The Role of Eukaryotic Initiation Factor 2 alpha Phosphorylation Pathway in Translational Control and Virus-mediated Oncogenesis

by

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Your file Votre référence
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To my daughter, the love and joy of my life

Abstract

Two important steps of translation initiation include the recognition of the mRNA cap structure by eIF4E and the recycling of eIF2. Each step is thought to be regulated independently through the interaction of eIF4E with 4E-BPs and the phosphorylation of the α subunit of eIF2 at serine 51. Phosphorylation of eIF2α by dsRNA-dependent protein kinase PKR inhibits protein synthesis in cells subjected to virus infection; therefore, most viruses have evolved mechanism to overcome the deleterious effects of PKR. The human papillomavirus (HPV) E6 oncoprotein contributes to virus-induced pathogenicity through multiple mechanisms including the inhibition of apoptosis and the blockade of interferon action. This study demonstrates a novel function of PKR providing a link between the two mechanisms of regulation of translation initiation. Activation of PKR induces the PI3K-PKB/Akt and FRAP/mTOR pathways leading to S6 and 4E-BP1 phosphorylation upon stress conditions and in response to growth stimuli. Induction of the PI3K pathway antagonizes the apoptotic effects of PKR activation, but does not intervene with its translational inhibitory activity. Investigating functional interaction of HPV E6 and PKR, we determined that HPV-18 E6 protein synthesis is regulated by eIF2α phosphorylation. On the other hand, E6 oncoprotein is able to rescue cells from PKR-mediated inhibition of protein synthesis and induction of apoptosis by promoting eIF2α dephosphorylation through physical association with GADD34/PP1 holophosphatase complex. These findings demonstrate, for the first time, the ability of PKR to activate a growth-stimulatory pathway; PI3K. Furthermore, it demonstrates role of oncogenic E6 in antagonizing signaling pathways induced by PKR including eIF2α phosphorylation and PI3K pathway.

Résumé

Les deux étapes importantes de l'initiation de la traduction sont la reconnaissance de la structure Cap de l'ARNm par le facteur d'initiation eIF4E, et le recyclage du facteur eIF2. Chacune de ces étapes est réglée indépendamment par l'interaction de eIF4E et de 4E-BPs d'une part et la phosphorylation de la sous-unité α du facteur eIF2 d'autre part. En réponse à une infection virale, les cellules active la protéine kinase PKR, qui phosphoryle eIF2α, et par conséquent réprime l'initiation de la traduction. Cependant, la majorité des virus ont développé des mécanismes leur permettant de contourner l'effet inhibiteur de PKR. Ainsi, l'oncoprotéine virale E6 contribue à la pathogénicité de HPV à travers différents mécanismes incluant l'inhibition de l'apoptose et le blocage de l'action des interférons (IFNs). Cette étude montre une nouvelle fonction de PKR en établissant un lien entre les deux mécanismes de régulation de l'initiation de la traduction. L'activation de PKR induit les voies PI3K et FRAP/mTOR, responsable de la phosphorylation des protéines S6 et 4E-BP en réponse aux stress cellulaires et aux facteurs de croissance. L'induction de la voie PI3K antagonise l'apoptose induite par PKR, mais n'intervient pas dans l'inhibition de la traduction. De plus, des expériences sur l'interaction fonctionnelle entre les protéines HPV-18 E6 et PKR, nous ont permis de déterminer que la synthèse de la protéine HPV-18 E6 est dépendante de la régulation de la phosphorylation de eIF2α. L'oncoprotéine E6 lève l'inhibition de la traduction et prévient l'induction de l'apoptose par PKR en provoquant la déphosphorylation de eIF2α suite à la liaison entre eIF2α et du complexe holophosphatase GADD34/PP1. Ces résultats montrent, pour la première fois, la capacité de PKR à activer la voie de

stimulation de la croissance PI3K et le rôle de l'oncoprotéine E6 comme antagoniste des voies eIF2α et PI3K induites par PKR.

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Preface

In accordance with "guidelines for thesis Preparation", the candidate has chosen the option of presenting her results in the classical format. A general introduction is presented in chapter I.

Materials and methods for research presented in this document are detailed in chapter II; the results described in chapter III have been published, in part, in the stated journal article, following subjection to the peer review process and in the manuscript, which is in the process of publication:

1. Shirin Kazemi, Suiyang Li, Stavroula Papadopoulou, Qiaozhu Su, Shuo Wang, Greg Matlashewski, Akihiko Yoshimura, Thomas E. Dever and Antonis E. Koromilas. (2004) Control of translation initiation factor eIF2α phosphorylation by the human papillomavirus-18 E6 oncoprotein: Implications for eIF2α-dependent gene expression and cell death. MCB 24: 3415-3429

2. Shirin Kazemi, Dionissios Baltzis, Zineb Mounir, Ana Maria Rivas-Estillas, Shirley Huang, Stavroula Papadopoulou, Like Qu, Qiaozhu Su, Shuo Wang, Jennifer F. Raven, Olivier Pluquet and Antonis E. Koromilas. (2006) Activation of the phosphoinositide-3 pathway by the protein kinase PKR provides a functional cross-talk between eIF2α and 4E-BP1 phosphorylation pathways. (manuscript under review)

All research presented in Chapter III was performed by the candidate, with the exception of these specific contributions:

Dr. Ana Maria Rivas-Estillas performed the experiment in Figure 15A.

Dr. Stavroula Papadopoulou contributed to Figure 15B.

Zineb Mounir participated in Figure 16A, C, D, Figure 17A, Figure 18C, and Figure 20.

Dr. Like Qu performed the experiment in Figure 18A.

Shirley Huang performed the experiment in Figure 18B.

Dionissios Baltzis has performed the experiment in Figure 28C.

Dr. Suiyang Li is responsible for cloning the FLAG-18 and 11 E6 expression plasmids and for generation of cell lines expressing these constructs (HT1080/FLAG-18 and 11 E6) used in this study.

The candidate was also involved in several collaborations with respect to research that is beyond the scope of this document. This research has been published in the following journal articles following subjection to the peer review process:

3. Wang S, Raven JF, Baltzis D, Kazemi S, Brunet DV, Hatzoglou M, Tremblay ML, Koromilas AE. (2006) The catalytic activity of the eukaryotic initiation factor-2α kinase PKR is required to negatively regulate Stat1 and Stat3 via activation of the T-cell protein-tyrosine phosphatase. JBC 281(14):9439-49

4. Robert F, Kapp LD, Khan SN, Acker MG, Kolitz S, Kazemi S, Kaufman RJ, Merrick WC, Koromilas AE, Lorsch JR, Pelletier J. (2006) Initiation of Protein

Synthesis by Hepatitis C Virus Is Refractory to Reduced eIF2*GTP*Met-tRNA$_i^{Met}$ Ternary Complex Availability. Mol Biol Cell. 2006 Aug 23 (Epub ahead of print)

5. Deborah Stewart, Shirin Kazemi, Suiyang Li, Paola Massimi, Lawrence Banks, Antonis Koromilas, and Greg Matlashewski (2004) Ubiquitination and proteasome degradation of the E6 proteins of human papillomavirus types 11 and 18. Journal of General Virology. 85: 1419-1426

6. Baltzis D, Pluquet O, Kazemi S, and Koromilas AE. (2006) The eIF2α kinases induce p53 degradation through the regulation of GSK3β upon ER stress independently of translational control. (Manuscript in preparation).

Table of Contents

List of Figures and Tables

Chapter I

General Introduction

Chapter I - General Introduction

1. Protein translation

Synthesis of protein from the messenger RNA (mRNA) is the final step of encoding the genomic information concealed in DNA. Protein translation consists of three steps: initiation, elongation and termination. Each step is processed by the help of designated factors in order to bring the three essential components, the ribosome, the mRNA and aminoacylated tRNAs together and mediate the synthesis of the polypeptide chain (1,2).

1.1 Introduction to translation

Translation of mRNA to protein is carried out by large ribonucleoproteins named ribosomes and is mediated by a large group of proteins termed translation factors. Translation is initiated with priming of the ribosome by the unique initiator methionyl-tRNA (Met-$tRNA_i^{Met}$), and its recruitment to the proper AUG start codon on the mRNA. The polypeptide chain is then extended by binding of aminoacyl-tRNAs, determined by the template mRNA, to the ribosome. The translation is terminated when a stop codon on mRNA is encountered (3).

1.1.1 Initiation of translation

Translation initiation is a complex process in which the initiator Met-$tRNA_i^{Met}$, small (40S), and large (60S) ribosomal subunits, are assembled by eukaryotic initiation factors (eIFs) into an 80S ribosome at the initiation codon (AUG) of mRNA (Fig.1).

The 40S ribosomal subunit initially forms the 43S pre-initiation complex, by binding to eIF3, 1, 1A, 5 and a ternary complex. The ternary complex is consisted of the Met-$tRNA_i^{Met}$ that recognizes the AUG codon during initiation, and eIF2 coupled to GTP. Binding of the pre-initiation complex to mRNA is mediated by interaction of eIF3 with the eIF4F protein complex associated with the 5' cap structure of mRNA. Cap is an inverted 7-methylguanosine that is attached to the first nucleotide structure located at the 5' end of the majority of eukaryotic mRNAs, which consists of m^7GpppN (where m^7G represents 7-methlguanylate, p represents a phosphate group and N represents any base) and together with the poly A tail at the 3' end form strong promoters of translation.

The eIF4F complex is comprised of several initiation factors including eIF4E, a cap-binding protein that mediates the interaction of eIFs and the ribosome with the 5' cap structure; eIF4A, a dead-box RNA helicase which serves to unwind the secondary structure at 5' UTR allowing the 43S complex to scan the mRNA for the start codon; and eIF4G, which acts as a scaffolding protein to recruit eIF4E, eIF4B and eIF3 (1,2). It is currently believed that the 43S complex scans the 5' UTR in a 5' to 3' direction until an appropriate start site for peptide synthesis is recognized through the formation of base pairs between the Met-$tRNA_i^{Met}$ and the start codon (4). Binding of 43S complex to the start codon AUG results in formation of a stable complex referred to as 48S initiation complex. Subsequently, eIF2-bound GTP undergoes hydrolysis that is catalyzed by eIF5 which is required for joining of the 60S to the initiation complex. At this step initiation factors including eIF2-GDP are dissociated from the small ribosomal subunit, leaving the initiator tRNA base-paired with AUG in ribosomal P-site. A second

step of GTP hydrolysis on eIF5B occurs to release eIF5B and render the ribosome competent for polypeptide synthesis. The large (60S) ribosomal subunit then joins the 40S subunit at this position to form the catalytically competent 80S ribosome (5).

Figure 1. Schematic representation of cap-dependent translation initiation

The eIF4F complex consisted of eIF4E, eIF4G and eIF4A is recruited to mRNA through association of eIF4E cap-binding protein with the cap structure. The RNA helicase eIF4A, assisted by eIF4B, unwinds the secondary structure at 5'UTR of the mRNA. The initiator Met-$tRNA_i^{Met}$ forms the ternary complex with eIF2-GTP, which together with 40S ribosomal subunit and eIF3 yield the 43S pre-initiation complex. eIF4F bridges the 43S pre-initiation complex to mRNA through interaction of eIF4G with eIF3. The pre-initiation complex scans the mRNA in a 5' to 3' direction until it identifies the initiation codon AUG, a process which is assisted by eIF1 and eIF1A. Stable binding of the 43S pre-initiation complex to the AUG codon forms the 48S initiation complex. AUG recognition triggers GTP hydrolysis on eIF2 and dissociation of initiation factors. Subsequently, joining of the 60S subunit results in formation of the 80S initiation complex which then proceeds to elongation.

Adapted from Gebauer and Hentze, Molecular Cell Biology 2004

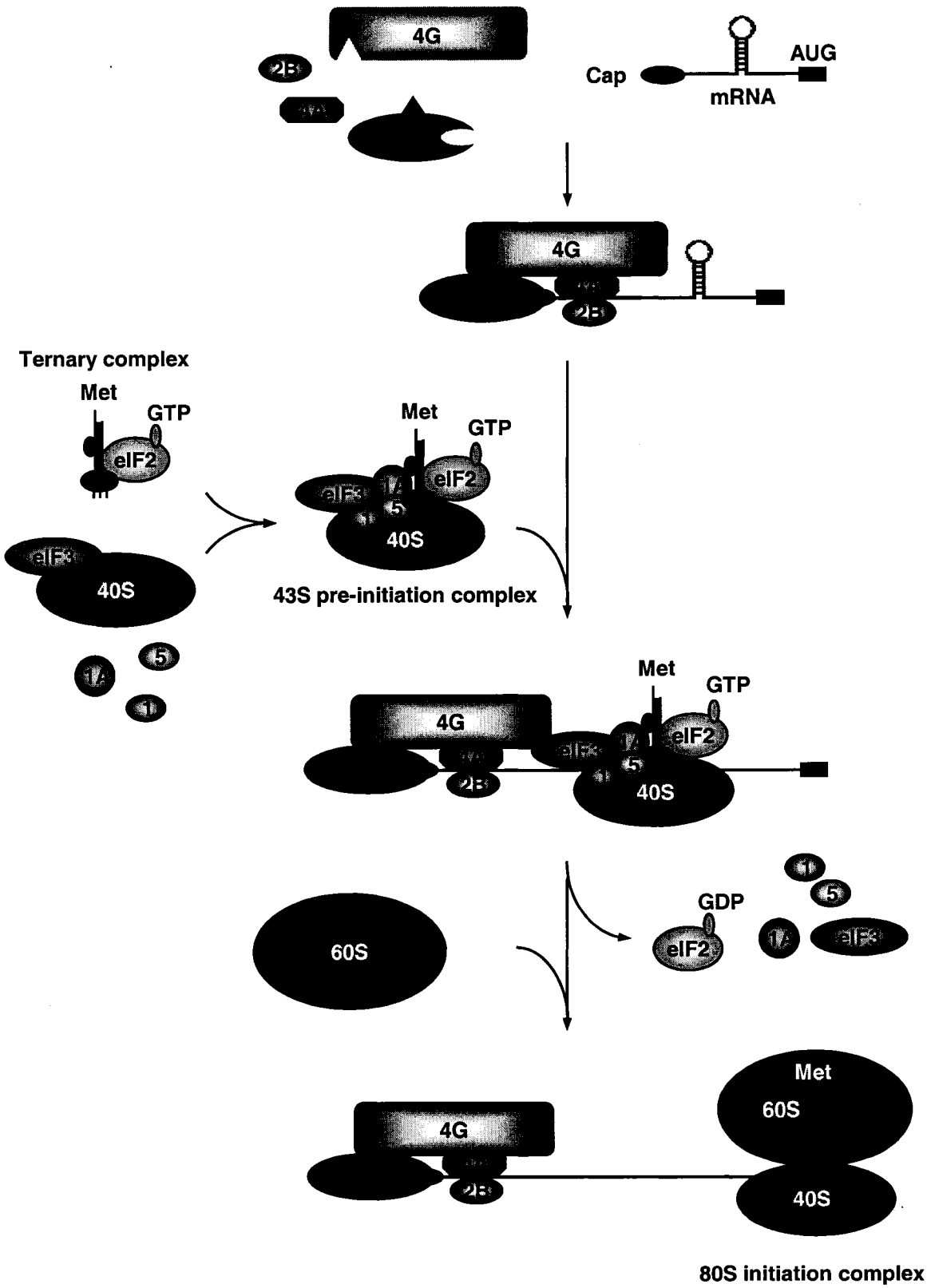
4G
2B
1A
Cap
AUG
mRNA
4G
2B
Ternary complex
Met
GTP
eIF2
40S
eIF3
5
1A
1
Met
GTP
eIF2
eIF3
1A
1
5
40S
43S pre-initiation complex
Met
GTP
4G
eIF2
eIF3
1A
1
5
2B
40S
60S
GDP
eIF2
1
5
1A
eIF3
Met
60S
4G
2B
40S
80S initiation complex

Figure 1

1.1.2 Translation initiation factors and their functions

Initiation of translation in eukaryotes is assisted by more than 25 polypeptides, termed eIFs each serving a specific function (2) (Table 1).

The initiation factor eIF4E is the rate-limiting factor in initiation and is characterized by its cap binding activity, which is crucial for cap-dependent translation. Therefore, the expression of eIF4E is necessary for active cell growth. Its structure resembles a cupped hand or a baseball glove forming a suitable binding site for the cap structure. Through this activity, eIF4E mediates correct positioning of 43S complex (6).

The role of eIF4G is scaffolding during initiation of translation, which recruits the initiation factors to the site of initiation. Moreover, eIF4G interacts with poly (A)-binding protein (PABP), which binds the poly A stretch at 3' UTR. This interaction mediates the circularization of the mRNA bringing the 3' UTR, in close proximity to the 5' end of the mRNA. The 3' UTR contains most translational regulatory sequences, and thus such circularization provides the means for synergistic stimulating effect of the cap structure and the poly-A tail on translation (1,2).

The RNA helicase eIF4A plays a major role in translation of highly structured mRNAs. eIF4A is an ATP-dependent RNA binding protein, an RNA-dependent ATPase and an RNA helicase. It belongs to a large family of DEAD box RNA helicases and is believed to unwind the secondary structure in the 5' UTR of capped mRNAs to facilitate ribosome binding and its subsequent scanning to reach the initiation codon (6). eIF4A, however, is an inefficient helicase and its function is enhanced by eIF4B and eIF4H. The role of eIF4B is not limited to its stimulatory effect on eIF4A helicase

activity. It may also contribute to the binding of ribosome to mRNA and to bridge eIF3 to 40S ribosomal subunit (7).

The pivotal role of eIF2 is the recruitment of the Met-tRNA$_i^{Met}$ to 40S ribosomal subunit, and due to this function it plays a major role in regulation of translation initiation. eIF2 consists of three non-identical subunits; α, β and γ (8). The α subunit contains the well-known serine (Ser) 51 phosphorylation site which is phosphorylated by the eIF2α kinases and is involved in translational control. The β subunit contains multiple phosphorylation sites for casein kinase II (CK-II), protein kinase C (PKC), cAMP- dependent protein kinase (PKA) and two of three consensus guanine nucleotide binding domains. It also contains the binding domain for eIF5B at its N-terminus and the guanine nucleotide exchange factor, eIF2B at its C-terminus. The γ subunit contains Met-tRNA$_i^{Met}$ binding site and all three consensus guanine nucleotide binding domain, which are the characteristic sequence of GTP-binding proteins. Thus, eIF2γ plays a major role in binding to GDP but it does not possess intrinsic GTPase activity. eIF5 is a GTPase-activating protein (GAP) specific for eIF2, which provides an "arginine finger" for the catalytic center of eIF2γ. Upon recognition of the start codon by the pre-initiation complex, eIF5 binds to eIF2 and mediates the hydrolysis of the GTP bound to eIF2 and eIF2-GDP is released from the initiation complex (9). Formation of another ternary complex requires the eIF2-bound GDP to be replaced by GTP. This reaction is catalyzed by eIF2B after each round of initiation (8).

The guanine exchange factor eIF2B consists of five non-identical conserved subunits α-ε, and is regulated by diverse signals (10). Phosphorylation of eIF2B by multiple kinases such as glycogen synthase kinase-3 (GSK-3), casein kinase (CK)-I,

CK-II and dual-specificity tyrosine phosphorylated and regulated kinase (DYRK) has a negative effect on its activity. Phosphorylation of eIF2B by GSK3 requires activity of DYRK as a priming kinase by phosphorylating eIF2B on Ser539. In response to insulin, GSK3 activity is inhibited and, thus, eIF2B becomes dephosphorylated and more active. In yeast, the activity of eIF2B is also regulated by fusel alcohols, such as butanol, which are products of amino acid breakdown as nitrogen source. The most important mechanism of regulation of eIF2B, however, is through phosphorylation of eIF2α in response to cellular stress. Once phosphorylated, eIF2 has higher affinity for eIF2B and results in inhibition of its nucleotide-exchange function (1,10).

eIF1 and eIF1A play an important role in translation initiation site specificity and ensure the correct arrangement of the components of 43S complex when binding to mRNA. They act synergistically to enable 43S to reach the initiation codon without arresting at the initial binding site (1). eIF1A enhances binding of 43S to mRNA and the scanning process, but is unable to promote reaching the first AUG. It also stabilizes binding of the ternary complex to 40S ribosomal subunit, which may play a role in correct positioning of Met-tRNA$_i^{Met}$ and mRNA during scanning. eIF1, on the other hand decreases formation of cap-proximal complex and promotes low levels of 48S complex formation (1). Together, eIF1 and eIF1A promote dissociation of 43S from cap-bound eIF4F complex and mediate binding of 43S to mRNA in order to scan to the initiation codon (1). In addition to its role in initiation codon selection during 48S complex formation, eIF1 also participates in maintaining the fidelity of the initiation process at a later stage, hydrolysis of eIF2-bound GTP, by inhibiting premature GTP hydrolysis and by linking establishment of codon-anticodon base-pairing with GTP

hydrolysis. Moreover, recent data demonstrate that full activation of eIF5B and efficient joining of 60S ribosomal subunit requires the extreme C-terminus of eIF1A which interacts with eIF5B (11).

eIF5 plays an essential role in initiation of protein synthesis in conjunction with GTP and other initiation factors. Following formation of the 48S initiation complex at the AUG codon of an mRNA, eIF5 interacts with the 48S initiation complex to promote the hydrolysis of eIF2-bound GTP, which results in the release of bound initiation factors from the 40S subunit, an event that is essential for the subsequent joining of the 60S ribosomal subunit to the 40S subunit resulting in formation of the functional 80S ribosome. Detailed characterization of the eIF5-promoted GTP hydrolysis reaction shows that eIF5 functions as a GTPase-activating protein (GAP) in translation initiation. It contains an arginine-finger motif consisting of an invariant arginine residue at its N-terminus, which is essential for its function. This invariant arginine residue is presumably involved in the stabilization of the transition state of the GTP hydrolysis reaction catalyzed by initiation factor eIF2 (1). Recent data reveal that hydrolysis of eIF2-bound GTP by eIF5 is necessary but not sufficient for joining of the 60S, and it requires the function of eIF5B (5).

eIF5B is a GTPase that facilitates joining of the 60S ribosomal subunit to the 40 S ribosomal subunit during translation initiation. Formation of the resulting 80S initiation complex triggers eIF5B to hydrolyze its bound GTP, reducing the affinity of the factor for the complex and allowing it to dissociate. The role of eIF5B could be to displace eIF3 and possibly eIF1 and eIF1A from the 40S subunit after release of eIF2 (9). The interaction of eIF1A with eIF5B promotes ribosomal subunit joining, and

possibly provides a checkpoint for correct complex formation, allowing full activation of GTP hydrolysis only upon formation of a properly organized 80 S initiation complex (11).

Table 1. Eukaryotic initiation factors and their functions

Translation initiation is a complex process which is assisted by more than 25 polypeptides termed initiation factors (eIFs for eukaryotic initiation factors), the most important and well characterized ones of which are listed in this table.

Adapted from Dever TE, Cell 2002

Table 1 : Eukaryotic initiation factors and their functions

Eukaryotic Initiation Factor	Function
eIF1	Fidelity of AUG codon recognition
eIF1A	Facilitate Met-tRNA$_i^{Met}$ binding to 40S subunit
eIF2	Bind Met-tRNA$_i^{Met}$ to 40S subunit; GTPase
eIF2B	Guanine-nucleotide exchange factor for eIF2
eIF3	Promote Met-tRNA$_i^{Met}$ and mRNA binding to 40S
eIF4A	Dead-box helicase
eIF4B	Promote eIF4A activity
eIF4E	m^{7}GpppN cap binding protein
eIF4F	Cap binding complex of eIFs 4A, 4E and 4G
eIF4G	Adaptor protein interacts with many other proteins
eIF4H	Similar to eIF4B
eIF5	AUG recognition and promote eIF2 GTPase activity
eIF5B	Subunit joining

1.2 Regulation of translation

Regulation of protein synthesis plays an important role in development, differentiation, cell cycle progression, cell growth, and apoptosis. In eukaryotic cells mRNA translation is affected by a wide range of environmental conditions. Protein synthesis is increased in response to hormones, growth factors, cytokines, nutrients and mitogens, whereas stress conditions such as virus infection, ER stress, DNA damage, oxidative and osmotic stress or withdrawal of nutrients lead to a decrease in protein synthesis (12).

Translational control involves regulation at various steps including initiation, elongation and termination (2). Considering that translation is one of the main energy-consuming processes, it is more efficient to regulate its onset than to disrupt it later at elongation or termination steps (12).

1.2.1 Regulation of translation initiation

Regulation of translation initiation is generally achieved by modifications in phosphorylation states of the initiation factors or their regulators. Two important steps of translation initiation include the recognition of the mRNA cap structure by eIF4E and the recycling of eIF2. Each step is thought to be regulated independently through the interaction of eIF4E with 4E-BPs and the phosphorylation of the α subunit of eIF2 at Ser 51.

