the unique mRNA fragments flanking the fusion point or truncation. Upon hybridization with mRNA, a dsRNA molecule will be generated that is sufficiently long to activate PKR within the cancer cell harboring the mutation (Fig. 3). On the other hand, in normal cells, where the chromosomal translocation or truncation does not occur, only short dsRNA species will be formed, with the two sequences which are far apart. These short dsRNA species, will not be long enough to activate PKR [23] thus the cells will not die. Namely, if such antisense RNA were to be expressed in all cells, only the cancer cells would die. This strategy could in principle be applied to many types of malignancies where such deletions or translocations are known to occur (Table 1) or can be identified.

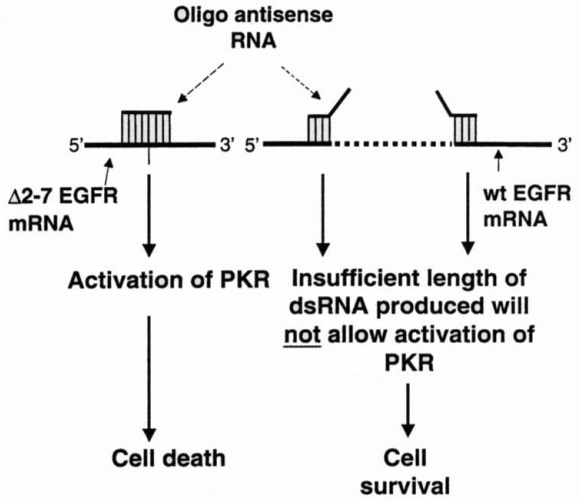

Fig. 3. Mechanism of specific activation of PKR in U87MGΔEGFR cells.

We utilized the U87MGΔEGFR cell line, which expresses a truncated form of EGFR-Δ(2–7) EGFR (Table 1), as a model system to examine this hypothesis [44]. Expression of a 39-nucleotide long antisense RNA complementary to the unique exon 1–8 junction, caused selective death of cells harboring the truncated EGFR both in vitro and in vivo but did not affect cells expressing wild type EGFR. A lentiviral vector expressing the 39 antisense sequence strongly inhibited gliobastoma growth within the mouse brain when injected subsequent tumor cell implantation. Treated mice survived whereas mice treated with sense expressing vector or untreated mice all died. The involvement of PKR in the selective death of U87MGΔEGFR cells was revealed in experiments demonstrating cell specific activation of PKR induced by ΔEGFR antisense, strong inhibition of total translation and rescue of the cells by treatment with a PKR inhibitor or the co-expression of PKR protein inhibitors or the dominant negative PKRΔ6 [44].

Table 1
Genetic abnormalities in cancers

Phenotype	Rearrangement	Genes involved	Reference
B cell NHL			
BL	t(8;14)(q24;q32)	MYC, IGH	[24]
	t(2;8)(p12;q24)	IGK, MYC	
	t(8;22)(q24;q11)	MYC, IGL	
FL, DLCL	t(14;18)(q32;q21)	IGH, BCL2	[24]
		CAAAAGCATTCTGAGAAGG***TG***AGATAAGAACTGAGT	
DLCL	t(3;22)(q27;q11)	BCL6, IGL	[24]
	t(3;14)(q27;q32)	BCL6, IGL	
	t(3q27)	BCL6	
MCL	t(11;14)(q13;q32)	CCND I, IGH	[24]
LPL	t(9;14)(p13;q32)	PAX5, IGH	[24]
		TGAATTTTATTTTTTTTGA***AG***GGGTCTTAGTGATGTCTGA	
SLL	t(14;19)(q32;q13.3)	IGH, BCL3	[24]
MALT	t(11;18)(q21;q21)		[24]
Variable	t(1;14)(p22;q32)		[24]
	t(1;14)(q22;q32)		
	t(3;14)(p21;q32)		
	t(10;14)(q24;q32)	LYT10, IGH	
	t(11;14)(q23;q32)	RCK, IGH	
	t(11;14)(q23;q32)	LPC, IGH	
T cell NHL			
ALCL (CD30+)	t(2;5)(p23;q35)	ALK, NPM	[24]
		GACAATTGATGACCTGGAA***GT***GTACCGCCGGAAGCACCAG	
CTCL	t(10q24)	LYT10	[24]
Variable	t(7;14)(q35;q11)	TCRB, TCRA	[24]
	t(11;14)(p13;q11)	RBTN2, TCRD	
	inv(14)(q11q32)	TCRA, TCL I	
	t(14;14)(q11;q32)		
Leukemia			
CML	t(9;22)	BCR, ABL	[25]
AML	t(7;11)(p15;p15)	NUP98, HOXA9	[26]
Other cancers			
Multiple liposarcoma	t(12;16)(q13;p11)	TLS/FUS, CHOP	[27]
Clear cell sarcoma	t(12;22)(q13;q12)	EWS, ATFI	[28]
DSRCT	t(11;22)	EWS, WT1	[29]
Synovial sarcoma	t(X;18)(p11;q11)	SYT-SSX1	[30]
Thyroid cancer	PTC5	RET, RFG5	[31]
Neuroblastoma, Ewing cancers	t(11.22)(q24.q12)	EWS, FLI1	[32]
Myxoid chondrosarcoma	t(9;22)(q22;q12)	EWS, TEC	[33]
Alveolar rhabdomyosarcoma	13q14 with either 2q35 or 1p36	FKHR, PAX7/PAX3	[34]
Prostate carcinoma	t(6;16)(p21;q22)	TPC/HPR	[35]
Gastric carcinoma	11q23	Duplication of ALL-1	[36]
Lung cancer	3p21.3	Alternative splicing of RBM6	[37]
Breast and ovarian cancers		Alternative splicing of TSG101	[38]
Pancreatic carcinoma	3p14	Alternative splicing of FHIT	[39]
Colorectal cancer		Alternative splicing of hMSH2	[40]
Wilms' kidney and thyroid tumors		Alternative splicing of PAX8	[41]
Retinoblastoma		Deletion in RB	[42]
Glioblastoma		Deletion in EGFR	[43]
		GGCTCTGGAGGAAAAGAAA***GG***TAATTATGTGGTGACAGAT	