1.2.2 Cap-dependent translation regulation through modulation of eIF4E function.

Regulation of cap-dependent translation initiation is achieved in part by modulating the function of the cap-binding initiation factor eIF4E. The majority of mRNAs that play a

role in cell proliferation and growth or those involved in cell survival or apoptosis have highly structured 5' UTRs which require formation of the eIF4F complex for their translation, which functions as a regulation mechanism to tightly control the expression of these genes (13). Binding of eIF4E to the 5' cap structure as a complex with eIF4G and eIF4A, results in unwinding of this secondary structures which facilitates the recruitment of the 43S pre-initiation complex to mRNA and the subsequent scanning process. Interaction of eIF4E and eIF4G requires a small domain in eIF4G which is shared by a family of proteins known as 4E-binding proteins (4E-BPs), which have been identified as important inhibitors of eIF4E. In quiescent cells 4E-BPs inhibit cap-dependent translation initiation by binding to eIF4E and preventing its association with eIF4G and thus assembly of a functional eIF4F complex (Fig.2).

Three 4E-BP proteins have been identified: 4E-BP1, 4E-BP2, and 4E-BP3, which share 56-59% homology, especially at their mid-region containing the eIF4E-binding site. Despite their high homology and identical functions, 4E-BP mRNAs are not expressed at the same levels in all tissues, which may explain the existence of three 4E-BPs with redundant function. The binding site to eIF4E is characterized by a conserved amino acid motif containing the core sequence YXXXXLΦ, where X is any amino acid and Φ is a residue with an aliphatic portion, most often lysine, but sometimes methionine or phenylalanine. Exposure of the cells to various types of extracellular stimuli such as hormones (insulin, angiotensin II, etc.) mitogens (TPA), growth factors (EGF, PDGF, NGF, IGFI, IGFII, etc.) and cytokines (IL-3, GMCSF, etc.), induces 4E-BP phosphorylation on several sites (14). This results in a decreased affinity of 4E-BP for eIF4E leading to their dissociation, thereby allowing translation

initiation to proceed. On the contrary, serum starvation, amino acid deprivation, and certain environmental stresses such as heat shock or osmotic shock, result in dephosphorylation of 4E-BP and inhibition of cap-dependent translation (15). To date, seven Ser and threonine (Thr) sites have been identified in 4E-BP1, two of which are situated at the amino-terminal side of the eIF4E-binding motif; Thr37 and Thr46 and the other five at the carboxy-terminal side; Ser65, Thr70, Ser 83, Ser101 and Ser 112. The first five phosphorylation sites are conserved among all 4E-BPs, but Ser101 and Ser112 only exist in 4E-BP1 (16,17). Hyperphosphorylation of 4E-BP occurs in two steps: phosphorylation of Thr37 and Thr46 acts as a priming event for Thr70 which occurs prior to Ser65. This priming may induce conformational changes in 4E-BP structure so that a second kinase is recruited and phosphorylates the other residues (16). This specific pattern of phosphorylation is required for disruption of the eIF4E/4E-BP interaction. Although Ser65 phosphorylation is the final step for release from eIF4E, phosphorylation of this residue is not sufficient to mediate the dissociation, suggesting that phosphorylation of the other sites is also required (18). The pathways leading to 4E-BP phosphorylation involve the phosphoinositide-3 kinase (PI3K) and mammalian target of Rapamycin (mTOR) (15,19) (Fig.3).

Figure 2. Regulation of cap-dependent translation initiation through modulation of eIF4E availability

The eIF4E-binding proteins share the same binding sites for eIF4E as eIF4G. Binding of 4E-BPs to eIF4E inhibits the formation of eIF4F complex and therefore initiation of translation. Phosphorylation of 4E-BPs on several residues results in its dissociation from eIF4E, which is now available to bind to eIF4G and proceed to initiate the translation.

Adapted from Gebauer and Hentze, Molecular Cell Biology 2004

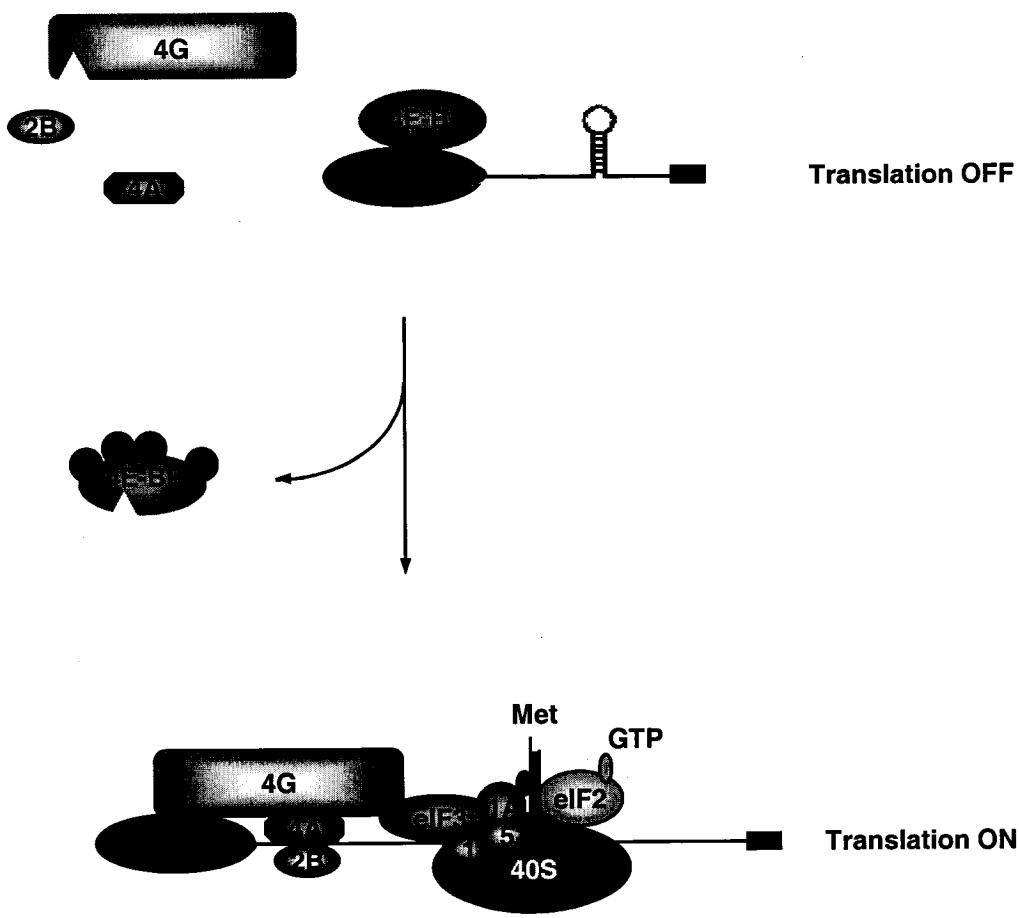

Figure 2

Figure 3. Schematic representation of the PI3K pathway

The best characterized pathway leading to phosphorylation of 4E-BP1 is the PI3K-FRAP/mTOR pathway. Exposure of the cells to extracellular stimuli such as growth factors, cytokines, hormones and mitogens induces PI3K signaling pathway, which results in cell growth, proliferation and survival. Upon binding of the ligands to the receptor tyrosine kinases, PI3K is recruited and activated through binding of its regulatory subunit p85 to the phosphorylated receptors. PI3K catalyzes the formation of PI(3,4,5)P3 from PI(4,5)P2 at the plasma membrane, which recruits proteins containing the pleckstrin homology (PH) domain such as PDK1 and PKB/Akt. This recruitment brings the two proteins in vicinity leading to phosphorylation and activation of PKB/Akt. Phosphorylation of PKB/Akt by FRAP/mTOR-Rictor results in its full activation which then leads to modulation of the activity of downstream effectors. Pharmacological inhibitors of the PI3K and FRAP/mTOR pathways; wortmannin, LY294002 and rapamycin are depicted in red.

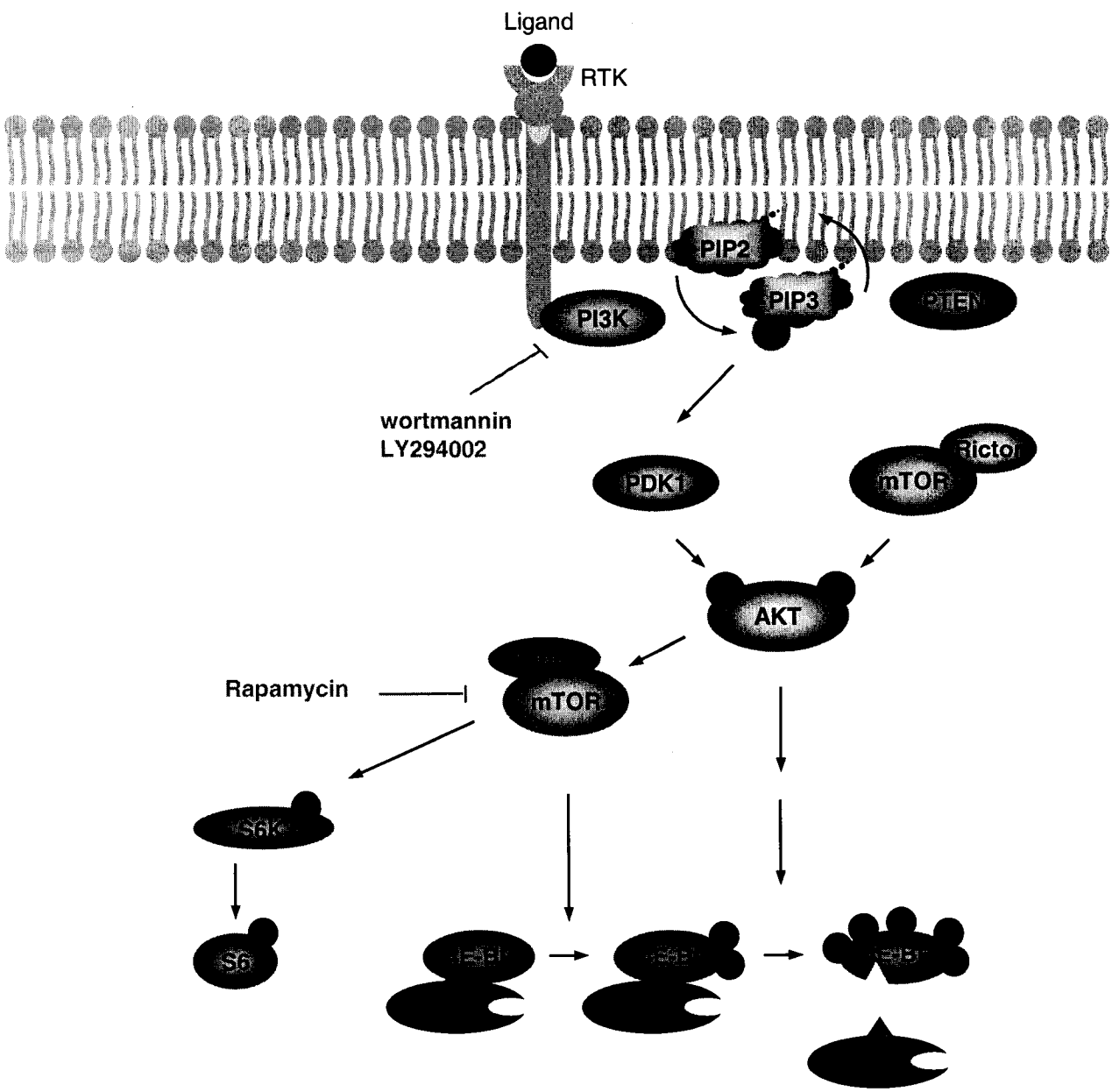
Ligand
RTK
PIP2
PIP3
PTEN
PI3K
wortmannin
LY294002
PDK1
Rictor
mTOR
AKT
Rapamycin
mTOR
S6K
S6

Figure 3

1.2.3 Regulation of translation initiation through modulation of eIF2 function.

Another step of regulation of translation initiation is phosphorylation of the translation initiation factor eIF2. As discussed earlier, eIF2 associates with 40S ribosomal subunit in its GTP-bound form as a part of the ternary complex to recruit the initiator tRNA. The GTP molecule is hydrolyzed upon recognition of the start codon leading to dissociation of eIF2-GDP from the initiation complex. To reconstitute a functional ternary complex for a new round of translation this GDP has to be replaced by GTP, a reaction catalyzed by eIF2B, a guanine nucleotide exchange factor (GEF) required for the recycling of eIF2-GDP to eIF2-GTP. Phosphorylation of the α subunit on Ser51 results in higher affinity of eIF2 for eIF2B, which sequesters eIF2-GDP and eIF2B in a complex with reduced GEF activity resulting in inhibition of the overall protein synthesis (20) (Fig.4). To date, four distinct eIF2α kinases have been identified. Functional analyses of these kinases have indicated that each enzyme provides the cell with a unique capability to modulate translation in response to specific forms of stress, including heme-regulated inhibitor (HRI), stimulated by heme depletion; general control non-derepressible-2 (GCN2), induced by amino acid deprivation; dsRNA activated protein kinase (PKR), activated upon virus infection; PKR-like endoplasmic reticulum-resident kinase (PERK), activated upon conditions of endoplasmic reticulum (ER) stress (7). The mechanism of function of these kinases is explained in section 3.

Figure 4. Regulation of translation initiation by eIF2α phosphorylation pathway

Initiation of translation requires hydrolysis of GTP to GDP at two steps; recognition of AUG initiation codon and joining of 60S subunit. The eIF2 binds to Met-tRNA$_i^{Met}$ in its active GTP-bound state to form the ternary complex. GTP is hydrolyzed upon recognition of AUG and eIF2-GDP leaves the initiation complex. The guanine nucleotide exchange factor eIF2B replaces GDP by GTP in order to reconstitute a functional ternary complex for a new round of translation initiation. Phosphorylation of the α subunit of eIF2 results in sequestering eIF2B in an inactive complex and as a result GDP-GTP exchange can no longer occur and global mRNA translation is inhibited. Four distinct kinases phosphorylate eIF2a in response to stress conditions such as heme deficiency, virus infection and ER stress, all resulting in inhibition of protein synthesis.

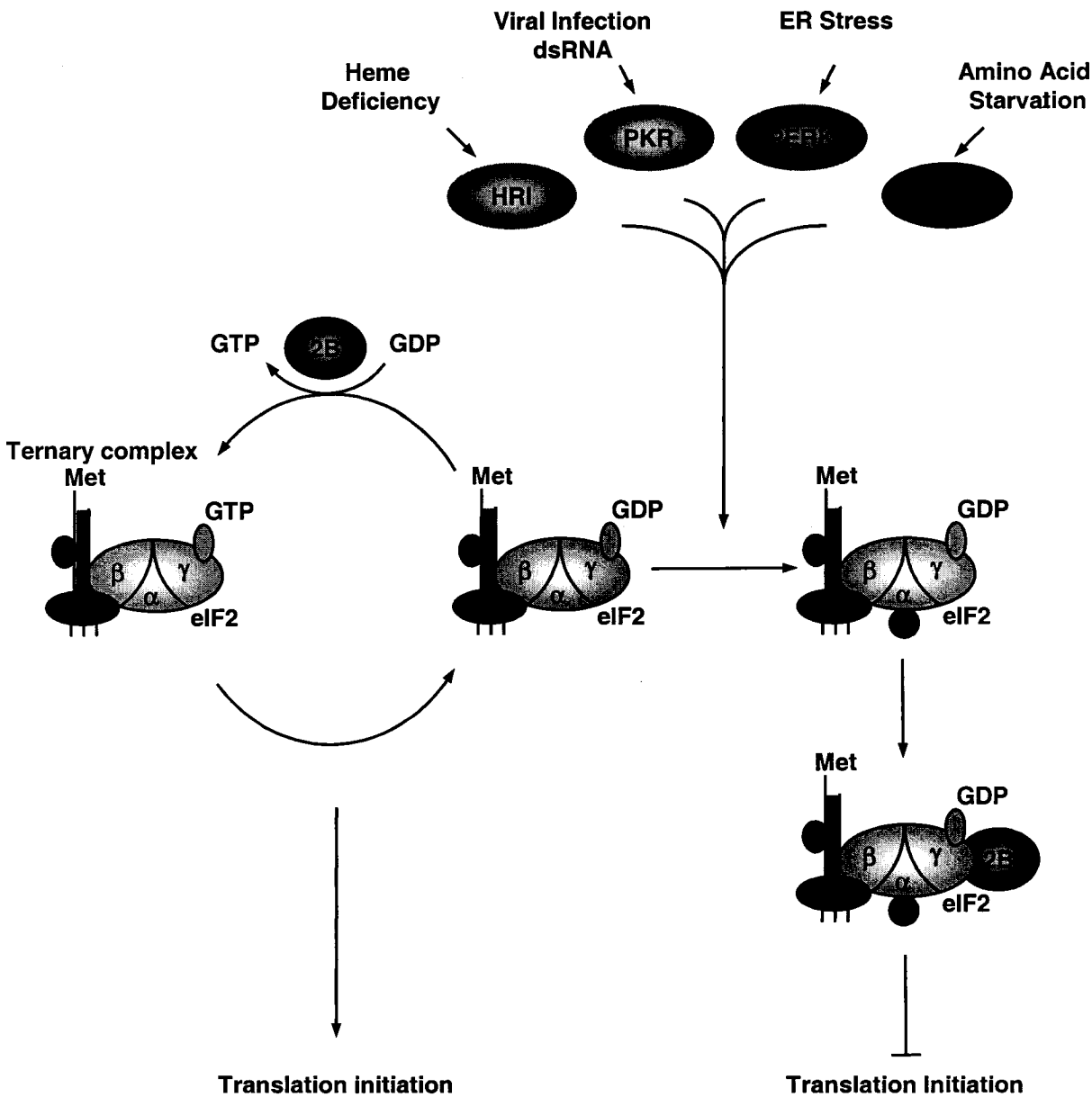
Viral Infection
dsRNA
ER Stress
Heme
Deficiency
Amino Acid
Starvation
PKR
HRI
GTP
GDP
Ternary complex
Met
GTP
eIF2
Met
GDP
eIF2
Met
GDP
eIF2
Met
GDP
eIF2
Translation initiation
Translation Initiation

Figure 4

2. PI3K Signaling Pathway

Phosphoinositide signaling plays a very important part in signal transduction in response to a wide range of cellular stimuli involved in numerous types of cellular processes, which underscores the significance of the lipid molecules as second messengers. Extracellular stimuli such as growth factors, cytokines and hormones bind to their cognate tyrosine kinase receptors (RTKs), resulting in phosphorylation of phosphatidylinositol lipids which initiates a set of events leading to cell growth, proliferation, cell cycle entry and cell survival. Various signaling proteins contain pleckstrin-homology (PH) domains which specifically bind to phosphorylated phosphoinositides (21). These proteins include serine threonine kinases, tyrosine kinases, and exchange factors that regulate heterotrimeric guanosine triphosphate (GTP)-binding proteins (G-proteins) and are located in the cytoplasm of unstimulated cells. Once translocated to the membrane where they associate with the phosphorylated lipids, these signaling proteins mediate recruitment of proteins required to initiate local responses leading to various signaling pathways. These signaling proteins coordinate complex events leading to changes in cell metabolism, cell growth, cell movement, and cell survival (22).

2.1 Phosphatidyinositol 3- Kinase PI3K

The enzymes that phosphorylate phosphatidylinositol and its derivatives are termed phosphoinositide kinases (PI3K). PI3Ks are dual specificity lipid and protein kinases that phosphorylate phosphatidylinositol-4,5-biphosphate [PI(4,5)P2] at its D-3 position of the inositol ring to form phosphatidyl-inositol-3,4,5-triphosphate [PI(3,4,5)P3] at the membrane (Fig.5A) (21). There are several forms of PI3K in higher

eukaryotes. Class Ia enzymes exist as a heterodimer consisted of a regulatory subunit (p85) and a catalytic subunit (p110) (Fig.5B). The mammalian p110 includes at least four different family members, p110α, β, γ, and δ, which are encoded by at least two genes (22). The regulatory subunit consists of five isoforms; p85α , p85β , p55α, p55γ and p50α (23-29). The p85β and p55γ are each products of a single gene whereas, p55α and p50α are products of alternative splicing of p85α gene (29). The structure of the regulatory subunit consists of two proline-rich motifs (PRMs), two Src Homology (SH2) domains and a p110 binding domain, which is situated between the two SH2 domains (IS) (Fig.5B) (22) . Through this domain, the regulatory subunit maintains the p110 catalytic subunit in a low-activity state in quiescent cells and mediates its activation by direct interaction with phosphotyrosine residues of activated growth factor receptors or adaptor proteins (21). The p85 subunits act as adaptor proteins, utilising the two SH2 domains for interactions with proteins containing phosphotyrosine in a specific motif, with a consensus sequence of YXXM. The p110γ does not associate with a p85 subunit but appears to be controlled by interactions with G-protein β and γ subunits (30). Moreover, a portion of growth factor-mediated PI3K activation has been shown to proceed through a direct interaction of a p21Ras effector domain with a specific site in the p110α or β subunits (31,32). In addition to being a lipid kinase, PI3K has been demonstrated to exhibit protein kinase activity (33). The crystal structure of PI3K shows the presence of a typical conserved protein kinase domain that shares similarity to the Src family of tyrosine protein kinases. However, PI3K has been shown to catalyze serine rather than tyrosine phosphorylation in the presence of Mn2+ ions (34). Indeed, the p110α catalytic subunit has been shown to phosphorylate the p85

regulatory subunit and the insulin receptor substrate-1 (IRS1) on serine residues (34-36).

2.2 Protein and lipid phosphatase PTEN

PTEN (Phosphatase and TENsin homolog on chromosome TEN) is a tumor suppressor, which is frequently mutated or lost in many tumor types (37,38). PTEN is a member of tyrosine phosphatase family with a unique lipid phosphatase activity that is not shared by other family members (39). It antagonizes PI3K signaling by dephosphorylating the D-3 position of the inositol ring in PI(3,4,5)P generated by PI3K (Fig.5A). PTEN phosphatase domain, located at the N-terminal half, has homology to both protein tyrosine phosphatase family and the nonphosphatases tensin and auxilin (37). The C-terminus contains a binding domain for a class of PDZ domain which plays a role in protein-protein interaction (40,41). In addition to its N-terminal domain, PTEN contains a calcium-independent C2 domain that mediates lipid binding and membrane localization. The phosphatase domain together with the C2 domain form a minimal catalytic unit, as truncation mutations in any of these regions leads to abolishment of the phosphatase activity (42). One important downstream target of PTEN is protein kinase PKB/Akt which is activated upon formation of phosphorylated lipids by PI3K.

Figure 5. The function and structure of PI3K

A. PI3K function. PI3K catalyzes the formation of PI(3,4,5)P3 from PI(4,5)P2 by phosphorylating the phosphoinositides at their D3 position of the inositol ring. This results in formation of the second messengers to initiate the downstream signaling pathways.

B. PI3K structure. Schematic diagram represents the structure of catalytic subunit and three regulatory subunit isoforms.