Refs. [24–35] indicate fusions of specified genes. Abbreviations: NHL, non-Hodgkin's lymphoma; BL, Burkitt's lymphoma; FL, follicular lymphoma; DLCL, diffuse large cell lymphoma; MCL, Mande cell lymphoma; LPL, lymphoplasmacytoid lymphoma; SLL, small lymphocytic lymphoma; MALT, mucosa-associated lymphoid tissue; ALCL, anaplastic large cell lymphoma; CTCL, cutaneous T cell lymphoma; DSRCT, desmoplastic small round cell tumor; CML, chronic myeloid leukemia; AML, acute myeloid leukemia. Sequences flanking the fusion point (bold and italics) are shown for some translocations.

The main advantage of this strategy is its high selectivity for the cancer cells expressing mutated genes. It should be noted that many unique mRNA species expressed specifically in tumors have not yet been identified. In order to implement the general strategy of dsRNA killing strategy (DKS) one can utilize any unique mRNA molecule, even those without a defined cancer promoting function. Given that a unique mRNA is transcribed in the target cells, an

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appropriate vector can be designed. This provides another significant advantage over the strategies targeting inhibition of expression or function of only one specific gene.

Thus the approach described here may be applicable to the treatment of a wide range of cancers and other proliferative disorders in which chromosomal translocation and truncational mutations occur. At this point it is not clear whether this approach will be widely successful. One should always consider the possibility that the AS-RNA produced will function as a classical antisense RNA and inhibit the expression of the target gene and not activate PKR. Another possibility is that the AS-RNA will form a three-dimensional structure incompatible with the formation of dsRNA. Yet another possibility is that the PKR activation pathway may be inhibited as a survival benefit for the cancer cell. In order to show feasibility of this strategy for other cancers we are currently examining the feasibility of the approach in other types of malignancies such as B and T cell lymphomas. We are utilizing two human non-Hodgkin lymphoma (NHL) cell lines, the Pfiefer B cell lymphoma which harbors a Bcl/IgH translocation and the Karpas 299 T cell lymphoma which harbors a NPM/ALK translocation (Table 1). Antisense and sense sequences corresponding to the translocation junction of NPM/ALK and Bcl-2/IgH were constructed in adenoviral and lentiviral vectors, respectively. At present we could not demonstrate cell death subsequent to antisense infection (unpublished data). There are a number of possibilities for a mutation in PKR or eIF-2α or inhibition of phosphorylation of PKR or/and eIF-2α. We examined the activation of PKR in vitro using synthetic dsRNA-pIC. In Pfiefer cells addition of pIC led to activation of PKR and phosphorylation of eIF-2α (unpublished data). In contrast, in Karpas 299 cells, although PKR was active, we did not detect any change in the phosphorylation of the eIF-2α.

Our assumption is that in Pfiefer cells, PKR activation is not sufficient to cause cell death because of the strong anti-apoptotic signaling in these cells, which may emanate from the high levels of Bcl-2 in these cells. The sequencing eIF-2α from Karpas 299 cells did not reveal any mutations. We currently suspect that in Karpas 299 cells, eIF-2α is protected by another protein. Indeed, the level of the p67 protein believed to be an inhibitor of eIF-2α phosphorylation [45], is significantly higher in Karpas 299 cells than in U87MG cells.

We are currently trying to overcome these obstacles in both cell lines. In the Pfiefer B cell lymphoma we would like to down-regulate Bcl-2 signaling. In the Karpas 299 T cell lymphoma, we are examining various approaches to reduce the level of p67.

5. Conclusions

The cancer specific activation of PKR could be a powerful and non-toxic tool for the treatment of various cancers. The proof of principle of the cancer specific activation of PKR strategy was shown on glioblastoma cells which harbor the truncated form of the EGFR. At the moment we are trying to apply this strategy for other cancers. Several obstacles could be encountered at two malignancies and others are being tested. Therefore feasibility of this approach for wide range of cancer remains to be established.

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