Adapted from Vivanco and Sawyers, Cancer 2002, and Funaki *et al.*, Cellular Signaling 2000

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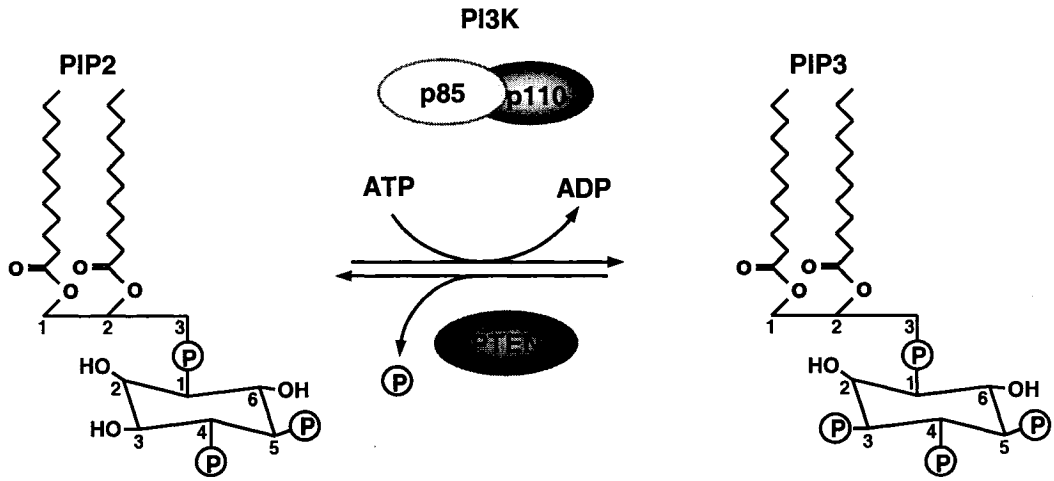
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PIP2
p85
p110
PIP3
ATP
ADP
HO
OH
HO

B

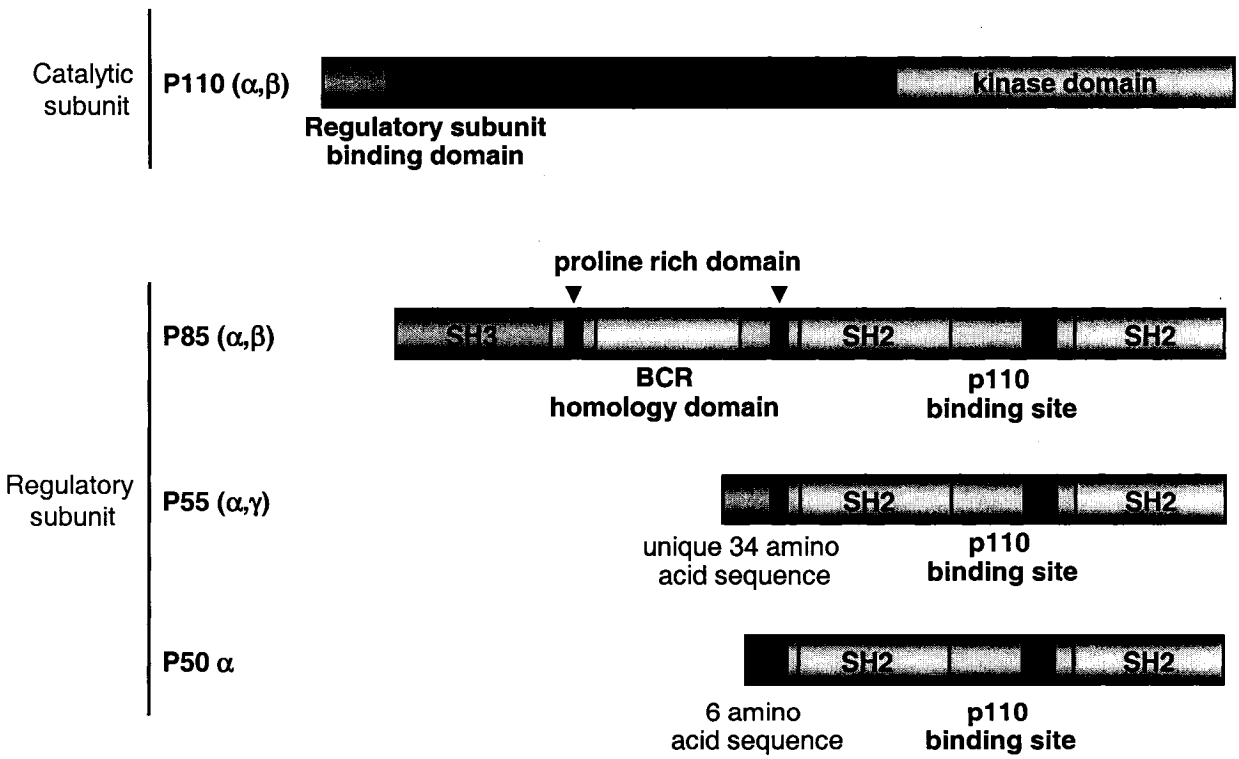
Catalytic subunit
P110 (α,β)
kinase domain
Regulatory subunit binding domain
proline rich domain
P85 (α,β)
SH3
SH2
SH2
BCR homology domain
p110 binding site
Regulatory subunit
P55 (α,γ)
SH2
SH2
unique 34 amino acid sequence
p110 binding site
P50 α
SH2
SH2
6 amino acid sequence
p110 binding site

Figure 5

2.3 Serine/threonine protein kinase PKB/Akt

Protein kinase B (PKB)/Akt was first identified as a homologue of the viral oncogene, *v-akt*. It is a Ser/Thr kinase, which belongs to the 'AGC' super family of protein kinases and shares similarity within its catalytic domain structure and in its mechanism of activation with protein kinase A (PKA) and protein kinase C (PKC) was thus named PKB (43). Three members of PKB/Akt family, termed PKB(α, β, γ) or Akt(1, 2, 3) are products of three distinct genes with 80% homology but different tissue distribution (44) (Fig.6A). The structure of PKB/Akt contains a PH domain at the N-terminus, a central kinase catalytic domain and a C-terminal hydrophobic motif (HM) (43,44). The PH domain is shared between numerous signaling proteins and provides a lipid binding module for the PI3K generated PI(3,4,5)P3 (45,46). The kinase domain contains a threonine residue in the activation loop (Thr308, Thr309 and Thr305 in PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3, respectively), phosphorylation of which results in activation of PKB/Akt by inducing conformational change that allows better substrate binding and higher rate of catalysis. The HM domain plays a dual role in regulating the kinase activity. It contains a docking site for PDK1 (3-Phosphoinositide-Dependent protein Kinase 1) and provides stability for the catalytic domain (44). It contains a second phosphorylation site, Ser473, Ser474, and Ser472 in PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3, respectively, which is required for full activation of PKB/Akt (43). Although PKB/Akt was originally suspected to play a role in signal transduction in response to growth factors, its significance was only demonstrated when it was shown to be a downstream target of PI3K (47,48). Activation of Class 1a and 1b PI3K is thought to induce PKB/Akt activity (Fig 3). Upon formation of PI(3,4,5)P3 at

the membrane as a result of PI3K activation, PKB/Akt is translocated to the membrane (49), where it is recruited to the vicinity of PDK1. This recruitment is believed to induce a conformational change in PKB/Akt, which enhances the accessibility of the threonine residue for phosphorylation by PDK1. Phosphorylation of PKB/Akt by PDK1 results in its activation, which in turn initiates signaling pathways affecting cell growth, cell cycle entry and cell survival (43). PDK1 is a Ser/Thr kinase that contains a C-terminal PH domain and interacts with phosphorylated lipids. PDK1 phosphorylates the threonine residue of PKB/Akt in the activation loop, which regulates access to the catalytic site of PKB/Akt (50-52). In order to achieve maximal activation, PKB/Akt requires phosphorylation at the serine site in its C-terminus. The kinase responsible for phosphorylation of this residue has been unknown. It has been believed that PDK1 itself (53), or another kinase termed PDK2 phosphorylate Ser473. It has also been suggested that modification of Ser473 occur through autophosphorylation (54). Recent data, however, demonstrates that FRAP/mTOR-Rictor complex phosphorylates the Ser473 residue (55). The most recent scenario suggests that PI3K-generated phosphorylated lipids recruit PKB/Akt and PDK1 to the plasma membrane. Phosphorylation of serine residue by FRAP/mTOR-rictor promotes PKB/Akt interaction with PDK1 through HM domain. This interaction results in activation of PDK1, which then phosphorylates PKB/Akt on the threonine residue. At this point the HM domain prefers intramolecular association and releases PDK1 and adopts a fully active state for PKB/Akt (44). Active PKB/Akt dissociates from the membrane and localizes to the cytoplasm and nucleus (56-58) where it phosphorylates the downstream targets such as glycogen synthase kinase-3 (GSK3) (59), Procaspase-9 (60), forkhead transcription factors (FKHR,

FKHRL1, AFX) (61-63), IKKα (64,65), Ask1 (66), BAD (67,68), CREB (69) and Mdm2 (70,71). Through modulating the activation of these proteins, PKB/Akt plays a role in regulation of apoptosis, proliferation and other processes (Fig.6B).

Most of the known protein targets of PKB/Akt become inactivated by the phosphorylation event. For example, phosphorylation of FKHRL1 results in their cytoplasmic localization and retention by a protein termed 14-3-3, which results in blocking transcription of genes normally stimulated by FKHRL1 in the nucleus. The same mechanism is applied to the proapoptotic protein BAD. Upon phosphorylation by PKB/Akt, BAD is sequestered in cytoplasm by 14-3-3 and can no longer bind to antiapoptotic proteins such as Bcl-2 or Bcl-X_L, which results in releasing them for a cell survival response (43).

A third target of PKB/Akt is GSK3 (59). This protein kinase was originally characterized as an important enzyme in regulation of glycogen synthesis in response to insulin before its diverse role in many cellular processes such as differentiation, proliferation, and transformation was determined (72). GSK3 is constitutively active in unstimulated cells and phosphorylates many proteins including glycogen synthase (GS) (73,74), c-Myc (75), β-catenin (76), p53 (77,78), and cyclin D1 (79) to keep them in inactive states or promote their degradation. The two isoforms of GSK3, α and β, are phosphorylated by PKB/Akt, a modification that turns off their catalytic activity and leads to the activation of pathways that are normally repressed by GSK3 (72).

Another downstream target of PKB/Akt is the PI3K-related protein kinase, FRAP/mTOR that plays a major role in regulating protein synthesis. It is phosphorylated directly by PKB/Akt (80,81).

Figure 6. PKB/Akt signaling pathway

A. The structure of PKB/Akt illustrates the plecksterin homology domain (PH), the kinase domain (KD) and the regulatory domain (RD). The structure of three members of the family is presented, indicating the Thr307 and Ser473 phosphorylation sites in kinase domain and regulatory domain, respectively.

B. The PKB/Akt signaling pathway and its downstream targets are shown. Upon activation of PI3K and formation of PIP3, PKB/Akt is phosphorylated and activated by its upstream kinases on Thr308 and Ser473. Activation of PKB/Akt results in phosphorylation and inactivation of proteins involved in apoptosis such as Bad, pro-caspase 9, FKHR, IKK and Mdm2 and activation of proteins involved in cell growth and proliferation such as GSK3, FRAP/mTOR, p21 and p27.

Adapted from Nicholson and Anderson, Cellular Signaling 2002, and Fresno Vara *et al.*, Cancer Treatment Reviews 2004

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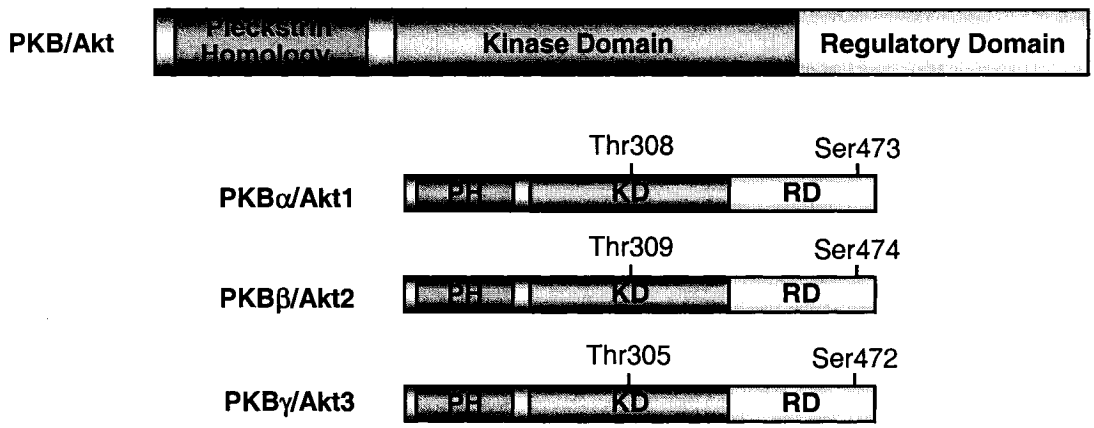
PKB/Akt
Pleckstrin Homology
Kinase Domain
Regulatory Domain
Thr308
Ser473
PKBα/Akt1
PH
KD
RD
Thr309
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PKBβ/Akt2
PH
KD
RD
Thr305
Ser472
PKBγ/Akt3
PH
KD
RD

B

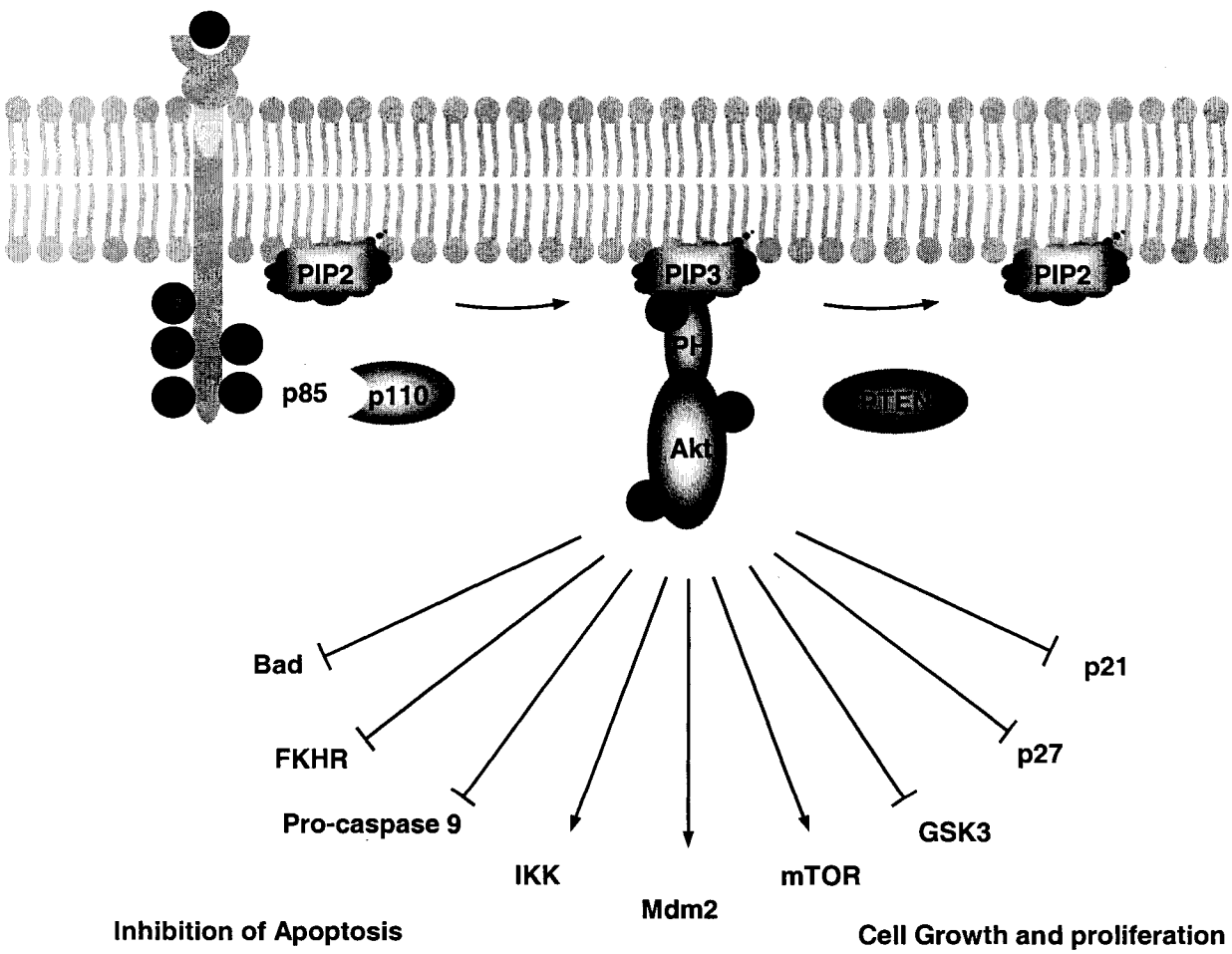
PIP2
PIP3
PIP2
p85
p110
PH
Akt
PTEN
Bad
FKHR
Pro-caspase 9
IKK
Mdm2
mTOR
GSK3
p27
p21
Inhibition of Apoptosis
Cell Growth and proliferation

Figure 6

2.4 Serine/threonine protein kinase FRAP/mTOR

The mammalian target of rapamycin (mTOR) also known as FRAP (FKBP-Rapamycin- Associated Protein), belongs to the PIKK (PI3K-related Kinases) family of kinases (82). The kinase domain of FRAP/mTOR is located at the C-terminus and has sequence similarities with the catalytic domain of PI3K (Fig.7A). To date, there is no evidence for lipid kinase activity of FRAP/mTOR. In this regard, FRAP/mTOR is similar to other members of the family, including ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3 related). Upstream to the catalytic domain is the FRB (FKB12-Rapamycin Binding) domain which mediates binding of FKB12 (FK506-Binding Protein) and rapamycin. FRAP/mTOR contains a large FAT (FRAP, ATM, TRRAP) domain which is shared between all PIKKs (83). The C-terminus end contains another FAT domain termed FATC, which is essential for FRAP/mTOR activity (84,85). FRAP/mTOR also possesses an autoinhibitory domain termed negative regulatory domain (NRD) or repressor domain between the catalytic and FATC domains. The N-terminus contains 20 tandem HEAT (Hungtington, EF3, A subunit of PP2A, TOR1) repeats, which are present in many proteins and are involved in protein-protein interactions (86).

FRAP/mTOR plays an important role in regulation of translation through phosphorylation and inactivation of 4E-BP1, as well as phosphorylation and activation of S6 kinase (S6K1) (87,88). In addition, FRAP/mTOR also modulates transcription by phosphorylating STAT3 and pRb (Fig.7B). The activity of FRAP/mTOR is regulated by growth and survival stimuli such as growth factors, cytokines and nutrients (89). Raptor (Regulatory Associated Protein of TOR) is a positive regulator of FRAP/mTOR and is

essential for its activity. It acts as an adaptor protein to recruit FRAP/mTOR substrates. At its N-terminus, Raptor contains a unique conserved domain followed by three HEAT repeats and seven WD-40 repeats at its C-terminus. The HEAT repeats in FRAP/mTOR and Raptor mediate the interaction of the two proteins (90). Raptor also binds to 4E-BP1 and S6K1 through interaction with a five amino acid motif termed TOS (TOR Signaling), present at the N-termini of both proteins (91,92). This interaction is necessary for phosphorylation of 4E-BP1 and S6K1 by FRAP/mTOR (93). Another positive regulator of FRAP/mTOR activity in response to nutrients, is the G protein β-subunit-like protein (GβL) (94). It contains seven WD-40 repeats and interacts with the kinase domain of FRAP/mTOR independently of Raptor. GβL stabilizes mTOR-Raptor complex, thus GβL, Raptor and FRAP/mTOR may represent a nutrient-sensitive complex (94). In response to nutrient condition such as amino acid availability mTOR-Raptor complex undergoes a conformational change that induces its efficient interaction with downstream targets leading to their phosphorylation (90,94).

FRAP/mTOR activation in response to growth factors and cytokines is mediated by PI3K (95,96). PKB/Akt plays an important role in regulation of mTOR activity. Two residues of FRAP/mTOR are directly phosphorylated by PKB/Akt; Thr 2446 and Ser 2448, from which Ser 2448 is phosphorylated by PKB/Akt *in vitro* and *in vivo* (81) (97). The significance of this phosphorylation in activation of FRAP/mTOR is not clear, since mutation of these sites does not affect FRAP/mTOR activity (80). Recent studies have demonstrated that regulation of FRAP/mTOR by PKB/Akt is mediated through modulation of TSC1 and TSC2 (Tuberous Sclerosis Complex 1 and 2) proteins that are upstream negative regulators of FRAP/mTOR. TSC1 and TSC2 interact through their

N-termini and function as a heterodimer to inhibit FRAP/mTOR activity (98,99). TSC1-TSC2 heterodimer acts as a GTPase activating protein (GAP). Rheb (Ras homolog enriched in brain) is a small GTPase and was characterized to function as an upstream positive regulator of FRAP/mTOR that acts downstream of PI3K, PKB/Akt and TSC1-TSC2. In fact, biochemical studies showed that TSC2 exhibits specific GAP activity toward Rheb (100,101). TSC2 is directly phosphorylated by PKB/Akt, which results in inactivation of TSC1-TSC2 by disrupting the complex and promoting degradation of TSC2 (102,103), resulting in the activation of FRAP/mTOR by Rheb.

The most important downstream targets of FRAP/mTOR are proteins involved in translation. The activation of several initiation factors including eIF4E, eIF4G and eIF4B is regulated directly or indirectly by FRAP/mTOR. Moreover, the S6K and its substrate S6 ribosomal protein and elongation factor 2 (eEF2), are also targets of this pathway (89).

FRAP/mTOR-mediated regulation of eIF4E is exerted through phosphorylation of 4E-BPs. Activation of FRAP/mTOR phosphorylates the two priming sites of 4E-BP1; Thr37 and Thr46, required for subsequent phosphorylation of 4E-BP1 on Thr 70, followed by Ser65, which ultimately results in its dissociation of eIF4E (18).

Mammalian cells contain two similar S6K proteins encoded by two different genes. They share a high homology and possess conserved phosphorylation sites. The S6 kinases play a role in regulation of cell growth and are direct targets of FRAP/mTOR (104). The generally accepted model proposed that activation of S6K leads to increased translation of certain mRNAs containing a terminal oligopyrimidine tract (TOP) at their 5' end, therefore termed 5'TOP mRNAs. These mRNAs encode for

proteins involved in translation machinery including all ribosomal proteins, the elongation factors and PABP (105,106). Recent studies, however, demonstrate that induction of 5'TOP mRNAs in response to amino acids, growth factors and mitogenic signaling relies only on PI3K and PKB/Akt and does not require S6K and S6 phosphorylation (107,108). On the other hand, mTOR signaling has been shown to regulate the activity of TIF-1, a transcription factor for RNA polymerase I (POL I), essential for rRNA synthesis. It has been demonstrated that activation of FRAP/mTOR results in a decrease in phosphorylation of TIF-1 at Ser199 residue and an increase at Ser44 residue, which leads to activation of TIF-1 (109). This finding provides strong evidence for a new role of FRAP/mTOR in cell growth and proliferation through regulation of rRNA synthesis.

Rapamycin is a macrocyclic antibiotic that was originally identified as an antifungal agent before its anti-tumor and immunosuppressive effects were appreciated. It possesses inhibitory role on cell growth in different cell types and induces G1 arrest due to inhibition of translation (110). It was isolated from a strain of *streptomyces hygroscopicus* found in a soil sample Rapa Nui (111). Currently rapamycin is used as an immunosuppressant to prevent graft rejection after organ transplantation; and its analogues, CCI-779, RAD001 and AP23841 are under clinical trial for anticancer application. The primary intracellular rapamycin receptor is FKBP12, an abundant, ubiquitously expressed protein with a possible role in protein folding (112). Rapamycin binds to FKBP12 forming a complex that interacts with FRB domain of FRAP/mTOR and inhibits the function of FRAP/mTOR (113).

Figure 7. FRAP/mTOR signaling pathway

A. Structural domains of FRAP/mTOR are illustrated. HEAT domain (Huntingtin, Elongation factor 3, A subunit of PP2A and Tor1p) is involved in protein-protein interaction. FAT domain (FRAP-ATM-TRRAP) is required for mTOR activity

FRB domain (FKBP12-Rapamycin Binding) mediates the binding of FKB12 and rapamycin to mTOR. The kinase domain shows sequence similarity to the catalytic domain of PI3K, but like other members of the PIKK family does not exhibit lipid kinase activity. FATC domain: FAT C-terminus

B. FRAP/mTOR is activated in response to nutrients and growth factors. Upon stimulation of cells with growth factors, activated PKB/Akt inhibits TCS1/TCS2 complex by phosphorylating TCS2, leading to activation of Rheb and subsequently activation of FRAP/mTOR. Downstream targets of FRAP/mTOR, include S6K1 and 4E-BP1 which play a role in translation. FRAP/mTOR also modulates transcription and regulates cell cycle by phosphorylating STAT3, pRb and p27.

Adapted from Asnaghi et al., Pharmacological Research 2004

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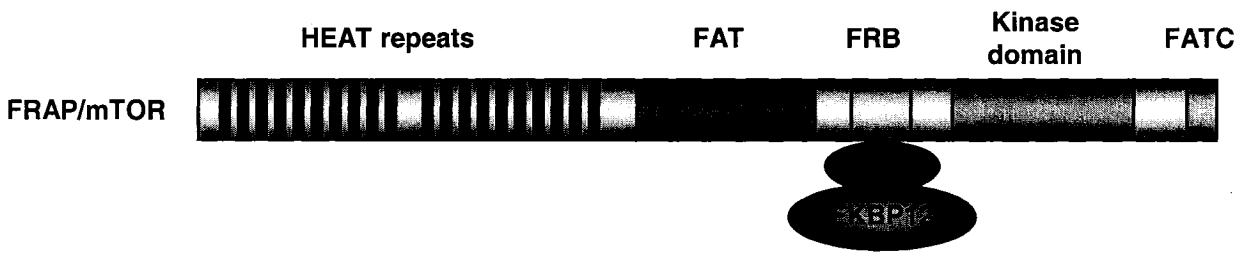
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FRB
Kinase domain
FATC
FRAP/mTOR

B

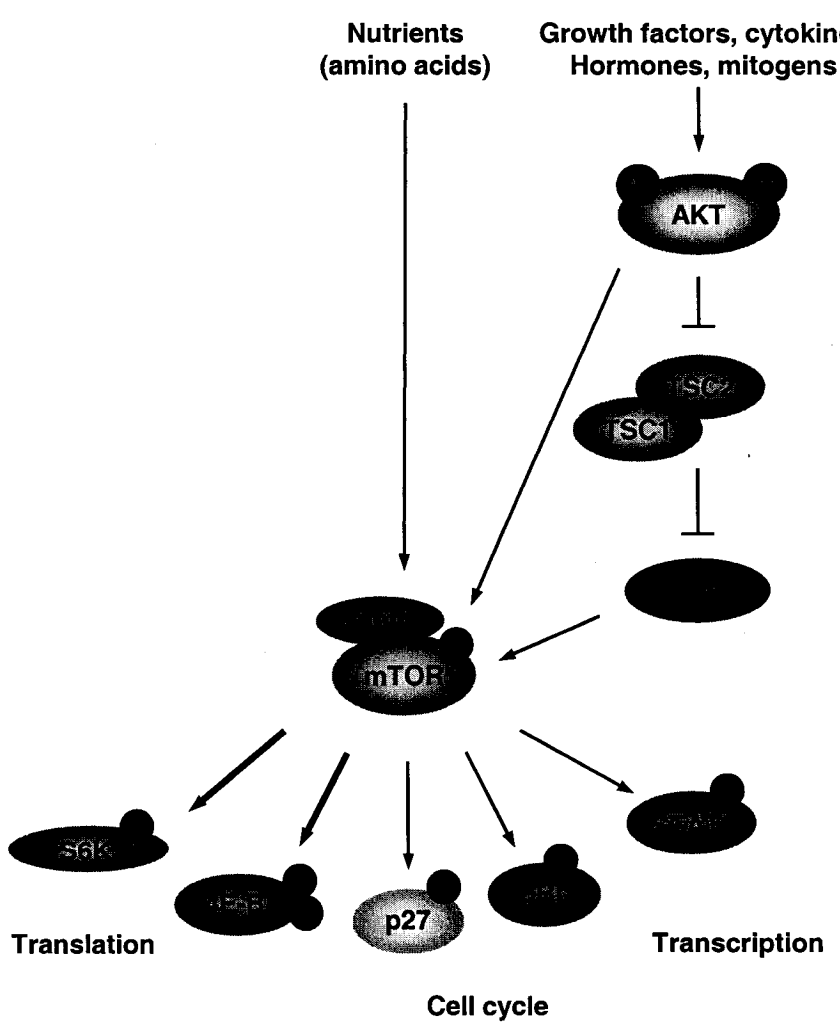

Nutrients (amino acids)
Growth factors, cytokines Hormones, mitogens
AKT
TSC2
TSC1
mTOR
S6K
4EBP1
p27
Translation
Transcription
Cell cycle

Figure 7

3. The eIF2α phosphorylation pathway

Eukaryotic cells respond to stress signals in part by inhibition of cellular protein synthesis to provide the cells with the means to restore the healthy state or by induction of apoptosis if the damage is beyond repair. An important pathway involved in this response is the eIF2α phosphorylation pathway (Fig.4). Phosphorylation of eIF2α on Ser51 residue by members of the family of eIF2α kinases results in inhibition of translation initiation by reducing the levels of functional eIF2B (114). Although phosphorylation of eIF2α results in inhibition of general translation, it stimulates expression of a specific class of genes. The best characterized examples of such genes are the yeast GCN4 protein, a transcriptional activator of amino acid biosynthetic genes whose production is increased under amino acid starvation conditions, and the mammalian ATF4, a transcriptional activator whose expression is increased under ER stress or amino acid starvation conditions (7).

3.1 The family of eIF2α kinases

To date, there are four distinct kinases with unique ability to respond to various stress conditions. They share a catalytic domain containing the conserved subdomains characteristics of all Ser/Thr protein kinases, but possess highly divergent regulatory domains (Fig.8). Specifically, these kinases show significant homology in subdomain V which may serve as the substrate binding domain (115,116).

Figure 8. The eIF2α kinase family

The structures of four eIF2α kinases are presented. The conserved kinase domains (KD) are depicted in red. The two heme-binding sites in HRI are marked in black. The dsRNA binding domains (dsRBD) in PKR are shown in orange. The N-terminal half of PERK, which resembles the corresponding domain of the ER stress-responsive IRE1 kinase, signal peptide (SP) and transmembrane (TM) domain of PERK are indicated. GCN2 structure consist of N-terminal GCN1 binding domain, charged region (+/–), pseudokinase domain (ΨKD), the regulatory histidyl-tRNA synthetase (HisRS) domain and the C-terminal ribosome binding and dimerization domain (RB/DD).

Adapted from Dever TE, Cell 2002

Figure8

Table 2. Activation of the eIF2α kinases

The activating condition or ligand for each kinase is indicated in Table 2.

Adapted from Dever TE, Cell 2002

Table 2: The eIF2α kinases and their activation conditions

eIF2α Kinase	Activation
HRI	Heme deficiency Oxidative stress
PKR	Viral infection dsRNA
PERK	ER stress Unfolded proteins
GCN2	Amino acid deprivation Uncharged tRNA

3.1.1 General Control Non-derepressible-2

GCN2 is a 180 kDa protein, which is activated by uncharged tRNA under conditions of amino acid starvation and shortage of purine. GCN2 contains a 110 amino acid insert between subdomains IV and V, and a 530 amino acid histidyl-tRNA synthetases (HisRS) sequence, which lies adjacent to the kinase domain and is required for GCN2 regulatory function. Binding of uncharged tRNA to the HisRS domain results in activation of the neighboring kinase domain leading to phosphorylation of eIF2α and inhibition of global protein synthesis. Despite this inhibitory effect translation of the transcription factor GCN4 is significantly enhanced. Upregulation of GCN4 promotes transcription of enzymes catalyzing amino acid and purine biosynthesis (115,117).

3.1.2 Heme-Regulated Inhibitor

HRI or HRC (Heme Control Repressor) is a 90 kDa protein that is activated under conditions of heme deficiency. Similar to GCN2, the structure of HRI contains an insert of 140 amino acids between subdomains IV and V. HRI contains two heme regulatory motifs (HRM) in its kinase domain. Upon heme deficiency, HRI is activated by dimerization and autophosphorylation leading to phosphorylation of eIF2α. Binding of hemin to HRI induces formation of disulfide bonds between the two monomers, which inhibits the activation of the kinase (118,119). Recent studies have implicated HRI in response to heat shock, osmotic stress and oxidative stress upon arsenite treatment (120).

3.1.3 PKR-like endoplasmic reticulum-resident kinase

PERK is a transmembrane protein residing on ER membrane. The N-terminal domain, which contains the regulatory domain, is located in the lumen of ER and senses the conditions of ER stress upon accumulation of misfolded proteins. The C-terminal cytoplasmic domain serves as a kinase which directly phosphorylates eIF2α. The regulatory domain of PERK is distantly related to that of IRE1 (Inositol-Requiring Enzyme 1), a different ER stress transducer that activates gene expression in the UPR (Unfolded Protein Response). PERK exists in an inactive form through binding of the chaperone protein BiP to its N-terminus. Upon conditions of ER stress, BiP dissociates from PERK to assist the proper folding of the misfolded proteins. Dissociation of BiP results in oligomerization, trans-autophosphorylation and activation of PERK, which leads to phosphorylation of eIF2α and inhibition of translation. Through this mechanism, PERK provides a balance between the capacity of ER and protein synthesis (121,122).

3.2 Double stranded RNA-activated protein kinase

PKR (Protein Kinase RNA activated) is a serine/threonine and tyrosine protein kinase with an important role in diverse processes such as anti-viral defense, signal transduction, cellular growth, differentiation and apoptosis (123-125). It is ubiquitously expressed in all eukaryotic cells at low levels and is transcriptionally induced by type I interferon (IFNα/β), a family of cytokines with anti-viral and anti-proliferative actions secreted from virus infected cells. PKR is unique among the eIF2α kinase family for its ability to respond to virus infection (123,126). It is activated by viral double stranded RNA (dsRNA) products. Binding of PKR to dsRNA produced during virus replication

induces dimerization and conformational changes that result in kinase activation by autophosphorylation on multiple sites (123). Activated PKR then phosphorylates the eIF2α at serine 51 causing the inhibition of protein synthesis. Through this capacity, the kinase functions as a mediator of the anti-viral and anti-proliferative effects of IFNs and as an inducer of apoptosis (Fig.9A).

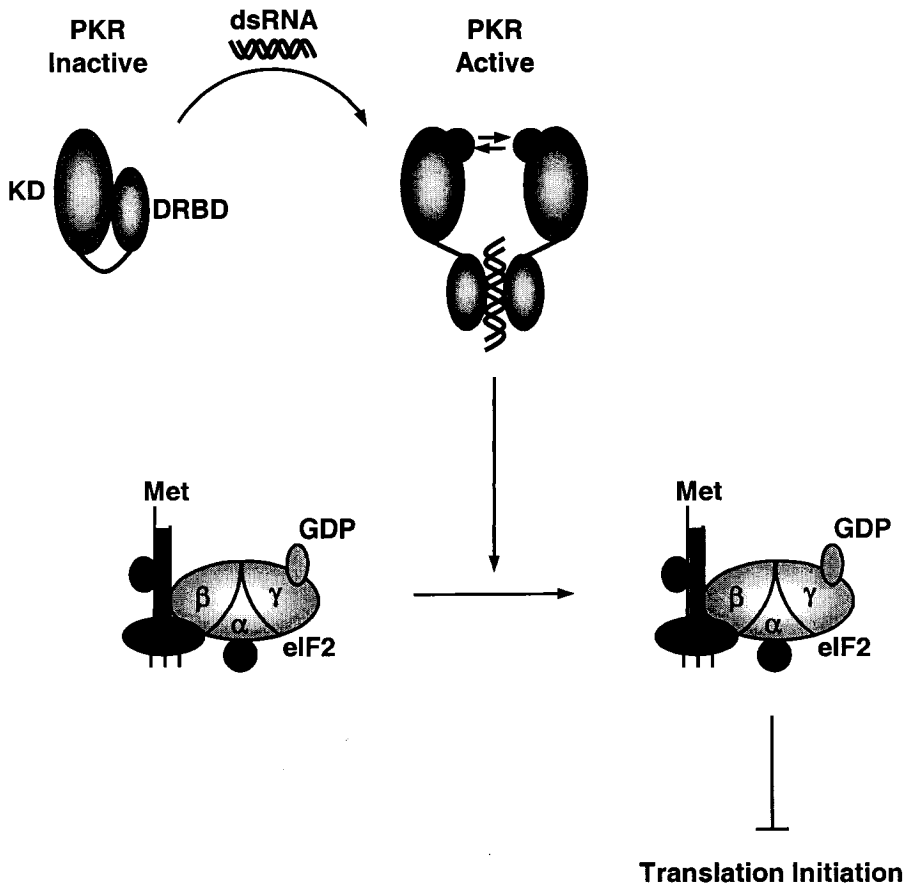
Figure 9. PKR activation and structure

A. Activation of PKR through dsRNA-mediated dimerization and autophosphorylation, results in phosphorylation of eIF2α and inhibition of translation initiation.

B. Structural representation of PKR showing the dsRNA binding motifs (dsRBMs) located at the dsRNA binding domain (dsRBD) or regulatory domain, the third basic region and eleven conserved kinase subdomains at the kinase domain (KD).

The structure of PKR was adapted from Williams B, Oncogene 1999

A


PKR
Inactive
dsRNA
PKR
Active
KD
DRBD
Met
GDP
β
γ
α
eIF2
Met
GDP
β
γ
α
eIF2
Translation Initiation

B

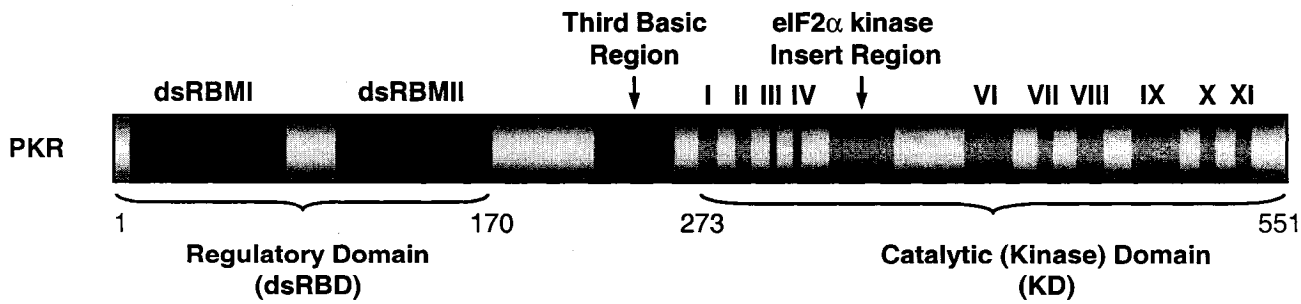

Third Basic
Region
eIF2α kinase
Insert Region
dsRBMI
dsRBMII
I II III IV
VI VII VIII IX X XI
PKR
1
170
273
551
Regulatory Domain
(dsRBD)
Catalytic (Kinase) Domain
(KD)

Figure 9

3.2.1 The structure of PKR

PKR is a 551 amino acid protein with two functionally distinct domains: an N-terminal regulatory domain or dsRNA binding domain; and a C-terminal kinase catalytic domain (127,128) (Fig.9B). The regulatory domain contains two dsRNA binding motifs (dsRBM) that are rich in basic residues and are separated by a 20 amino acid linker. Although both dsRBMs are required, the dsRBMI shows a greater importance in dsRNA binding. These 65 amino acid motifs exist in several dsRNA-binding proteins. The two dsRBM of PKR show 49% homology with 29% identity in their sequence (129). In addition to regulation of PKR activity, the regulatory domain serves to target PKR to ribosomes (130). The C-terminus of PKR contains a conserved protein kinase consisting of eleven conserved kinase subdomains that characterize the members of serine/threonine kinase subfamily (124,131). This region which extends from amino acid 273 to 551 contains the ATP-binding region with the lysine residue at position 296 which is essential for the phosphate transfer reaction. Mutation of this residue to arginine, proline or histidine renders the kinase catalytically inactive (132,133). The interaction between PKR and eIF2α is mediated by a region located at amino acids 367-551 (134).

3.2.2 Regulation of PKR

Activation of PKR is subjected to regulation at several levels: transcriptional activation, binding to dsRNA, autophosphorylation and association with cellular regulator proteins (135-138).

The promoter of PKR contains a number of regulatory elements such as IFNα/β responsive ISRE, IFNγ responsive GAS, IL-6 sensitive NF-IL6, the acute phase

responsive factor (APRF) and NF-κB elements (139,140). The structure of PKR promoter, however, suggests that other signaling pathways are also involved in regulation of transcription. It has been demonstrated that IRF-1 plays a role in induction of PKR transcription through activation of the ISRE element. TNFα and TGFβ are also involved in modulation of PKR (141). Specifically, TNFα induces both mRNA and protein levels of PKR and (142) and TGFβ can inhibit the induction of PKR that occurs during myogenesis (143).

Activation of PKR is triggered by binding to dsRNA. In the inactive form, the dsRNA binding domain of PKR interacts with the substrate binding domain exerting an inhibitory effect on the catalytic activity. Upon viral infection, dsRNA is produced as a byproduct of viral replication. Binding of dsRNA to PKR dsRBMs, induces a conformational change, in which the dsRBD swings away from the rest of the protein leading to exposure of the C-terminus for autophosphorylation. Dimerization of PKR is essential for its autophosphorylation and activation. PKR homodimer is formed either by direct interaction of its dsRBD and part of the catalytic domain (amino acids 244-296), or by dsRNA bridging the two proteins. PKR homodimer is then autophosphorylated in *trans* at Thr446 and Thr451 residues located between kinase subdomains VII and VIII, which are essential for PKR kinase activity. Other phosphorylation sites include Ser342, Thr255 and Thr258, which play a less important role on PKR activity (144-146). Phosphorylation within the third basic region results in securing PKR in an active conformation, while phosphorylation in the activation loop facilitates substrate binding and catalysis (136).

3.2.3 Cellular inhibitors and activators of PKR

PKR activity can also be modulated by cellular proteins. PKR can directly bind to inhibitory proteins such as $P58^{IPK}$, TRBP (TAR RNA Binding Protein), p67 and dRF. $P58^{IPK}$ belongs to eukaryotic stress response protein family and is involved in stress pathways. It is activated by influenza virus infection and appears to bind to the third basic domain of PKR and inhibits both autophosphorylation and phosphorylation of eIF2α (147). TRBP is another cellular inhibitor of PKR, which like PKR, contains dsRBM and forms nonfunctional heterodimers by interacting with dsRBMs of PKR or through a direct protein interaction (148-150). p67 is an eIF2α associated glycoprotein, which binds to eIF2α and prevents its phosphorylation by PKR (151). An additional PKR inhibitor is dRF, which inhibits binding of dsRNA to PKR and its autophosphorylation (152). TRBP may inhibit PKR function by sequestering dsRNAs In addition to the inhibitory proteins, cellular proteins exist that activate PKR. PACT (Protein ACTivator of PKR) is one such example, which is a dsRNA binding protein and acts as an activator of PKR (153).

3.2.4 Viral inhibitors of PKR

Because of the deleterious effects of host's protein synthesis inhibition, many viruses have evolved distinct mechanisms to counteract PKR activation and eIF2α phosphorylation as a means to evade, at least in part, the antiviral action of IFN. These mechanisms include direct inhibition of the kinase, down-regulation of PKR protein, regulation of eIF2α phosphorylation or translational pathways downstream of eIF2α phosphorylation (154,155) (Fig.10).

Some viruses inhibit PKR activation through inhibition of dsRNA binding to PKR or by sequestering dsRNA. Adenovirus, Epstein-Barr virus and human immunodeficiency virus (HIV) synthesize inhibitory dsRNAs. Adenovirus VAI RNA (Virus-Associated RNA) is a 260 nucleotide RNA transcribed by host RNA polymerase III. It accumulates in the cytoplasm of the infected cells and inactivates PKR through a direct interaction with the kinase at its dsRBMs, thus acting as a competitive inhibitor (156). Epstein-Barr virus-encoded RNAs (EBERs) are similar to VAI RNA in size, mode of transcription and function. EBER RNAs are found at high levels and bind to and inactivate PKR (157,158). HIV transactivator responsive region (TAR) RNA was shown to bind to PKR at low levels and activate the kinase, whereas high levels of this RNA inactivates PKR (159,160). TAR RNA can also recruit a cellular protein termed TRBP that can interact with PKR and inhibit its function (149). Vaccinia virus, a member of poxvirus family, encodes two proteins termed E3L and K3L, which inhibit PKR through distinct mechanisms (161). E3L binds to dsRNA with high affinity and belongs to the family of dsRNA binding proteins. It inhibits PKR activation by sequestering dsRNA activators of PKR that are produced as a result of viral replication (162). Influenza virus nonstructural (NS) 1 protein also acts as an antagonist of the dsRNA-PKR binding reaction. NS1 is an RNA binding protein, which binds to both poly(A) RNA and dsRNA; however, unlike E3L, it does not possess any dsRBM. NS1, which is a nuclear protein, sequesters the primary viral transcription products in the nucleus and prevents their transport to cytoplasm where they bind and activate PKR (163). Reovirus σ3 protein, a structural component of the reovirus outer shell, is a member of the dsRNA binding protein homology family and similar to E3L, it inhibits

PKR by sequestering dsRNA (164). Influenza virus activates the cellular protein $P58^{IPK}$, which forms a physical complex with PKR by binding to its dimerization domain and inhibits PKR autophosphorylation and activation (165). Hepatitis C virus nonstructural (NS) 5A protein binds to PKR and inactivates the kinase (166). Vaccinia virus K3L inhibits PKR by acting as a pseudosubstrate. K3L binds to catalytic domain of PKR between amino acids 366-415 (167). K3L shows sequence similarities with eIF2α and since it acts as a pseudosubstrate, it lacks the Ser51 phosphorylation residue (168). HIV transactivator (Tat) protein physically interacts with PKR and acts as both a substrate and an inhibitor of the kinase (169). Some viruses impede PKR function by affecting its protein levels. Several studies suggest that HIV Tat protein expression could result in reduction of PKR protein levels. This could also partly explain the inhibition of PKR activity by Tat (170,171).

Poliovirus infection also decreases PKR levels, which is speculated to be mediated by degradation of the kinase (172). In herpes simplex virus (HSV)-infected cells, despite PKR activation, eIF2α phosphorylation is impaired (173). HSV encodes a protein which is a product of *γ1 34.5* gene, which is functionally related to the cellular GADD34 (Growth Arrest and DNA Damage Gene product 34). Dephosphorylation of eIF2α in cells subjected to ER stress is mediated by protein phosphatase 1 (PP1)-GADD34 holophosphatase complex, where PP1 is the catalytic subunit and GADD34 is the regulatory subunit. HSV *γ1 34.5* gene product functions as GADD34 and recruits PP1 to dephosphorylate eIF2α (174). Simian virus 40 (SV40) T antigen is capable of relieving translation inhibition by activated PKR. Since eIF2α phosphorylation occurs at

elevated levels in SV40-infected cells, this regulation is likely to be exerted at a step downstream of eIF2α phosphorylation, which remains to be identified (175,176).

Figure 10. Regulation PKR expression and function and its viral inhibitors

PKR is transcriptionally induced by type 1 interferons (IFNs) and activated through dsRNA-dependent dimerization and autophosphorylation, leading to phosphorylation of eIF2α. Viral products inhibit PKR expression and function through distinct mechanisms, which are indicated at each level.

Adapted from Gale and Katze, Pharmacology and Therapeutics 1998

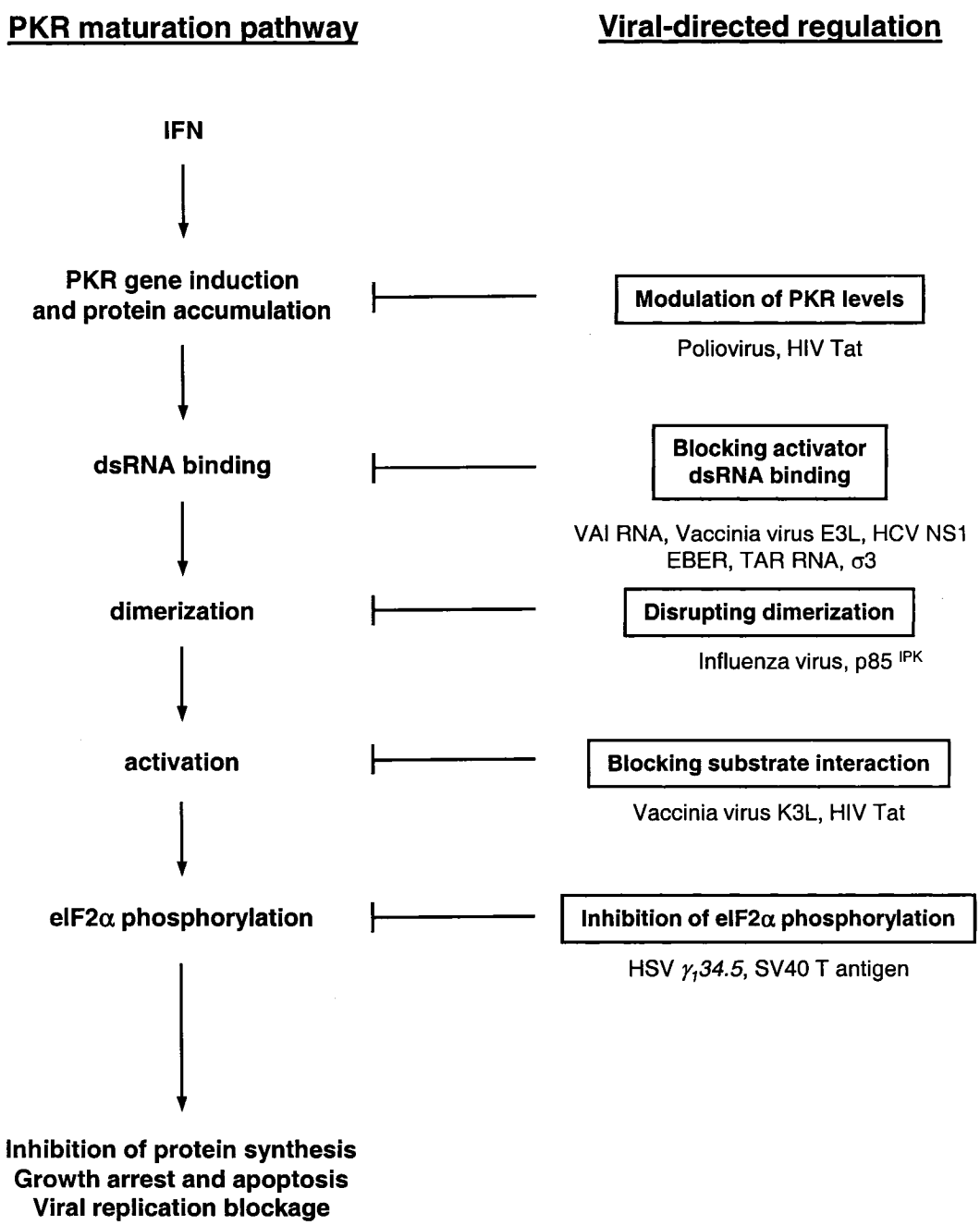

PKR maturation pathway
Viral-directed regulation
IFN
PKR gene induction and protein accumulation
Modulation of PKR levels
Poliovirus, HIV Tat
dsRNA binding
Blocking activator dsRNA binding
VAI RNA, Vaccinia virus E3L, HCV NS1
EBER, TAR RNA, σ3
dimerization
Disrupting dimerization
Influenza virus, p85 IPK
activation
Blocking substrate interaction
Vaccinia virus K3L, HIV Tat
eIF2α phosphorylation
Inhibition of eIF2α phosphorylation
HSV γ1 34.5, SV40 T antigen
Inhibition of protein synthesis
Growth arrest and apoptosis
Viral replication blockage

Figure 10

3.2.5 Substrates of PKR

The best characterized substrate of PKR is eIF2α, which is phosphorylated at Ser51. This phosphorylation site is located in a basic region and is flanked by four arginine residues on its C-terminal side (177). PKR induces NF-κB (Nuclear Factor κ-B) activity through inactivation of its kinase IκB. Phosphorylation of an unknown protein in IκB kinase complex by PKR results in degradation of IκBα (178). PKR also enhances transcription activity of p53 (179). PKR physically interacts with p53 and phosphorylates Ser392 residue (180). In a recent report, we have shown that PKR negatively regulates Stat1 and Stat3 through phosphorylation and activation of TC-PTP, a tyrosine phosphatase which targets active Stat1 and Stat3 and decreases their cytokine-induced phosphorylation and transcriptional activities (181).

3.2.6 Biological functions of PKR

Although PKR was originally characterized as a translational inhibitor in an antiviral pathway regulated by IFNs (182), there is increasing evidence indicating the role of PKR in other signal transduction pathways. PKR is involved in signaling pathways such as NF-κB (183), interleukine 3 (IL-3) (184) and IFNβ (185,186). It is also implicated in the activation of immediate early gene expression by platelet-derived growth factor (PDGF) (187). PDGF activates PKR autophosphorylation, which can be blocked by activated p21 ras (188). PKR has been demonstrated to play a role in regulating transcription of genes such as immunoglobin κ (189), IRF1 (190), IFNα and IFNβ (191), the vascular epithelial cell adhesion molecule (VCAM1) (192), major histocompatibility complex (MHC) class I (193), Fas ligand and other members of the

apoptotic pathway (194,195). Furthermore, activation of PKR has been shown to play a role in differentiation of myoblasts and preadipocytes (196,197).

PKR is also involved in regulation of cell growth and proliferation and, thus, is suspected to exert an anti-tumor function. Overexpression of PKR results in inhibition of growth in yeast, mouse and insect cells and induces apoptosis in human cells. Expression of mutant forms of PKR which inactivates the kinase, as well as overexpression of PKR inhibitors result in exhibition of a transformed phenotype (141). This phenotype results from reduction of eIF2α phosphorylation, enhanced translation of critical regulatory gene products and deregulation of cell growth. PKR is speculated to inhibit cell proliferation and growth by inhibiting protein synthesis and thereby targeting proteins involved in cell division or by interacting with the ligands of growth factors (198). PKR is also an inducer of apoptosis in response to various stimuli such as LPS (199), dsRNA (200), serum deprivation or TNFα treatment (201).

Following dsRNA treatment, protein levels of Fas and Bax are upregulated, which are mainly regulated at posttranscriptional level. Phosphorylation of eIF2α is suspected to be involved. Although phosphorylation of eIF2α results in inhibition of global protein synthesis, the translation of several mRNAs, including those involved in apoptosis is increased. Bax mRNA is one example which resembles yeast GCN4 mRNA translation in response to amino acid starvation and eIF2α phosphorylation. The 5' UTRs of these mRNAs have several open reading frames (ORFs) (4 in GCN4 and 3 in Bax), which facilitates their translation upon eIF2α phosphorylation (117).

4. Human papillomaviruses

Papillomaviruses are small DNA viruses which induce papillomas (or warts) in a variety of higher vertebrates including human (202). Discovery of the major role of human papillomaviruses (HPV) in human cancers, most notably human cervical carcinoma, has drawn great attention to the study of HPVs and their mechanism of action.

4.1 Classification

Papillomaviruses were originally classified in *papovaviridea* family of viruses. The name papovavirus is derived from its members, papillomavirus and poliovirus and simian vacuolating virus. This classification was based on the fact that all members have small size, a nonenveloped virion, an icosahedral capsid, a double-stranded circular DNA genome, and utilize the nucleus as a site of multiplication. As it was later recognized that these virus groups have different genome sizes, completely different genome organizations, and no major nucleotide or amino acid sequence similarities, they are now officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families, *Papillomaviridae* and *Polyomaviridae* (203). Papillomaviruses infect a wide range of animals from birds to mammals, including humans, and are highly species specific, i.e. cross-species transmission of the virus is very rare. The majority of papillomaviruses have a specific tropism for squamous epithelial cells. Some HPVs preferentially infect cutaneous epithelia, whereas another groups target mucosal epithelia (204). To date, over 100 different HPV types have been identified, the classification of which is based on the sequence of their L1 genes (205). HPVs are divided into two groups: the low-risk HPVs, such as types 6 and 11, which

are rarely found in malignant tumors but induce benign genital warts, and the high-risk types, such as 16 and 18, which are frequently detected in cervical carcinoma (206,207).

4.2 Virion structure

Papillomavisuses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. Papillomavirus particles measure 55 nm in diameter. The capsid consists of 72 pentameric capsomers arranged on a T=7 surface lattice, which wraps around the genome; a single circular double-stranded DNA of approximately 8000 bp. The capsid contains two structural proteins, which are encoded by the virus (208,209).

4.3 The genome

Papillomavirus genome is consisted of three regions; two coding regions and a non-coding regulatory region (Fig.11). The open reading frames are divided into early (E) and late (L) followed by a number which represents their coding potential; the lower the numbers, the longer the open reading frames. The early region encodes 8 open reading frames which are involved in regulatory functions required for invasion of the host cell by the virus and the production of the progeny virus (206). The late open reading frames encode genes for viral capsid proteins. Some early proteins such as E2 and E4 also play a role in the late stages of viral life cycle. All open reading frames (ORFs) and, thus, all genes are located on one strand of the viral DNA indicating that only one strand serves as the template for transcription. The non-coding regulatory region of papillomavirus genome, also known as the long control region (LCR), upstream regulatory region (UUR) or non-coding region (NCR), does not contain any

major open reading frames but includes the origin of DNA replication, and a large number of binding sites for cellular and viral transcription factors (206).

4.3.1 The early region

The genes located at the early region encode for viral factors involved in viral plasmid replication, regulation of viral transcription and cellular transformation (204). Transcription of the early genes is initiated at a promoter located upstream of E6 ORF. The papillomavirus E1 is a nuclear phosphoprotein which is involved in initaition of DNA replication (210). It is an ATPase and has ATP dependent helicase activity which unwinds the dsDNA. It interacts with DNA polymerase α-primase and recruits it to the origin of replication. E1 binds the origin of replication forming a trimeric ring-like structure. E1 also binds to E2, an interaction that is essential for viral DNA replication (211). The E2 regulatory protein is a viral transcription factor with important role in viral transcription and replication (212). The structure of E2 protein consists of a transactivating domain at its N-terminus and a sequence-specific DNA binding and dimerization domain at its C-terminus (202). E2 exists as a dimer and is speculated to mediate the binding of E1 to the origin of replication. Most importantly, expression of E2 acts as a transcriptional repressor of E6 an E7 and is believed to tightly regulate the expression of these proteins, which play a role in viral oncogenesis (210). Although located in the early region, the E4 protein is expressed at a later stage of infection. It associates with the keratine cytoskeleton and has been shown to induce the collapse of the cytokeratin network suggesting a role in release of viral particles from the cell (202,213).

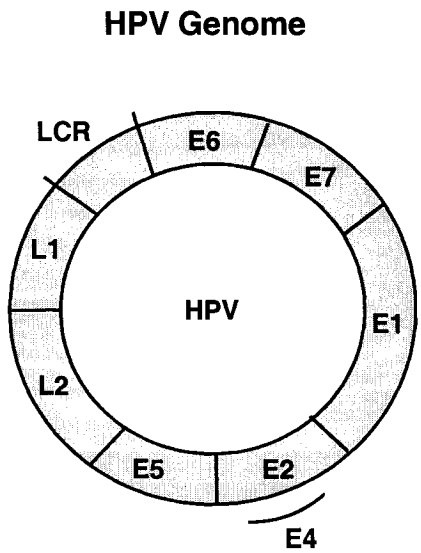
Figure 11. Human papillomaviruses genome and life cycle

A. Schematic structure of HPV-16 genome. The LCR (Long Control Region) is the non-coding regulatory region which contains the viral origin of replication. The early region contains 6 open reading frames all of which fulfill regulatory functions for viral replication and production of progeny virus. The late region encodes for the two viral capsid proteins.

B. HPV life cycle is illustrated in epithelial cells. Normal epithelial cells division and differentiation is compared to abnormal differentiation of HPV-infected cells. HPV infection occurs through microtramas in the basal layer of the epithelium. HPV genome replicates with each cell division and the infected daughter cells migrate to the suprabasal layers and start differentiation. Unlike, normal keratinocytes, the infected differentiated cells enter S phase which results in vegetative viral DNA replication. Subsequently virions are assembled and released into environment.

Adapted from Munger K, Frontier in Biosciences 2002, and Fehrmann and Laimins LA, Oncogene 2003.

A


HPV Genome
LCR
E6
E7
L1
HPV
E1
L2
E5
E2
E4

B

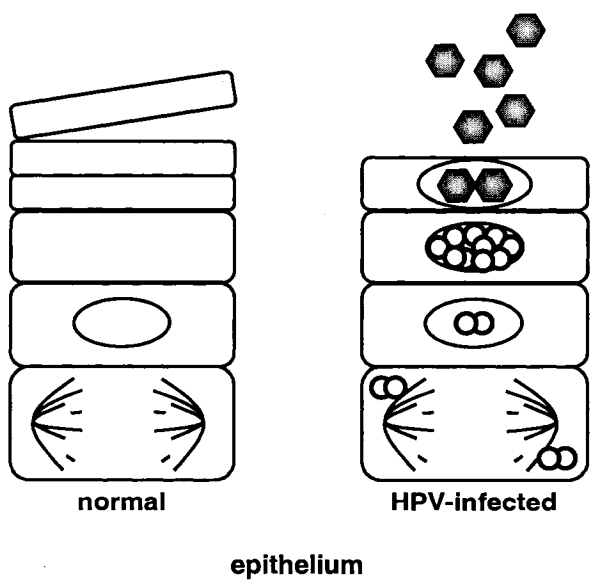

normal
HPV-infected
epithelium

Figure 11

4.3.2 The HPV oncoproteins

The ability of HPV infection to progress to malignancy is attributed to the transforming properties of three proteins; E5, E6 and E7 (214).

E5 is a highly hydrophobic protein, which is localized at the endosomal membranes, Golgi apparatus and plasma membrane. Unlike bovine papillomavirus (BPV) E5 which plays a central role in transformation, HPV E5 only exerts weak transforming activity (215). HPV E5 increases cell growth and proliferation possibly through regulation of epidermal growth factor (EGF) and its receptor (EGFR). It has been demonstrated that E5 interacts with a vacuolar ATPase and interferes with the acidification of the endosomes. The inhibition of PH change in endosomes results in rapid turnover of EGFR to the plasma membrane and prolongs the signaling mediated by EGF (216). Recent studies have shown an important role of E5 in regulation of viral late gene expression and amplification (214).

E6 is one of the first genes expressed during viral infection and plays an important role in cell immortalization (217). The E6 protein lacks any intrinsic enzymatic activities and exerts its function through interaction with key regulatory cellular proteins. E6 is a 150 amino acid protein containing two zinc finger domains. These domains are formed as a result of disulfide bonds between four C-x-x-C motifs and are involved in protein-protein interaction. One of the most important and the best characterized target of E6 is p53 (218) (Fig. 12). The tumor suppressor p53 is a transcription factor that mediates cell cycle arrest or apoptosis in response to DNA damage, and acts as a guardian of the genome by inducing growth arrest to allow cells to repair the damage or apoptosis, if the damage is too severe and beyond repair. Thus,

although it is not required for normal cell proliferation, it plays a central role in cellular response to a variety of stress conditions, which may affect genomic integrity and ultimately lead to the abnormal cell proliferation. It is thus not surprising that mutational inactivation of p53 is detected in more than 50% of human cancers (219). In the absence of DNA damage, Mdm2 binds to p53 and promotes its degradation. In response to DNA damage, the protein kinase ATM binds to p53 and phosphorylates serine 15, which prevents Mdm2 binding. In cells infected with high-risk HPV types, E6 oncoprotein binds to p53 and mediates its ubiquitination and degradation through recruitment of a cellular ubiquitin ligase E6AP (E6 Associated Protein) (218) .

E6 also interacts with paxillin, a cytoplasmic protein involved in actin organization and attachment of cells to the extracellular matrix via focal adhesion proteins, such as FAK (Focal Adhesion Kinase) and vinculin (220). E6 proteins from high-risk HPVs can bind paxillin, and it is therefore possible that abrogation of paxillin binding to vinculin by E6 allows these papillomaviruses to interfere with the differentiation program of the host cells (221). E6 oncoprotein is also able to bind to the PDZ domain-containing proteins such as hDlg, hScrib, Mupp1, MAGI-1, -2, -3, leading to their ubiquitination and degradation (214). PDZ domain is found in proteins involved in cell-cell contact and it may play a role in signal transduction. E6-induced degradation of these proteins results in disruption of cell contact and contributes to neoplastic transformation. E6 from high risk HPVs has also been shown to disrupt the transcriptional machinery through its association with the transactivator CBP/p300 protein, a function with important implications in cellular immortalization and transformation (204,222). E6 oncoprotein is capable of activating telomerase, a multi-subunit enzyme responsible for replicating

telomeric DNA at the ends of the chromosomes, activation of which is a critical step in cellular transformation. Under normal conditions, the telomerase is inactive and cell division results in shortening of telomeres, which will eventually lead to senescence and cell death (223-225). Therefore, in most tumor cells telomerase is activated to maintain the telomere length. E6 oncoprotein is speculated to upregulate the catalytic subunit of telomerase by transcriptional activation of its promoter (226). Furthermore, E6 from the high-risk HPVs can efficiently immortalize human mammary epithelial cells and induce epithelial hyperplasia and skin tumors in transgenic mice (204,204). In addition to oncogenesis, E6 has a significant contribution in altering the immune response through its ability to inhibit apoptosis and suppress IFN action. This may account, at least in part, for the poor responsiveness of HPV infected cells to IFN treatment in vitro and in vivo (227).

Figure 12. HPV E6-mediated degradation of p53

The tumor suppressor p53 induces cell cycle arrest at G1 phase in response to DNA damage or under cellular stress conditions. The best characterized function of HPV E6 oncoprotein is to inactivate p53. E6 oncoprotein binds to a cellular ubiquitin ligase termed E6-associated protein (E6AP). The E6-E6AP complex then targets p53 for degradation by recruiting the ubiquitin complex of enzymes, which ubiquitinates lysines on p53 and promotes its proteasome-dependent degradation.

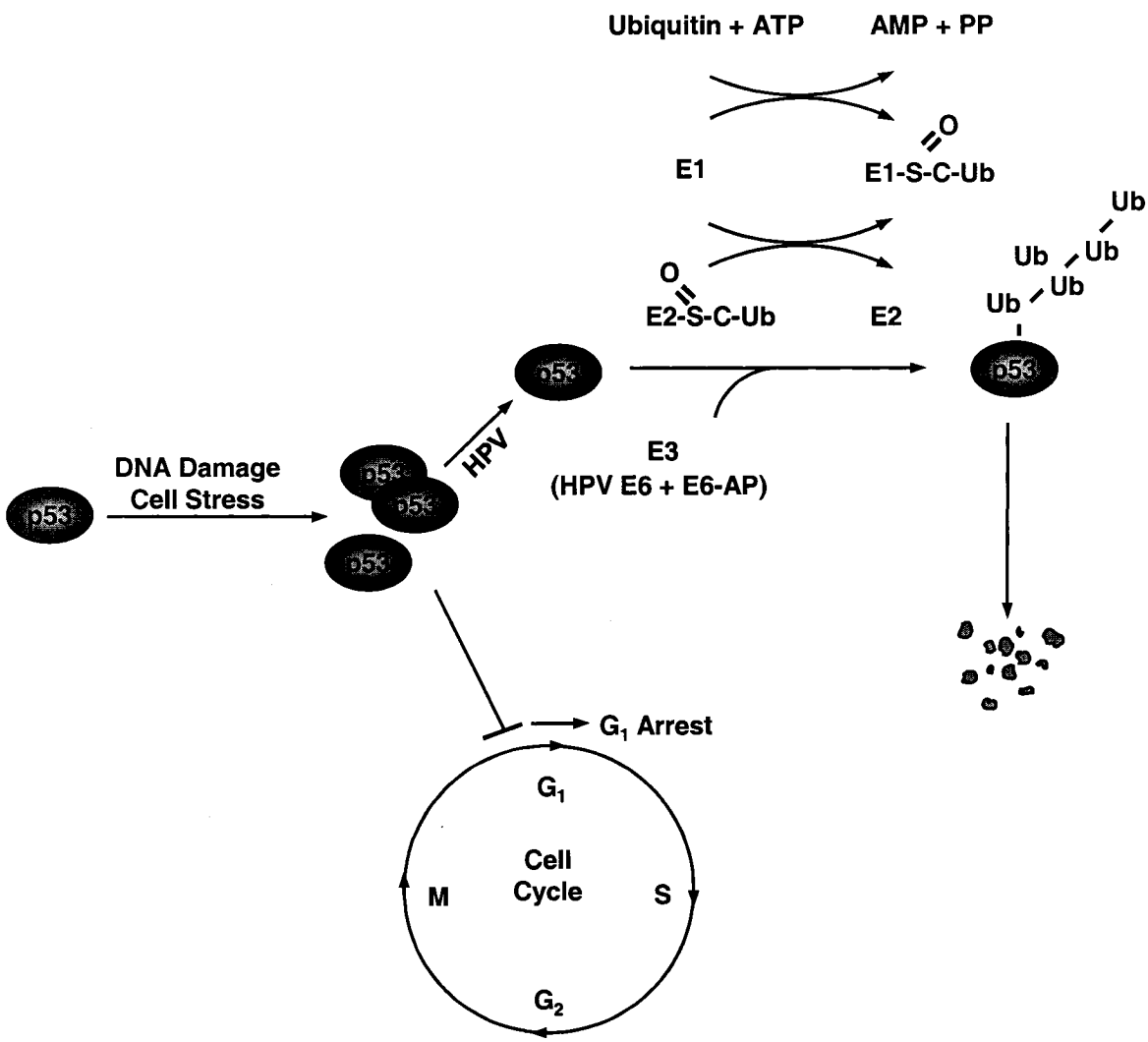

Ubiquitin + ATP
AMP + PP
E1
E1-S-C-Ub
O
E2-S-C-Ub
E2
Ub
Ub
Ub
Ub
Ub
p53
DNA Damage
Cell Stress
HPV
E3
(HPV E6 + E6-AP)
G1 Arrest
G1
Cell
Cycle
M
S
G2

Figure 12

E7 is a 100 amino acid, nuclear protein, containing a zinc finger motif at its C-terminus. The best characterized target of E7 is the tumor suppressor retinoblastoma protein (pRb) (Fig.13). The pRb is an important regulator of cell cycle entry. Hypophosphorylated pRb binds E2F transcription factor and suppresses its transcription activity. pRb is hyperphosphorylated at early G1 and towards S phase, allowing E2F to induce the transcription of several genes involved in DNA synthesis, such as DNA polymerases and thymidine kinases (228,229). E7 promotes cell cycle progression by binding to hypophosphorylated pRb and inhibiting its interaction with E2F (230). Inactivation of pRb by E7 is also important in induction of replication of viral DNA in differentiated cells. Normal, uninfected epithelial cells exit cell cycle upon differentiation, a regulation which is attributed to pRb function. Binding of E7 to pRb, causes the differentiated cells to undergo cell division, which provides the optimal environment for viral replication and amplification (231). E7 is also capable of binding to two members of the same family, p107 and 130, which also negatively regulate E2F (232). Recent studies suggest a negative effect of E7 on cyclin-dependent kinase inhibitors (CDKs) such as p21 (233,234) and p27 (235), which results in inhibition of p53-dependent cell cycle arrest (236).

Figure 13. HPV E7 inactivates pRb to promote cell cycle progression.

The pRb tumor suppressor regulates cel cycle progression from G1 to S phase. Under normal conditions, pRb is hypophosphorylated in early G1 and becomes hyperphosphorylated towards S phase. Hypophosphorylated pRb binds to E2F transcription factors and inhibits the transcription of genes required for DNA synthesis. HPV E7 oncoprotein binds to hypophosphorylated pRb and inhibits its interaction with E2F allowing for cell cycle progression in differentiated epithelial cells, which leads to productive replication of HPV genes.

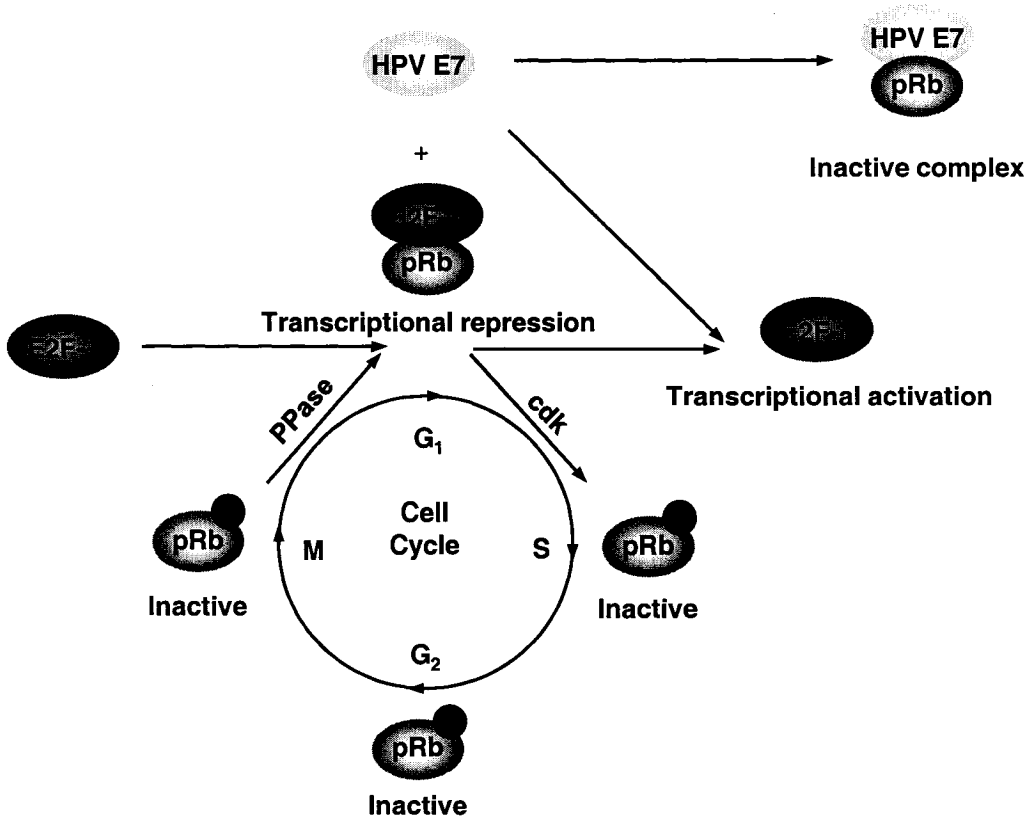

HPV E7
HPV E7
pRb
Inactive complex
+
pRb
Transcriptional repression
Transcriptional activation
PPase
cdk
G1
Cell
Cycle
M
S
G2
pRb
Inactive
pRb
Inactive
pRb
Inactive

Figure 13

4.3.3 The late region

The genes located at the late region encode for the capsid proteins L1 and L2. The major capsid protein (L1) is a 55 kDa protein, which represents 80% of the total viral proteins. The minor capsid protein (L2) is 70 kDa. The late mRNAs are transcribed from a specific promoter, termed late promoter (P_L), which is only active in terminally differentiated cells (208,209).

4.3.4 The long control region

The LCR is located at the 5' of the early genes and 3' of the late genes and regulates viral DNA replication and transcription. It contains a transcription enhancer which is only activated in epithelial cells (202).

4.4 Viral life cycle

The life cycle of the virus can be divided into early and late stages. These stages are linked to the differentiation state of the epithelial cells. HPVs rely on the host cell machinery for synthesizing their genomes and the replication takes place in squamous epithelial cells. Initial infection of papillomaviruses occurs through the basal cells, a single layer of undifferentiated proliferating cells at the basal levels of a stratified squamous epithelium, which are protected with several layers of non-dividing differentiated cells (Fig.11B). Vegetative viral replication including DNA synthesis, production of viral capsid proteins and viral assembly, however, are all restricted to the terminally differentiated cells. The DNA of the virus can only be detected in the middle and upper layers of squamous epithelium but not within the cells at the lower layer or in the fibroblasts in the dermis (213). The cellular receptors and the mode of viral entry is

mainly unknown; however, it has been shown that papillomaviruses can bind a variety of cell types, suggesting that the receptor is a widely expressed and evolutionary conserved protein and that the specificity of the virus is not controlled at the levels of entry (202). The infection may also occur through microtraumas in the epithelium, which expose the basal cells to viral entry. Following entry, the viral DNA is stablished as an episome with approximately 50 copies per cell. Expression of early genes involved in replication, E1 and E2, and those encoding the oncoproteins, E5, E6 and E7, is essential for the establishment and maintenance of the viral genome. The viral DNA replicates in synchrony with cellular DNA replication (237). Thus, upon cell division, the infected daughter cells carrying copies of viral DNA, migrate to the suprabasal level and begin to differentiate. Uninfected keratinocytes exit cell cycle once they reach the suprabasal layer. The HPV-infected cells, however, enter the S phase, which allows the replication of viral DNA to thousands of copies per cell (238). At this stage, the late genes are expressed, virus particles are assembled, and released with the upper layer of the epithelium (239).

4.5 HPV and cancer

Infection with high-risk HPVs is associated with tumors in various tissues and organs and is clearly implicated in the development of cervical cancer with an incidence rate of ~500,000 cases per year, globally (206,207). High risk HPVs are found in 100% of cervical cancer, which provides strong evidence that these viruses are the major etiological cause of such malignancies (206,207). In addition, HPVs are associated with head and neck cancer and cutaneous tumors, in particular epidermodysplasia verruciformis and non-melanoma skin cancers (206).

In early infection and in low-grade cervical lesions, the viral genome is present as an episome. In later stages of cervical carcinomas, the high-risk HPV genome is found integrated in the host DNA. The outcome of this integration, which is the hallmark of malignant progression, is the loss of E2 regulatory genes, which under normal conditions control the expression of the viral oncoproteins (240). The loss of such control results in aberrant expression of the E6 and E7 oncoproteins, which contributes to cellular transformation and eventually leads to cancer (241,242). In general, integration of the HPV genome into a host cell chromosome is the first step in initiation of HPV-associated cancers. This process is an accidental event and is terminal for the viral life cycle. Even though integration does not occur at specific chromosomal hot spots in the human genome, it follows a consistent pattern with respect to the viral genome (214). Malignant progression often occurs in conjunction with other risk factors such as decreased immune function, or often a long latency period after other genomic alterations in the host cell DNA have occurred (206).

Recent studies have characterized the cellular targets of HPV oncoproteins; however more effort is nessecary to fully understand the mechanism of action of these viruses, in order to design strategies to combat HPV-associated diseases.

Specific Research aims

The objective of this research was to characterize the signaling pathways induced upon activation of PKR and to study the link between the two mechanisms involved in regulation of translation initiation, i.e. 4E-BP1 phosphorylation and eIF2α phosphorylation pathways. Although believed to possess anti-proliferative properties, several studies have suggested a role for PKR in signaling pathways leading to induction of growth and proliferation. In this regard, experiments were performed to explore the role of PKR in induction of PI3K pathway. These studies identified a novel property of PKR to induce PI3K activity leading to phosphorylation of 4E-BP1 and S6 ribosomal protein. Furthermore, this study focused on the functional interactions between HPV-18 E6 and PKR. Initial studies have investigated the application of IFNs in therapies against HPV-associated malignancies. However, the effectiveness of the IFN treatment appears to be dependent on the HPV subtype. Therefore, experiments were conducted to investigate the molecular mechanisms of HPV E6 oncoprotein to evade the anti-viral and anti-proliferative effects of IFNs. These studies identify functional interaction of HPV E6 with several members of the IFN signaling pathway including its downstream effector, PKR. Further experiments were performed to characterize the mechanism of action of HPV E6 to combat PKR and eIF2α phosphorylation pathway.

Chapter II

Materials and Methods

Chapter II - Materials and Methods

1. Plasmid Constructs

FLAG-tagged HPV-18 E6 and HPV-11 E6 in pcDNA3.1/Zeo vector (Invitrogen, Inc.) were constructed by cloning the CMV-FLAG polylinker region of the pFLAG-CMV-2 vector (Sigma) into the multiple cloning region of pCDNA3.1/Zeo (Invitrogen) and the E6 sequences were then cloned between the *Eco*RI and *Eco*RV restriction sites of the newly generated vector. GyrB-PKR cDNAs in pSG5 vector (Stratagene, Inc.) were provided by Dr. T. Dever (243). pCMV-FLAG wild-type PTEN construct was kindly provided by Dr. Georgescu (42). pMV7/HA-eIF4E and pMV7 were described elsewhere (244). Dominant negative p85 expressing adenovirus or control adenovirus, were provided by Dr. Mossman (McMaster University). FLAG-GADD34 constructs were kind gifts of Dr. Shenolikar (245).

2. Cell culture and reagents

2.1 Cell lines

To generate HT1080 cells expressing GyrB-PKR or GyrB-PKRK296H, cells were transfected with either GyrB-PKR or GyrB-PKRK296H cDNA in pSG5 vector together with pcDNA3.1/Zeo vector at a ratio 5:1. GyrB-PKR or GyrB-PKRK296H cells expressing the HPV E6 proteins were generated by transfecting HT1080 cells with either GyrB-PKR or GyrB-PKRK296H cDNA together with either FLAG-18 E6 or FLAG-11 E6 in pcDNA3.1/Zeo vector at a ratio 5:1. The generation of HT1080 cells

expressing FLAG-18 E6 was performed using FLAG-18 E6 in pcDNA3.1/Zeo vector (246). Transfection was performed with Lipofectamine reagent (Invitrogen, Inc) according to manufacturer's instructions. Cells were selected in the presence of 200 μg/ml of Zeocin (Invitrogen), and clones were isolated, expanded and characterized. To generate cells expressing HA-eIF4E, HT1080/GyrB-PKR cells were transfected with either pMV7/HA-eIF4E or pMV7 vector (generous gift from Dr. N. Sonenberg's lab) and were selected in the presence of 400 μg/ml of G418 (Bioshop). Spontaneously immortalized mouse embryonic fibroblasts (MEFs) from isogenic $PKR^{+/+}$ and catalytic $PKR^{-/-}$ mice (198,198) were generated as described (247).

2.2 Cell culture

The human fibrosarcoma HT1080 cells (ATCC CCL-121), and $PKR^{+/+}$ and $PKR^{-/-}$ MEFs, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and antibiotics (penicillin-streptomycin, 100 U/ml; ICN Biomedicals, Inc.). Human kidney embryonic (HEK) 293T cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM L-glutamine (Cellgro) and antibiotics.

2.3 Transfection

Transfections were performed using 3 μl FuGENE 6 (Roche), Lipofectamine Plus (Invitrogen) or Lipofectamine 2000 (Invitrogen) reagents according to manufacturers' instructions. Briefly, 6 x 10^5 cells were seeded in 6-cm plates. The day after, cells were incubated with reagents and 2 μg of vector DNA in serum-free medium

at 37°C for 5 h. The medium was then replenished with 10% serum and cells were incubated for an additional 24 hours.

2.4 RNA Interference

For siRNA transfection, 1 x 10^5 cells were seeded in 6-cm plates. The following day, cells were transfected with 200 pmoles siRNA (scrambled (SCR) or human PIK3R1 (Dharmacon) using 4 μl Lipofectamine 2000 (Invitrogen) in medium lacking serum. Six hours post-transfection, the plates were washed with serum-free DMEM and replenished with medium containing 10% serum. Cells were incubated at 37°C for an additional 72 hours before being treated with coumermycin.

2.5 Biochemical reagents

For IFN treatment, 100 IU/ml of mouse IFNγ (Cedarlane, CL9209R) was used. Treatment with coumermycin (Biomol, GR-317) was performed at a concentration of 100 ng/ml. Rapamycin (Bioshop, RAP004), LY294002 (Sigma, L-9908) and Wortmannin (Bioshop, WOR222) were used at a concentration of 20 nM, 20 μM and 10 μM, respectively. MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) (Calbiochem, 474790) was used at 40 μM. For thapsigargin treatment 1 μM was used (sigma, T9033).

2.6 Adenovirus infection

For virus infection, 2 x 10^5 cells were seeded in 6-cm plates. The following day, cells were infected with control adenovirus (Ad BHGdelE1, E3) or dominant negative p85 expressing adenovirus (Ad5dnp85) (MOI: 250) in serum-free medium. Cells were incubated at 37°C for 24 hours before being treated with coumermycin.

3. Protein analysis

3.1 Protein extraction

Cells were washed twice with ice-cold phosphate buffer saline (PBS) solution (140mM NaCl, 15mM KH_2PO_4 pH 7.2, 2.7 mM KCl), and proteins were extracted with a lysis buffer containing 10 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 1% Triton-X-100, 1mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. After incubation on ice for 20 min, the lysates were centrifuged at 14,000x g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and the protein concentration was measured by Bradford assay (BioRad). Samples were stored at –85°C.

3.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.) according to standard protocol.

3.3 Isoelectric focusing and two-dimensional (2D) gel electrophoresis

Cells were lysed with 8 M urea, 4% (wt/vol) CHAPS(248), 65 mM DTT, and 0.5% (vol/vol) IPG buffer (pH 3 to 10 NL or pH 4 to 7) (Bio-Rad). Isoelectric focusing (IEF) step was performed by using an Ettan IPGphor IEF unit (Amersham) and 7-cm strips at pH 3 to 10 NL or pH 4 to 7 (Bio-Rad). The strips were passively rehydrated with 125 μl of rehydration buffer containing 80 μg of the protein extracts, 8 M urea, 2% (wt/vol) CHAPS, 10 mM DTT, 0.5% (vol/vol) IPG buffer (pH 3 to 10 NL or pH 4 to 7),

and a trace amount of bromophenol blue for 10 hours. IEF was performed at 150 V for 40 min, 500 V for 40 min, 1,000 V for 40 min, and 5,000 V for 2.5 hours. The strips were then equilibrated for 12 minutes in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% (wt/vol) DTT, and a trace amount of bromophenol blue. For the second-dimension analysis, the strips were incubated for 5 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 2.5% (wt/vol) iodoacetamide, and a trace amount of bromophenol blue. The equilibrated strips were then subjected to SDS-10% PAGE, followed by immunoblot analysis.

3.4 Immunoblot analysis

Immunoblottings were performed according to the standard protocol (249). Briefly, the membranse were blocked in phosphate-buffered saline (PBS) or Tris-buffered Saline (TBS) (according to antibody specification sheet) containing 0.5% Tween-20 (PBST or TBST) and 5% milk for one hour at room temprature. The primary antibody was diluted in PBST or TBST containing 5% BSA and added to the membrane for 16 hours at 4°C. After six 5-minute washes with PBST or TBST, the membrane were incubated in peroxidase-conjugated secondary antibody diluted in PBST or TBST containing 5% milk for one hour at room temperature. Subsequently, the membrane was subjected to another six 5-minute washes and the reaction was visualized with enhanced chemiluminescence (ECL) detection system as detailed by manufacturer (Perkin Elmer). Quantification of the bands in the linear range of exposure was performed by densitometry using the NIH Image 1.54 software.

3.4.1 Primary antibodies

The primary antibodies were as follows: anti-FLAG M2 mouse monoclonal antibody (Sigma; 2 μg/ml), anti-human PKR mouse monoclonal antibody [clone F9 or E8 (250); 1 μg/ml], anti-GyrB mouse monoclonal antibody (clone 7D3, John Innes Enterprises; 0.2 μg/ml), anti-human eIF2α rabbit polyclonal antibody (Cell signaling; 1 μg/ml), phosphospecific antibodies against eIF2α-pSer51 [(251); 1 μg/ml], anti-Bik mouse monoclonal antibody (clone C33-1, BD Biosciences; 1 μg/ml), anti-p53 mouse monoclonal antibody (Ab-2, Oncogene Science; 1 μg/ml). The following antibodies were used from the APOPTOPAK miniature set from Upstate Biotechnology: Anti-Bcl2 mouse monoclonal IgG (clone 124, 1 μg/ml), anti-Bak rabbit polyclonal IgG (1 μg/ml) and anti-Bax rabbit polyclonal IgG (1 μg/ml). The following antibodies were purchased from Santa Cruz Biotechnology: Anti-GADD153 (CHOP) rabbit polyclonal IgG (sc-575; 1μg/ml), anti-PP1 mouse monoclonal IgG (sc-7482; 2 μg/ml), anti-FAS rabbit polyclonal IgG (sc-715; 1 μg/ml), phosphospecific antibodies against 4EBP-Thr37/46, -Ser65 and -Thr70 (Cell Signaling; 1 μg/ml), anti-4EBP rabbit polyclonal antibody ((Sonenberg);1 μg/ml), anti-S6-Ser235/236 rabbit polyclonal antibody (Cell Signaling; 1 μg/ml), anti-S6 rabbit polyclonal antibody (Cell Signaling; 1 μg/ml), anti-PKB/Akt-Thr308 rabbit polyclonal antibody (Cell Signaling; 1 μg/ml), anti-PKB/Akt-Ser473 rabbit polyclonal antibody (Cell Signaling; 1 μg/ml), anti-PKB/Akt rabbit polyclonal antibody (Cell Signaling; 1 μg/ml), anti-Ras mouse monoclonal antibody (upstate, 1 μg/ml), anti-4E rabbit polyclonal antibody (Sonenberg; 1 μg/ml), anti-4GI rabbit polyclonal antibody (Sonenberg; 1 μg/ml), anti-HA rabbit antibody (Upstate, 1 μg/ml), anti-actin mouse monoclonal IgG (ICN; 0.1 μg/ml).

3.4.2 Secondary antibodies

The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody or HRP-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech.; dilution 1:1000).

3.3 Immunoprecipitation and pull-down assays

3.3.1 Immunoprecipitation

Equal amounts of protein extracts were incubated with 2 μg of the specified antibody for 2 hours in 4°C with rotation. Proteins were then immunoprecipitated using 50 ml of a 50% suspension of anti-mouse IgG (whole molecule) agarose beads (sigma) or protein A sepharose beads (Pharmacia). The samples were then rotated for additional 2 hours at 4°C. The immunopercipitates were washed three times in the lysis buffer, and subjected to SDS-PAGE and immunoblot analysis.

3.3.2 GST pull-down assay

For GST pull-down assays equal amounts of GST fusion proteins were precipitated with glutathione Sepharose 4B beads, while incubated in 4°C for 2 hours, washed with lysis buffer and resuspended in 200 μl lysis buffer. The protein extract from transfected cells were subsequently added to the suspension and rotated for 2 hours at 4°C. The beads were then washed with lysis buffer and precipitated proteins were subjected to SDS-PAGE.

3.3.3 Cap-binding assay

Cap binding assay was performed as described by Lekmine et al. (252). Cells were subjected to the indicated treatment, and lysed in cold cap binding buffer (100 mM KCl, 20 mM HEPES, pH 7.6, 7 mM β-mercaptoethanol, 0.2 mM EDTA, 10% glycerol, 50 mM β-glycerol phosphate, 50 mM NaF, 100 μM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) with 4 cycles of freeze and thaw. Protein extracts (250 μg) were subsequently incubated with m^7GTP-agarose resins at 4°C. The resin was then washed with cap-binding buffer, once with 500 ml and twice with 1 ml, resuspended in Laemmli sample buffer, boiled and resolved on SDS-PAGE.

3.3.4 Ras activation assay

Ras activation was assessed using Ras activation assay kit (upstate, 17-218). Briefly, 500 μg of protein extracts were incubated with Raf-1 RBD (Ras Binding Domain) agarose beads to pull down activated Ras. The precipitates were then subjected to immunoblotting with anti-Ras antibody to assess the levels of activated Ras.

4. PI3K protein and lipid kinase assay

The PI3K assay was performed as described (253) with some modifications. Briefly, cells were subjected to indicated treatments and proteins were extracted in lysis buffer containing 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20mM Tris-HCl pH 7.4, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM NaF, 1mM sodium pyrophosphate, 2 μg/ml leupeptin and aprotinin. Protein extracts (500 μg) were subjected to immunoprecipitation with anti-PI3K p85 (Upstate)

in presence of protein A-agarose. The samples were centrifuged at 13,000 rpm and sedimented beads were washed once with lysis buffer, twice with PBS containing 1% NP-40 and 100 μM sodium orthovanadate, three times with 100 mM Tris-HCl pH 7.4, containing 5 mM LiCl and 100 μM sodium orthovanadate, twice with TNE (10 mM Tris-HCl pH 7.4, 150 mM Nacl, 5 mM EDTA and 100 μM sodium orthovanadate). The last traces of buffer were completely removed and the pelleted beads were resuspended in 50 μl fresh TNE. To the resuspended pellet, we added 10 μl of 100 mM $MgCl_2$ and 20 μg of L-α-Phosphatidylinositol (PI) (Jena Biosciences) which were previously sonicated in a water bath sonicator for one hour. The reaction was started by adding 10 μCi [γ-^{32}P] ATP, and incubated at 23°C for 15 min with continuous agitation. The reactions were stopped with 20 μl 6N HCl. Extraction of the lipids were done by adding 160 μl of chloroform:methanol (1:1) and the samples were vortexed and centrifuged at room temperature to separate the phases. The lower organic phase (30 μl) was spotted onto the silica-coated glass TLC plates (Sigma Aldrich) precoated with 1% potassium oxalate. The spots were allowed to dry and resolved chromatographically with 2M acetic acid/isopropanol (1:2). The plates were dried and exposed to film, and the autoradiographic signals were quantified using Scion Image 4.0.3.2 software. The lipid standards were run as a separate lane on the TLC plate to identify the migration of PIP3 (Echelon). TLC plates were stained with iodine to identify the formation of phosphorylated lipid products. For the protein kinase assay H2B was added to the resuspended pellet as substrate The reaction was started by adding 10 μCi [γ-^{32}P] ATP, and incubated at 23°C for 15 min and radioactive protein were visualized by subjecting the supernatant to SDS-PAGE.

5. eIF2α dephosphorylation assay

Purified recombinant histidine-tagged eIF2α was prepared and phosphorylated by a purified GST-fusion protein of human PKR in vitro based on a previously published protocol (254). The unincorporated [γ-^{32}P] ATP was removed using MicroSpinTM G-25 columns (Amersham Pharmacia Biotech). An aliquot of ^{32}P-eIF2α was then incubated with anti-FLAG immunoprecipitates in a 10-μl dephosphorylation reaction (dephosphorylation buffer: 20 mM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.8 mM ATP). The reaction was incubated at 30°C for the specified time and was terminated by boiling the sample in an equal volume of 2% SDS loading buffer. Phosphorylated eIF2α was detected by SDS-PAGE and autoradiography.

6. *In vivo* ^{35}S labeling

For *in vivo* ^{35}S labeling, cells were subjected to the indicated treatment in DMEM plus 10% calf serum for the appropriate time. The medium was subsequently replaced by DMEM lacking methionine and supplemented with 10% dialyzed fetal bovine serum for 2 hours in the presence of the treatment. Tran^{35}S-label (ICN) was then added to the cells at a concentration of 100 μCi per 10^6 cells and culture continued for an additional 2 hours. Protein extracts were subjected to SDS-PAGE and radioactive proteins were visualized by autoradiography.

7. Cell staining and flow cytometry analysis

Cells were prepared for flow cytometry analysis as described (255) with a few modifications. Briefly, approximately 10^6 cells per 10-cm-diameter dish were detached

in PBS plus 0.1 mM EDTA and washed in ice cold PBS. Following centrifugation at 900xg for 5 min, cells were suspended in 0.5 ml of cold PBS and fixed by adding 4.5 ml of ice cold ethanol drop wise with gentle mixing. Fixed cells were stored at −20°C for at least 8 hours. For staining, pelleted cells were washed once with PBS, and suspended in 0.5 ml of PBS containing 50 μg/ml of propidium iodide (Sigma) and 20 μg/ml of RNase (Sigma). Cells were incubated at 37°C for 30 min and maintained at 4°C for 8 hours before subjected to flow cytometry analysis on a FACScan.

8. Immunofluorescence microscopy

Cells grown on 22-mm coverslip (Fisher) were treated as indicated, washed with PBS, fixed with 1% formaldehyde for 15 min at room temperature, and then blocked with 5% BSA, PBS, 0.1% TX-100 for 1 hour. To detect p53, cells were stained with a mixture of 1:200 diluted indicated antibodies. Cells were incubated with primary antibodies for 16 hours at 4°C, washed with PBS, and incubated for 1 hour with Alexa Fluro-488-conjugated secondary antibody or Alexa Fluro-546-conjugated secondary antibody (Molecular Probes). To visualize the nucleus, cells were counterstained with 1 μg/mL 4, 6-diamidino-2-phenylindole (DAPI, Sigma). After mounting, cells were analyzed with Carl Zeiss (axioskop 40) fluorescence microscope and AxioVision Rel. 4.5 software.

9. Cell proliferation assay

The proliferation rate of cells were assessed using the CellTiter 96® AQ_{ueous} One solution assay kit (Promega), a colorimetric method for determining the number of viable cells in culture in multiwell plate format. Briefly, cells were grown for the

indicated time and the assay was performed by adding a small amount of the CellTiter 96® AQ_{ueous} One Solution Reagent directly to culture wells, incubating for 1-4 hours and then recording absorbance at 490nm with a spectrophotometric plate reader. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

10. Microarray analysis

For cDNA microarray analysis, total RNA was isolated by lusing cells in Trizol reagent (Gibco BRL)(256). Generation of cDNA, fluorescent labeling and hybridization to the Affymetrix human U133A cDNA chip, which covers 22,000 genes(257), were performed by Genome Quebec (Montreal, Quebec). For each cell line, the values obtained after coumermycin treatment were normalized to those in the absence of the antibiotic. We focused on those genes whose expression was either induced or suppressed at a minimum of 5-fold in GyrB-PKR cells and remained unaffected in GyrB-PKRK296H cells (less than 3-fold induction or suppression) after coumermycin treatment.

Chapter III

Results

Chapter III - Results

1. PKR inducible system

PKR is believed to exist in latent form in cells. Upon virus infection, dsRNA produced as a byproduct of viral replication, binds to dsRNA binding domain of PKR and activates the kinase through its dimerization and autophosphorylation. In most *in vitro* assays virus infection or dsRNA transfection is used as a means to activate PKR. Employing these approaches; however, does not only result in activation of PKR; rather, it leads to induction of a variety of signaling pathways such as toll-like receptors 3 (TLR3) and RNase L pathway. On the other hand, considering that the antiviral, anti-proliferative and tumor suppressor functions of PKR were assigned by *in vitro* experiments, generation of the PKR knockout ($PKR^{-/-}$) mice was initially considered as an optimal system to examine these effects *in vivo*. Characterization of the two different PKR knockout mice; however, did not yield the anticipated results. In addition, experiments performed using cells from these $PKR^{-/-}$ mice have led to contradictory and confusing results. Recently, experiments done in our laboratory demonstrated that neither of the $PKR^{-/-}$ mice are completely devoid of PKR, and that truncated forms of the kinase is still expressed in both mice (258).

To overcome these obstacles and to generate a proper system to study the biological functions of PKR, we established an inducible system to manipulate PKR activity by employing an alternative approach: GyrB-PKR.

1.1 GyrB-PKR fusion proteins

To study the role of dimerization on PKR autophosphorylation and activation, Dever's group generated constructs to express fusion proteins consisting of the first 220 amino acid of the B subunit of bacterial enzyme DNA Gyrase (GyrB) and the wild-type kinase domain of PKR (residues 258-551) or the same domain containing the inactivating K296H mutation (GyrB-PKR or GyrB-PKRK296H) (243) (Fig.14A). DNA gyrase is a topoisomerase expressed in Escherichia Coli, which introduces negative superhelical turns into double-stranded closed circular DNA and therefore plays a role in bacterial DNA replication. It has been documented that the presence of coumermycin, leads to dimerization of DNA Gyrase. It has also been reported that coumermycin-mediated dimerization of Raf1-GyrB fusion protein results in activation of the Raf1 kinase, through induction of dimerization (259). Addition of coumermycin will, therefore, induce dimerization of GyrB-PKR fusion proteins. This mimics dimerization of PKR by dsRNA and leads to its autophosphoryaltion and activation (Fig.14B).

1.2 Generation of GyrB-PKR stable cell lines

GyrB-PKR constructs were primarily used in transient transfection system to analyze the role of dimerization in PKR activation (243). Since this system was proven to be functional, we decided to generate stable cell lines which permanently express the GyrB-PKR fusion proteins. These cell lines establish an optimum system to study kinase-dependent functions of PKR, without activation of other pathways, which normally occurs when dsRNA is used to activate PKR.

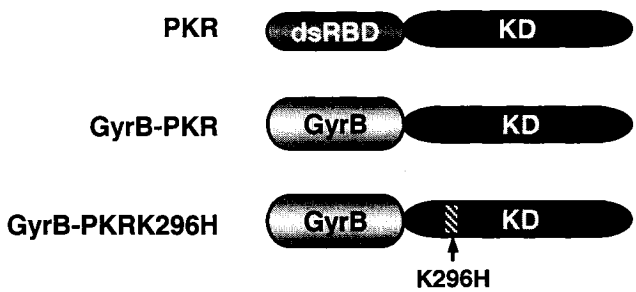
Figure 14. The GyrB-PKR inducible system

A. A schematic representation of PKR and GyrB-PKR proteins. The dsRNA binding regulatory domain of PKR wild-type or a kinase dead mutant (bearing a mutation at lysine 296 in the ATP-binding pocket) was replaced by the N-terminus of Gyrase B from E.Coli to form the GyrB-PKR fusion protein.

B. In the presence of antibiotic coumermycin, GyrB domain mediates cross-linking of the GyrB-PKR fusion protein, mimicking dsRNA-mediated dimerization of PKR. This results in PKR autophosphorylation and activation.

C. HT1080 human fibrosarcoma cells were stably transfected with GyrB-PKR constructs. Cells were treated with coumermycin (100 ng/ml) and expression and activation of PKR was assessed. Cell extracts were subjected to immunoblot analysis with immunoblotting with anti-GyrB antibody (a), phosphospecific antibody against eIF2α-pSer51 (b) and anti-eIF2α antibody (c).

A



B

C

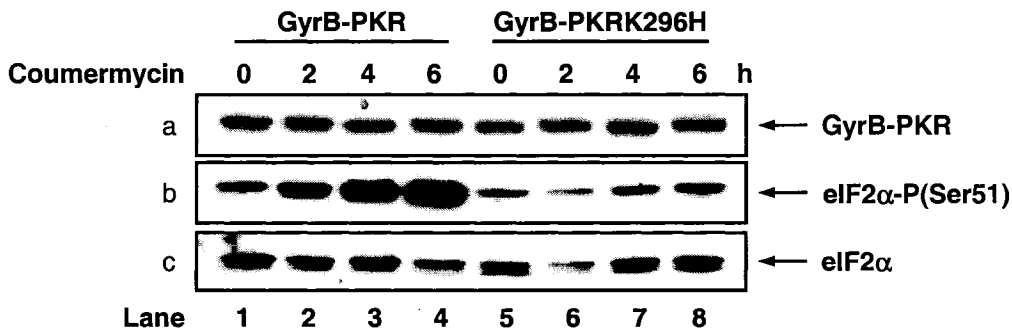


Figure 14

1.3 Characterization of HT1080/GyrB-PKR cell lines

To verify that the GyrB-PKR inducible system is functional, HT1080 cells expressing GyrB-PKR and GyrB-PKRK296H cells were treated with coumermycin for 6 hours and phosphorylation of eIF2α was assessed (Fig.14C). We observed an induction in PKR activation in GyrB-PKR expressing cells as indicated by upregulation of eIF2α phosphorylation, whereas no induction was observed in GyrB-PKRK296H cells (panel b). Immunoblot analysis with an anti-GyrB antibody showed an equal expression of GyrB-PKR and GyrB-PKRK296H (panel a).

2. Signaling properties of PKR

Given that PKR is a serine/threonine and tyrosine kinase (125), the only well characterized substrate of PKR so far is eIF2α. PKR, however, has been demonstrated to be involved in other signaling pathways through interaction with their components. Therefore, we sought to identify other pathways which lie downstream targets of PKR.

2.1 Activation of PI3K and mTOR pathways by PKR

Translational control at initiation step proceeds through two distinct pathways including phosphorylation of 4E-BP1, which controls the availability of eIF4F complex and phosphorylation of eIF2α, which controls the availability of the ternary complex. Our studies started from an initial hypothesis and preliminary observation and led to investigation of any relationship between the two pathways involved in this regulation.

2.1.1 Activation of GyrB-PKR induces the phosphorylation of 4E-BP1.

Investigating the signaling properties of GyrB-PKR, we made the observation that activation of GyrB-PKR led to a transient induction of 4E-BP1 phosphorylation (Fig.15A). Specifically, treatment of GyrB-PKR-expressing cells with coumermycin significantly induced the phosphorylation of 4E-BP1 at Thr 37/46 and Thr 70 (panels a and c, lanes 1-5). Activation of GyrB-PKR also enhanced the phosphorylation of Ser65 of 4E-BP1 but to a lesser extent than the phosphorylation at the threonine sites (panel b, lanes 1-5). In fact, the basal levels of Ser65 phosphorylation were elevated in HT1080 cells possibly due to their transformed phenotype (compare lane 1 in panels a, b and c). Interestingly, coumermycin treatment also induced 4E-BP1 phosphorylation in cells containing the catalytically inactive mutant GyrB-PKRK296H, which was expressed at equal levels to GyrB-PKR (260) (panels a-c, lanes 6 to 10). Phosphorylation of 4E-BP1 by GyrB-PKRK296H; however, took place to a lesser extent than phosphorylation by active GyrB-PKR (compare lanes 1-5 with lanes 6-10). Because PKR can exhibit kinase-independent properties by acting as a scaffold protein for other kinases (186,261,262), we speculate that dimerization of GyrB-PKRK296H upon cross-linking of the GyrB domain by coumermycin may be capable of inducing a pathway(s) leading to the phosphorylation of 4E-BP1. Immunoblotting with an anti-4E-BP1 antibody further demonstrated the phosphorylation of the protein in cells expressing either GyrB-PKR or GyrB-PKRK296H as documented by the slow migration of the hyperphosphorylated forms of 4E-BP1 (panel d, lanes 2, 3, 7 and 8). Interestingly, prolonged treatment with coumermycin resulted in the dephosphorylation of 4E-BP1 in both GyrB-PKR and GyrB-PKRK296H-expressing cells (panels a-c, lanes 4, 5, 9 and

10) and in the upregulation of total 4E-BP1 levels only in cells expressing GyrB-PKR (panel d, lanes 4 and 5). These data indicated the presence of both positive and negative regulatory mechanisms of 4E-BP1 phosphorylation in GyrB-PKR-expressing cells.

Figure 15. Conditional activation of GyrB-PKR induces the phosphorylation of 4E-BP1 via activation of PI3K and FRAP/mTOR pathways.

A. Protein extracts (50 μg) from HT1080 cells expressing either GyrB-PKR or GyrB-PKRK296H, treated with coumermycin (100 ng/ml) for 0-24 hours, were subjected to immunoblotting with phosphospecific antibodies against 4E-BP1-pThr37/46 (a), -pSer65 (b), -pThr70 (c) or anti-4E-BP1 antibody (d). Actin levels show an equal loading (e).

B. HT1080 cells expressing GyrB-PKR were left untreated or treated with coumermycin (100 ng/ml) in the absence or presence of rapamycin (20 nM) and/or LY294002 (20 μM), as indicated. Protein extracts (50 μg) were subjected to immunoblotting with phosphospecific antibodies against 4E-BP1-pThr37/46 (a), -pSer65 (b), -pThr70 (c), anti-4E-BP1 antibody (d) or phosphospecific antibody against eIF2α-pSer51 (e).

C. Protein extracts (80 μg) were subjected to 2D gel electrophoresis followed by immunoblotting with antibodies against total 4E-BP1. Differences in migration of 4E-BP1 are indicated by arrows in panel c and d. (C: coumermycin, R: rapamycin, LY: LY294002)

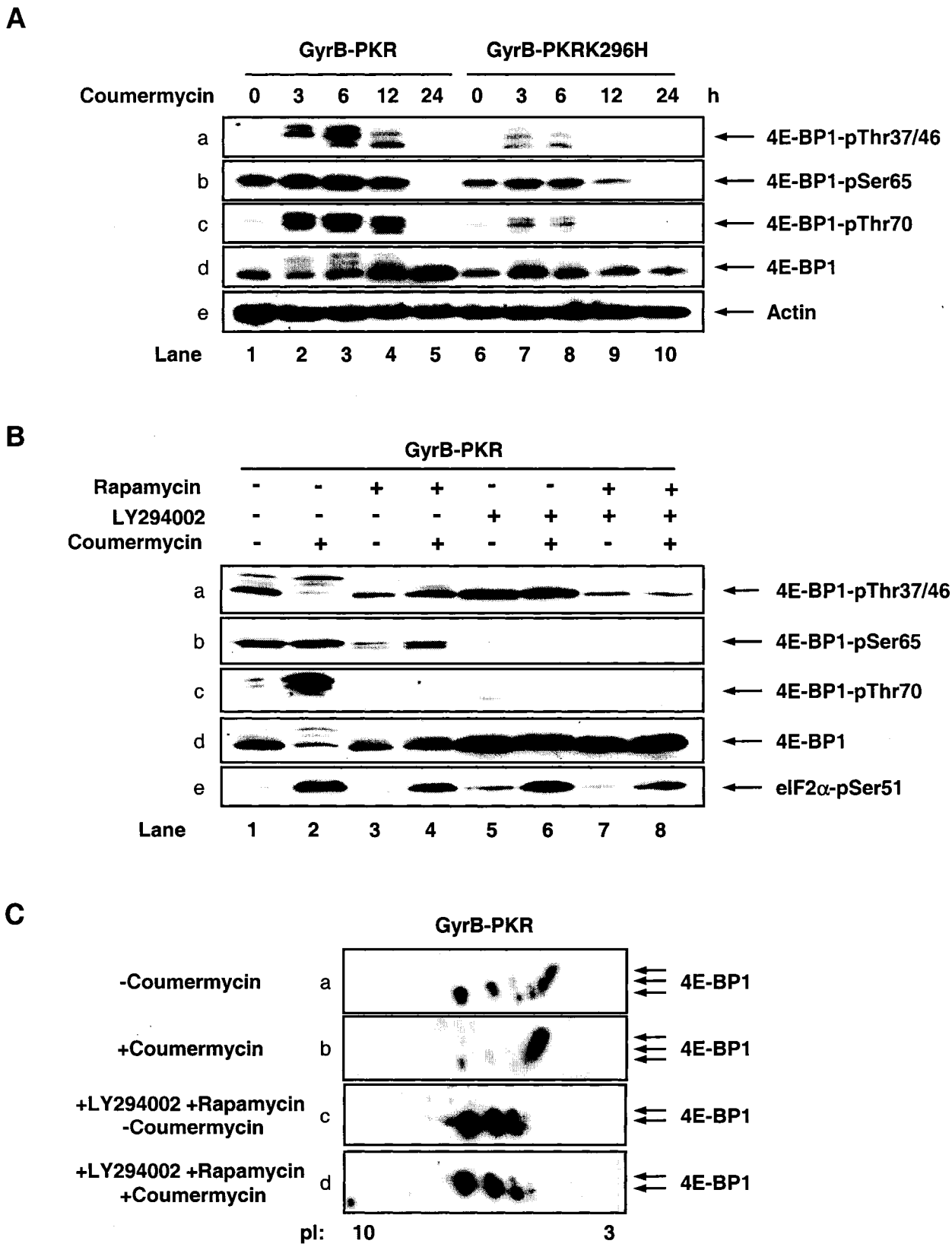

A
GyrB-PKR
GyrB-PKRK296H
Coumermycin 0 3 6 12 24 0 3 6 12 24 h
a
b
c
d
e
4E-BP1-pThr37/46
4E-BP1-pSer65
4E-BP1-pThr70
4E-BP1
Actin
Lane 1 2 3 4 5 6 7 8 9 10
B
GyrB-PKR
Rapamycin - - + + - - + +
LY294002 - - - - + + + +
Coumermycin - + - + - + - +
a
b
c
d
e
4E-BP1-pThr37/46
4E-BP1-pSer65
4E-BP1-pThr70
4E-BP1
eIF2α-pSer51
Lane 1 2 3 4 5 6 7 8
C
GyrB-PKR
-Coumermycin
+Coumermycin
+LY294002 +Rapamycin -Coumermycin
+LY294002 +Rapamycin +Coumermycin
a
b
c
d
4E-BP1
pI: 10 3

Figure 15

2.1.2 PKR-mediated phosphorylation of 4E-BP1 proceeds through PI3K and mTOR pathways.

Since 4E-BP1 is a downstream target of PI3K and FRAP/mTOR, we wished to determine whether phosphorylation of 4E-BP1 by GyrB-PKR is mediated through the PI3K pathway. When GyrB-PKR cells were treated with LY294002; the inhibitor of PI3K, or rapamycin; the inhibitor of FRAP/mTOR, we noticed a reduction in the basal levels of phosphorylated 4E-BP1 (Fig.15B, panels a-c, lanes 3 and 5). The basal levels of 4E-BP1 phosphorylation were further reduced when both inhibitors were used (lane 7). When cells were treated with coumermycin in the presence of rapamycin, we observed that activated GyrB-PKR was partially capable of inducing the phosphorylation of 4E-BP1 at Thr37/46 and Ser65 (panels a and b, lane 4). This indicates that GyrB-PKR can affect the phosphorylation of these sites either independently of FRAP/mTOR or through a rapamycin-insensitive function of FRAP/mTOR as recently reported (55,263). When the PI3K inhibitor was used; however, we noticed that induction of 4E-BP1 phosphorylation at Thr37/46 by GyrB-PKR was not possible (panel a, compare lane 5 with lane 6) whereas phosphorylation of 4E-BP1 at Ser65 and Thr70 was below detectable levels (panels b and c, lanes 5 and 6). Similar results were obtained when both LY294002 and rapamycin were used (panels a-c, lanes 7 and 8). Immunoblot analysis with anti-4E-BP1 antibody showed the lack of the hyperphosphorylated forms of the protein in the presence of the kinase inhibitors (panel d) confirming the phosphorylation pattern of 4E-BP1 obtained with the phosphospecific antibodies. Significantly, the presence of LY294002 and rapamycin

did not affect GyrB-PKR activity, since induction of eIF2α phosphorylation took place efficiently in cells treated with the inhibitors (panel e, lanes 2, 4, 6 and 8).

Phosphorylation of 4E-BP1 was further confirmed by isoelectric focusing (IF) and two-dimensional) gel electrophoresis (2DGE) (Fig.15C). Specifically, we saw that treatment with coumermycin yielded more acidic forms of 4E-BP1 (panel b) compared to untreated cells (panel a) indicative of phosphorylation. Cells treated with both LY294002 and rapamycin (panel c) produced 4E-BP1 forms that were shifted to the basic region of the gel compared to 4E-BP1 in untreated cells (panel a). This was consistent with the ability of both inhibitors to reduce the basal levels of 4E-BP1 phosphorylation in the absence of coumermycin (Fig.15B). Activation of GyrB-PKR by coumermycin in the presence of both inhibitors resulted in some minor differences in the migration of the 4E-BP1 forms as indicated (Fig.15C, compare panels c and d) suggesting that induction of 4E-BP1 phosphorylation by GyrB-PKR is mainly through the activation of the PI3K pathway. The additive effects of both inhibitors indicated that the PI3K-PKB/Akt and FRAP/mTOR form a branched rather than a linear pathway leading to 4E-BP1 phosphorylation in HT1080 cells. Also, phosphorylation of Thr70 and Ser65 was much more sensitive to inhibition by rapamycin than Thr37/46 phosphorylation as shown in previous studies (18,263,264).

2.1.3 Activation of GyrB-PKR results in induction of PI3K pathway.

To date, the best characterized pathway leading to phosphorylation of 4E-BP1 involves the activation of PI3K and FRAP/mTOR, which also results in the phosphorylation of PKB/Akt and the ribosomal S6 protein (15). First we assessed the effect of PKR activation on PI3K pathway and verified PKB/Akt phosphorylation. We

observed that phosphorylation of PKB/Akt at Thr308 and Ser473 was induced by activated GyrB-PKR (Fig.16A, panel a and b). Treatment of GyrB-PKRK296H-expressing cells with coumermycin; however, did not exhibit similar effects. Induction of both PKB/Akt in GyrB-PKR cells occurred in a time-dependent manner and was reduced after 24 h of treatment (panel a, lane 5) following a pattern similar to 4E-BP1 phosphorylation (Fig.15A). Consistent with the activation of PKB/Akt, induction of GyrB-PKR resulted in phosphorylation of GSK3β at Ser9 (Fig.16B).

2.1.4 Activation of GyrB-PKR results in induction of mTOR pathway.

Next, we examined if PKR was able to activate mTOR pathway. We observed that S6 phosphorylation at Ser235/236 was induced upon GyrB-PKR activation (Fig.16C, panel a) in a time-dependent manner. In fact, S6 phosphorylation declined after 24 hours of treatment (lane 5) following a pattern similar to 4E-BP1 phosphorylation (Fig. 16C). When we assessed the eIF2α phosphorylation levels, we found that Ser51 phosphorylation of eIF2α reached a peak at 6 hours after coumermycin treatment and remained high as long as the treatment lasted (Fig. 16D). This showed that the decrease in the phosphorylation levels of 4E-BP1 and S6 was not due to a downregulation of the GyrB-PKR activity. It is noteworthy that, although the above experiments were performed in the absence of serum to diminish the background effects of growth factors on protein phosphorylation, GyrB-PKR is capable of inducing the phosphorylation of PKB/Akt, S6 and 4E-BP1 in the presence of serum (Fig.17A). Furthermore, treatment of parental HT1080 cells with coumermycin did not induce PKB/Akt, S6, eIF2α or 4E-BP1 phosphorylation (Fig.17B), demonstrating the lack nonspecific effects of coumermycin.

Figure 16. Conditional activation of GyrB-PKR induces the PI3K/mTOR pathway. HT1080 cells expressing GyrB-PKR were left untreated or treated with coumermycin (100 ng/ml) for 0-24 hours. Protein extracts (50 μg) were subjected to SDS-PAGE and immunoblotting with the following antibodies;

A. phosphospecific antibody against PKB/Akt-pThr308 (a), phosphospecific antibody against PKB/Akt-pSer473 (b) or anti-PKB/Akt antibody (c)

B. phosphospecific antibody against GSK3β-pSer9 (a) or anti-GSK3β antibody (b)

C. phosphospecific antibody against S6-pSer 235/236 (a) or anti-S6 antibody (b)

D. phosphospecific antibody against eIF2α-pSer51 (a) or anti-eIF2α antibody (b)

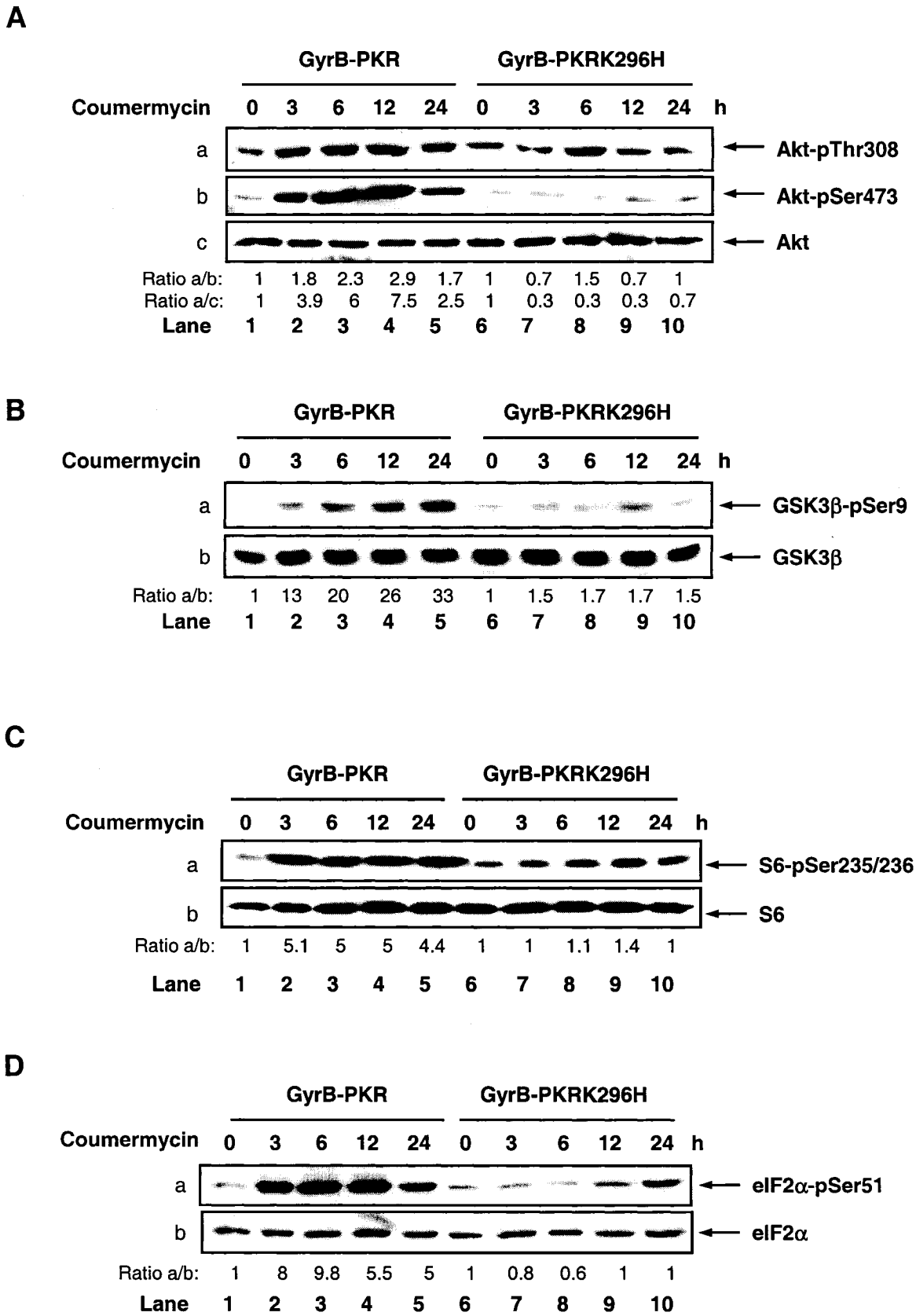

A
GyrB-PKR
GyrB-PKRK296H
Coumermycin 0 3 6 12 24 0 3 6 12 24 h
a
b
c
Akt-pThr308
Akt-pSer473
Akt
Ratio a/b: 1 1.8 2.3 2.9 1.7 1 0.7 1.5 0.7 1
Ratio a/c: 1 3.9 6 7.5 2.5 1 0.3 0.3 0.3 0.7
Lane 1 2 3 4 5 6 7 8 9 10
B
GyrB-PKR
GyrB-PKRK296H
Coumermycin 0 3 6 12 24 0 3 6 12 24 h
a
b
GSK3β-pSer9
GSK3β
Ratio a/b: 1 13 20 26 33 1 1.5 1.7 1.7 1.5
Lane 1 2 3 4 5 6 7 8 9 10
C
GyrB-PKR
GyrB-PKRK296H
Coumermycin 0 3 6 12 24 0 3 6 12 24 h
a
b
S6-pSer235/236
S6
Ratio a/b: 1 5.1 5 5 4.4 1 1 1.1 1.4 1
Lane 1 2 3 4 5 6 7 8 9 10
D
GyrB-PKR
GyrB-PKRK296H
Coumermycin 0 3 6 12 24 0 3 6 12 24 h
a
b
eIF2α-pSer51
eIF2α
Ratio a/b: 1 8 9.8 5.5 5 1 0.8 0.6 1 1
Lane 1 2 3 4 5 6 7 8 9 10

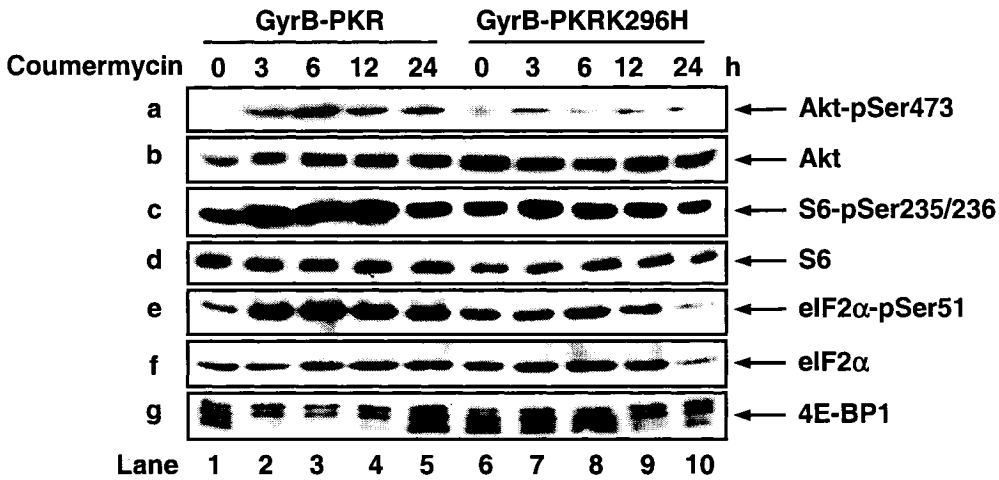
Figure 16

Figure 17. Phosphorylation of components of the PI3K pathway in GyrB-PKR and HT1080 parental cells

A. Protein extracts (50 μg) from HT1080 cells expressing either GyrB-PKR or GyrB-PKRK296H, untreated or treated with coumermycin (100 ng/ml) in the presence of serum for up to 24 hours, were subjected to immunoblotting with phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b), phosphospecific antibody against S6-pSer 235/236 (c) or anti-S6 antibody (d), phosphospecific antibody against eIF2α-pSer51 (e) or anti-eIF2α antibody (f) and anti-4E-BP1 antibody (g).

B. Serum-deprived HT1080 parental cells were left untreated or treated with of coumermycin in the absence of serum for up to 24 hours. Protein extracts (50 μg) were subjected to immunoblotting with a phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b), phosphospecific antibody against S6-pSer 235/236 (c) or anti-S6 antibody (d), phosphospecific antibody against eIF2α-pSer51 (e) or anti-eIF2α antibody (f) and anti-4E-BP1 antibody (g).

A


GyrB-PKR
GyrB-PKRK296H
Coumermycin 0 3 6 12 24 0 3 6 12 24 h
a
b
c
d
e
f
g
Akt-pSer473
Akt
S6-pSer235/236
S6
eIF2α-pSer51
eIF2α
4E-BP1
Lane 1 2 3 4 5 6 7 8 9 10

B

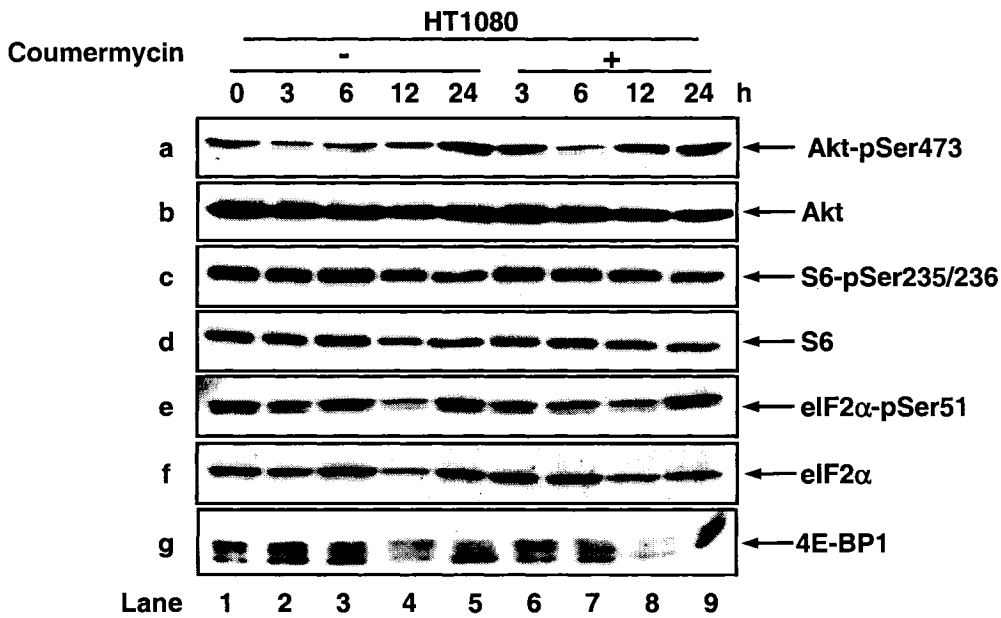

HT1080
Coumermycin
-
+
0 3 6 12 24 3 6 12 24 h
a
b
c
d
e
f
g
Akt-pSer473
Akt
S6-pSer235/236
S6
eIF2α-pSer51
eIF2α
4E-BP1
Lane 1 2 3 4 5 6 7 8 9

Figure 17

2.1.5 PKR acts upstream of PI3K to induce PKB/Akt and S6 phosphorylation.

We further tested the effects of LY294002 and rapamycin on PKB/Akt and S6 phosphorylation, respectively. Induction of PKB/Akt phosphorylation at Ser473 in cells with activated GyrB-PKR was undetectable in the presence of LY294002 (Fig.18A) indicating that PKR functions upstream of PI3K. Similarly, induction of S6 phosphorylation by activated GyrB-PKR was not possible in the presence of rapamycin indicating that PKR acts upstream of the FRAP/mTOR kinase (Fig.18B). To further confirm these observations, we used wortmannin, which at concentration of 10 μM inhibits PI3K without affecting FRAP/mTOR activity (265). We noticed that wortmannin eliminated the induction of PKB/Akt and S6 phosphorylation by activated GyrB-PKR (Fig.18C, panels a and c) without affecting eIF2α phosphorylation levels (panel e).

Collectively, these data demonstrated that induction of 4E-BP1 phosphorylation by GyrB-PKR proceeds through the activation of the PI3K and its downstream effectors PKB/Akt and mTOR kinases.

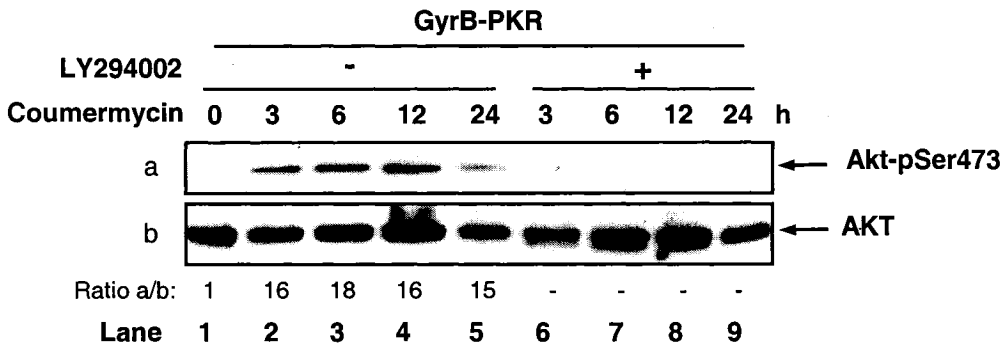
Figure 18. GyrB-PKR acts upstream of PI3K

A. Serum-starved HT1080 cells expressing GyrB-PKR were left untreated or treated with coumermycin (100 ng/ml) in the absence or presence of LY294002 (20 μM) for 0-24 hours. Protein extracts (50 μg) were subjected to immunoblotting with phosphospecific antibody against PKB/Akt-pSer473 (a) or anti-PKB/Akt antibody (b).

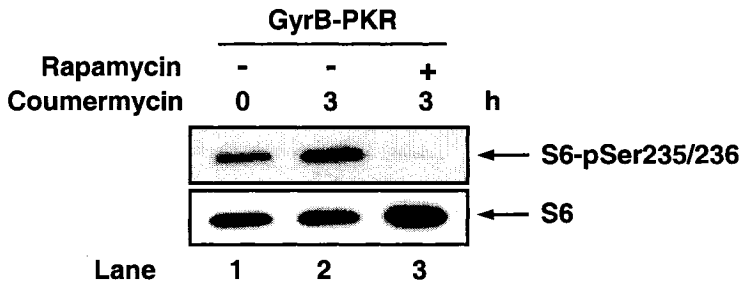
B. HT1080 cells expressing GyrB-PKR were left untreated or treated with coumermycin (100 ng/ml) in the absence or presence of Rapamycin (20 nM) for 3 hours, in the absence of serum. Protein extracts (50 μg) were subjected to immunoblotting with phosphospecific antibody against S6-pSer 235/236 (a) or anti-S6 antibody (b).

C. Serum-deprived HT1080 cells expressing GyrB-PKR were left untreated or treated with coumermycin (100 ng/ml) in the absence or presence of wortmannin (10 μM) for 0-24 hours. Protein extracts (50 μg) were subjected to immunoblotting with a phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b), phosphospecific antibody against S6-pSer 235/236 (c), anti-S6 antibody (d), phosphospecific antibody against eIF2α-pSer51 (e) or anti-eIF2α antibody (f).

A



B



C

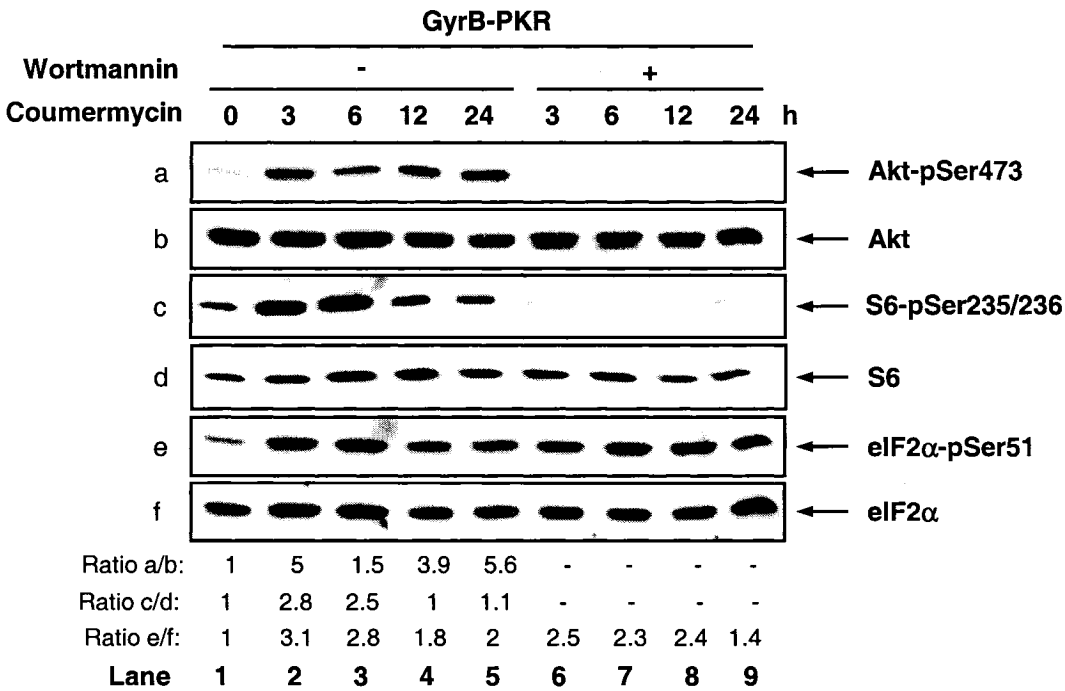


Figure 18

2.2 Induction of PI3K activity by PKR

Given that PKR activation induces phosphorylation of the components of PI3K and mTOR pathways, we wished to examine if PI3K activity is affected upon activation of PKR.

2.2.1 PKR induces PI3K lipid and protein kinase activity.

PI3K activation was assessed by testing the phosphorylation of phosphatidylinositols (PIs) in GyrB-PKR cells upon activation of the eIF2α kinase (266). A significant induction of PI3P formation was observed in GyrB-PKR cells compared to GyrB-PKRK296H cells after coumermycin treatment (Fig.19A).

PI3K has been shown to possess protein kinase activity (33). To verify further the effect of PKR, PI3K activity was assessed in extracts from GyrB-PKR-expressing cells using histone 2B (H2B) as substrate. Stimulation of GyrB-PKR activity by coumermycin induced H2B phosphorylation by ~50% (Fig.19B, panel a), which was equivalent to the induction of H2B phosphorylation by PI3K from serum-stimulated HEK293 cells.

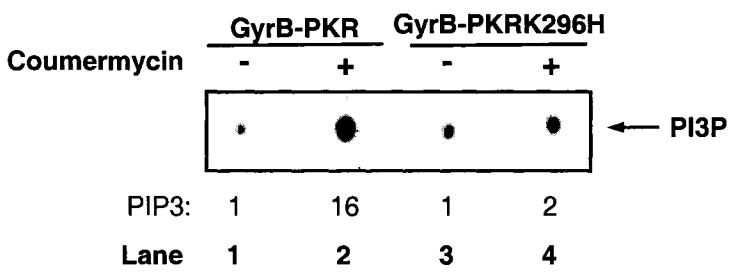
Collectively, the above data demonstrate that GyrB-PKR acts upstream of PI3K and induces both lipid and protein kinase activity.

Figure 19. Activation of GyrB-PKR results in induction of PI3K activity.

A. Serum-starved GyrB-PKR or GyrB-PKRK296H-expressing cells were left untreated or treated with coumermycin (100 ng/ml) for 6 hours. Protein extracts (500 μg) were subjected to immunoprecipitation with an anti-PI3K p85 antibody followed by *in vitro* lipid kinase assay in the presence of [^{32}P-γ] ATP and phosphatidylinositol (PI) as substrate. Radioactive PI3P was visualized by thin layer chromatography (TLC) and autoradiography.

B. Human embryonic kidney cells (HEK) 293T cells or HT1080 cells expressing GyrB-PKR were deprived from serum for 16 hours. 293T cells were left untreated or treated with 10% serum for 1 hour and GyrB-PKR-expressing cells were left untreated or treated with coumermycin (100 ng/ml) for 6 hours in the absence of serum. Protein extracts (500 μg) were subjected to immunoprecipitation with an anti-PI3K p85 antibody followed by *in vitro* protein kinase assay in the presence of [^{32}P-γ] ATP and Histone 2B (H2B) as substrate. Radioactive H2B was visualized by autoradiography, whereas H2B and PI3K p85 protein levels were detected with anti-H2B and anti-p85 antibodies (b and c, respectively).

A


GyrB-PKR
GyrB-PKRK296H
Coumermycin
- + - +
PI3P
PIP3: 1 16 1 2
Lane 1 2 3 4

B

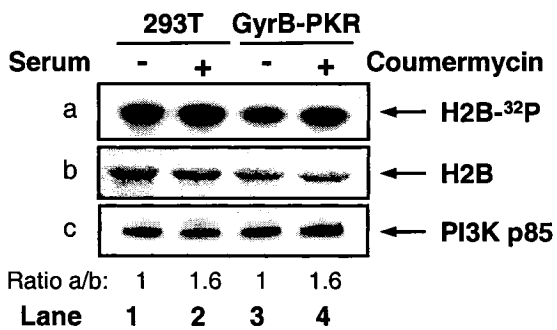

293T
GyrB-PKR
Serum
- + - +
Coumermycin
a
H2B-32P
b
H2B
c
PI3K p85
Ratio a/b: 1 1.6 1 1.6
Lane 1 2 3 4

Figure 19

2.2.2 Activation of PI3K is required for induction of PKB/Akt phosphorylation.

To address whether PI3K is required for PKR-mediated induction of phosphorylation of the components of the pathway, several experiments were performed. First, GyrB-PKR-expressing cells were transfected with RNAi against the p85 subunit of PI3K. Downregulation of the p85 subunit by RNAi prevented the induction of PKB/Akt phosphorylation at Ser473, upon coumermycin treatment, suggesting a role of p85 in PKR-mediated activation of the components in PI3K pathway (Fig.20A). Moreover, overexpression of PTEN in cells expressing GyrB-PKR, which results in dephosphorylation of the phosphorylated lipids, resulted in inhibition of PKR-mediated PKB/Akt phosphorylation (Fig.20B). This suggests that induction of PKB/Akt phosphorylation by PKR is dependent on formation of PIP3 and therefore on PI3K activity. Furthermore, infection of cells containing GyrB-PKR with adenovirus expressing a dominant negative mutant of the p85 subunit prevented the induction of PKB/Akt phosphorylation at Ser473 by activated GyrB-PKR, suggesting that p85 function is required for PKR-dependent phosphorylation of PKB/Akt. (Fig.20C). In these experiments GyrB-PKR activity was not affected as indicated by the induction of eIF2α phosphorylation.

Collectively, these data demonstrate the role of PI3K in PKR-dependent activation of PKB/Akt. Based on these data it is concluded that GyrB-PKR acts upstream of PI3K as an activator of the pathway.

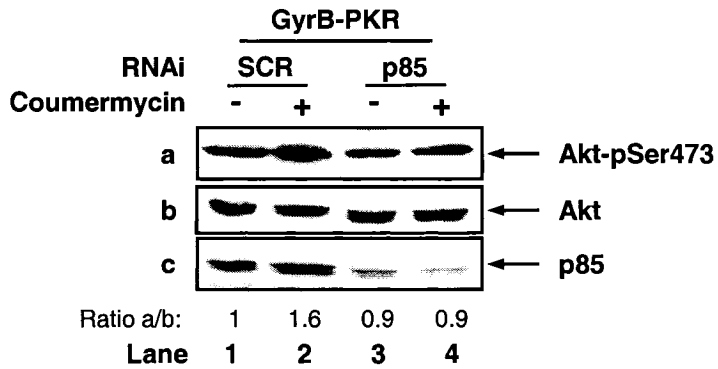
Figure 20. PKR-mediated induction of PKB/Akt phosphorylation requires PI3K.

A. HT1080 cells expressing GyrB-PKR were transiently transfected with scrambled (SCR) or PI3K p85 siRNA for 72 hours. Cells were then treated with coumermycin (100 ng/ml) for 6 hours. Protein extracts (30 μg) were subjected to immunoblot analysis with a phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b) or anti-p85 antibody (c).

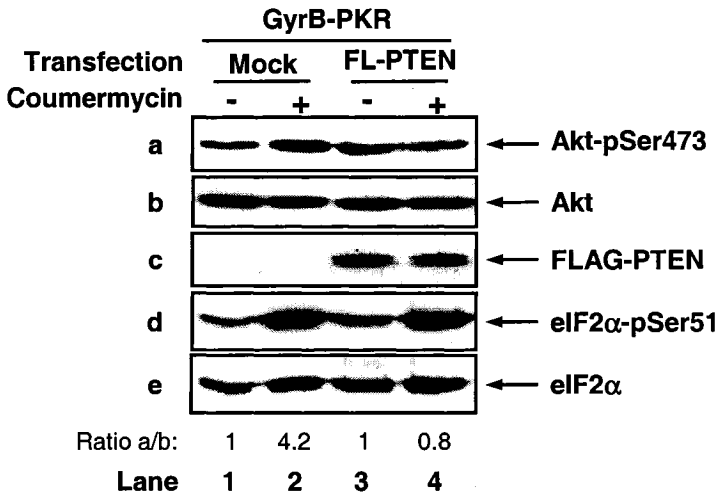
B. GyrB-PKR-expressing cells were transiently transfected with pCMV/FLAG wild-type PTEN or the empty vector for 24 hours, followed by coumermycin treatment (100 ng/ml) for 6 hours. Protein extracts (30 μg) were subjected to immunoblotting with phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b), anti-FLAG antibody (c), phosphospecific antibody against eIF2α-pSer51 (d) or anti-eIF2α antibody (e).

C. HT1080 cells expressing GyrB-PKR were infected with adenovirus expressing dominant negative p85 (dnp85) or the control virus (MOI 500). 24 hours post-infection cells were treated with coumermycin (100 ng/ml) for 6 hours. Protein extracts (30 μg) were subjected to immunoblotting with phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b), anti-p85 antibody (c), phosphospecific antibody against eIF2α-pSer51 (d) or anti-eIF2α antibody (e).

A



B



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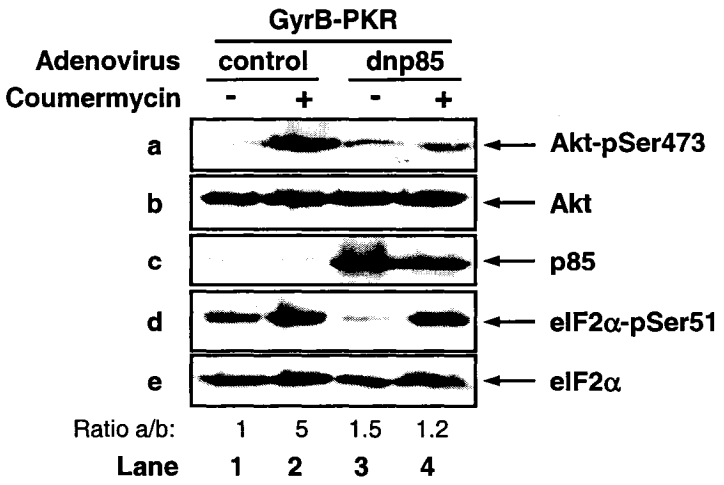


Figure 20

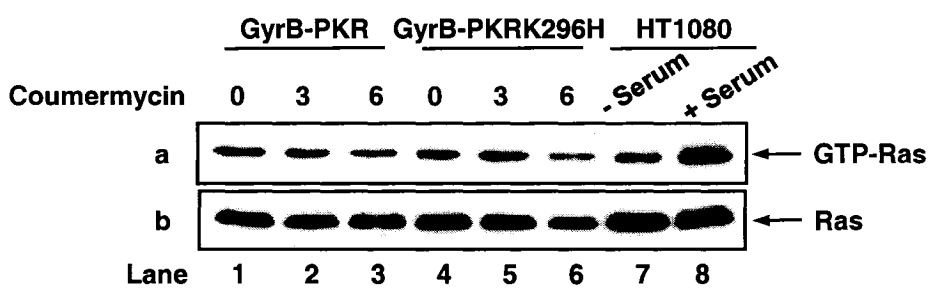
2.2.3 Activation of PI3K by PKR is not mediated by Ras.

The HT1080 cells contain an active form of N-Ras (267). Given the ability of Ras to activate PI3K through a direct interaction with its catalytic subunit, p110 (31,32), we wished to determine whether GyrB-PKR had an effect on Ras activity. To this end, we assessed the levels of activated Ras by measuring the amount of Ras bound to c-Raf1 (Fig.21, panel a) versus the total Ras protein (panel b). We did not notice any significant differences in Ras activation in GyrB-PKR or GyrB-PKRK296H cells before or after coumermycin treatment (panel a) suggesting that PKR does not modulate Ras activity. HT1080 cells were not refractory to signaling leading to Ras activation since serum stimulation of the parental cells increased the levels of activated Ras bound to c-Raf1 (panel a, compare lanes 7 and 8).

Furthermore, conditional media from GyrB-PKR-expressing cells was not able to mimic the effects of activated GyrB-PKR excluding the possibility of secretion of a growth factor or a cytokine leading to the activation of the PI3K in an autocrine fashion.

Figure 21. PKR-mediated PI3K activation is independent of Ras.

HT1080 cells expressing GyrB-PKR or GyrB-PKRK296H were treated with coumermycin (100 ng/ml) in the absence of serum. Protein extracts (500 μg) were subjected to pull-down assay with Raf-1 RBD agarose beads followed by immunoblotting with anti-Ras antibody (a). Total Ras protein levels were assessed in 50 μg of protein extracts using anti-Ras antibody (b). Extracts from HT1080 cells incubated in serum-free medium or stimulated with 20% serum were used as negative and positive controls.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GyrB-PKRK296H
HT1080
Coumermycin
0
3
6
0
3
6
- Serum
+ Serum
a
b
GTP-Ras
Ras
Lane
1
2
3
4
5
6
7
8

Figure 21

2.3 Functional interaction between PKR and PI3K pathway

Since PKR possesses inhibitory effect on translation and proliferaion, induction of PI3K pathway, which has positive effect on translation and proliferation, was intriguing. To address the functional interaction between the two pathways, the effect of PI3K activation by PKR on translation and apoptosis was assessed.

2.3.1 PKR activation does not disrupt 4E-BP1 interaction with eIF4E.

Sequential phosphorylation of 4E-BP1 at Thr37/46, Thr70 and Ser65 is required for its dissociation from eIF4E in response to mitogenic signaling (16). To examine the physiological relevance of 4E-BP1 phosphorylation in cells with activated GyrB-PKR, we assessed the interaction of eIF4E with 4E-BP1 in binding assays to the cap analogue (252). We found that activation of GyrB-PKR by coumermycin did not cause the dissociation of 4E-BP1 from eIF4E (Fig. 22, panel a, lane 2) despite the induction of 4E-BP1 phosphorylation as described above. Treatment of GyrB-PKR-expressing cells with rapamycin led to a higher amount of eIF4E/4E-BP1 complex formation (panel a, lane 3) as previously demonstrated (268,269) indicating that HT1080 cells do not contain lesions that affect the eIF4E pathway. When the GyrB-PKRK296H-expressing cells were used, we found that eIF4E/4E-BP1 interaction was also unaffected by coumermycin treatment (panel a, lanes 5). We observed, however, that the background binding of 4E-BP1 to eIF4E was higher in GyrB-PKRK296H than in GyrB-PKR-expressing cells (compare lane 4 to 2). It is not immediately clear what caused these differences in 4E-BP1/eIF4E complex formation between the two cell lines. One possibility is that the higher background phosphorylation of Ser65 in GyrB-PKR than in GyrB-PKRK296H-expressing cells (Fig. 15A, panel b) caused the decrease in

eIF4E/4E-BP1 association since previous reports indicated that Ser65 phosphorylation may play a role in the dissociation of eIF4E and 4E-BP1 (270,271).

From the cap-binding assays we also observed that phosphorylated eIF2α at Ser51 was part of the eIF4E/4E-BP1 complex, whose presence in the complex was enhanced after GyrB-PKR activation (Fig. 22, panel c). This was an unexpected finding, which we further pursued by immunofluorescence microscopy. We examined the localization of eIF4E and 4E-BP1 before and after activation of GyrB-PKR by coumermycin. We observed the formation of distinct cytoplasmic foci upon activation of GyrB-PKR, which consisted of eIF4E and 4E-BP1 (Fig. 23A) as well as phosphorylated eIF2α (Fig. 23B). We also assessed the localization of eIF4E with eIF4G in cells treated with coumermycin and observed that eIF4E co-localizes with eIF4G in the distinct cytoplasmic foci (Fig. 24). The cytoplasmic granules were not detectable in coumermycin treated GyrB-PKRK296H-expressing cells (Fig. 25A and B) suggesting that their formation is dependent on the catalytic activity of PKR.

Collectively, these data show that activation of GyrB-PKR does not cause the dissociation of 4E-BP1 from eIF4E. In fact, it appears that induction of eIF2α phosphorylation by GyrB-PKR results in the formation of cytoplasmic granules consisting of eIF4E, 4E-BP1 and eIF4G.

Figure 22. The effect of PKR-mediated 4E-BP1 phosphorylation on eIF4E/4E-BP1 association

Serum-deprived GyrB-PKR and GyrB-PKRK296H-expressing cells were left untreated or treated with either coumermycin (100 ng/ml) or rapamycin (20 ng/ml) for 6 hours in the absence of serum. Cap binding assay was performed to assess the eIF4E/4E-BP1 interaction. Protein extracts (250 μg) were subjected to 7-methyl GTP-sepharose chromatography m^7GTP-agarose resins, and immunoblotting with antibodies against 4E-BP1 (a), eIF4E (b) or eIF2α-pSer51 (c).

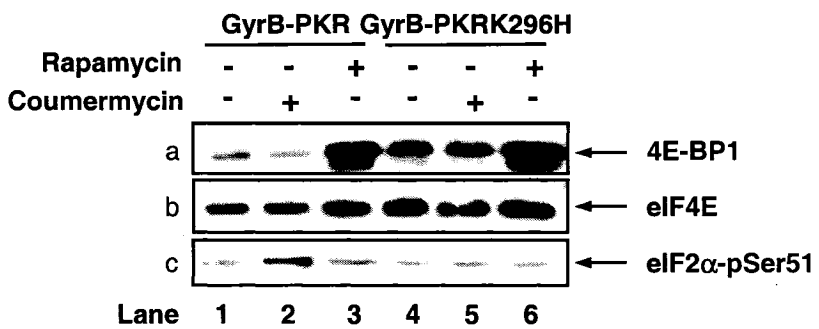

GyrB-PKR
GyrB-PKRK296H
Rapamycin - - + - - +
Coumermycin - + - - + -
a
b
c
4E-BP1
eIF4E
eIF2α-pSer51
Lane 1 2 3 4 5 6

Figure 22

Figure 23. Activation of PKR results in formation of stress granules containing eIF4E, 4E-BP1 and phosphor-eIF2α.

HT1080/GyrB-PKR cells were grown on 22-mm coverslips, left untreated or treated with coumermycin for 6 hours and subjected to immunofluorescence analysis.

A. Cells were immunostained with anti-eIF4E and anti-4E-BP1 antibodies.

B. Cells were subjected to immunostaining with anti-eIF4E and phosphospecific antibody against eIF2α-pSer51.

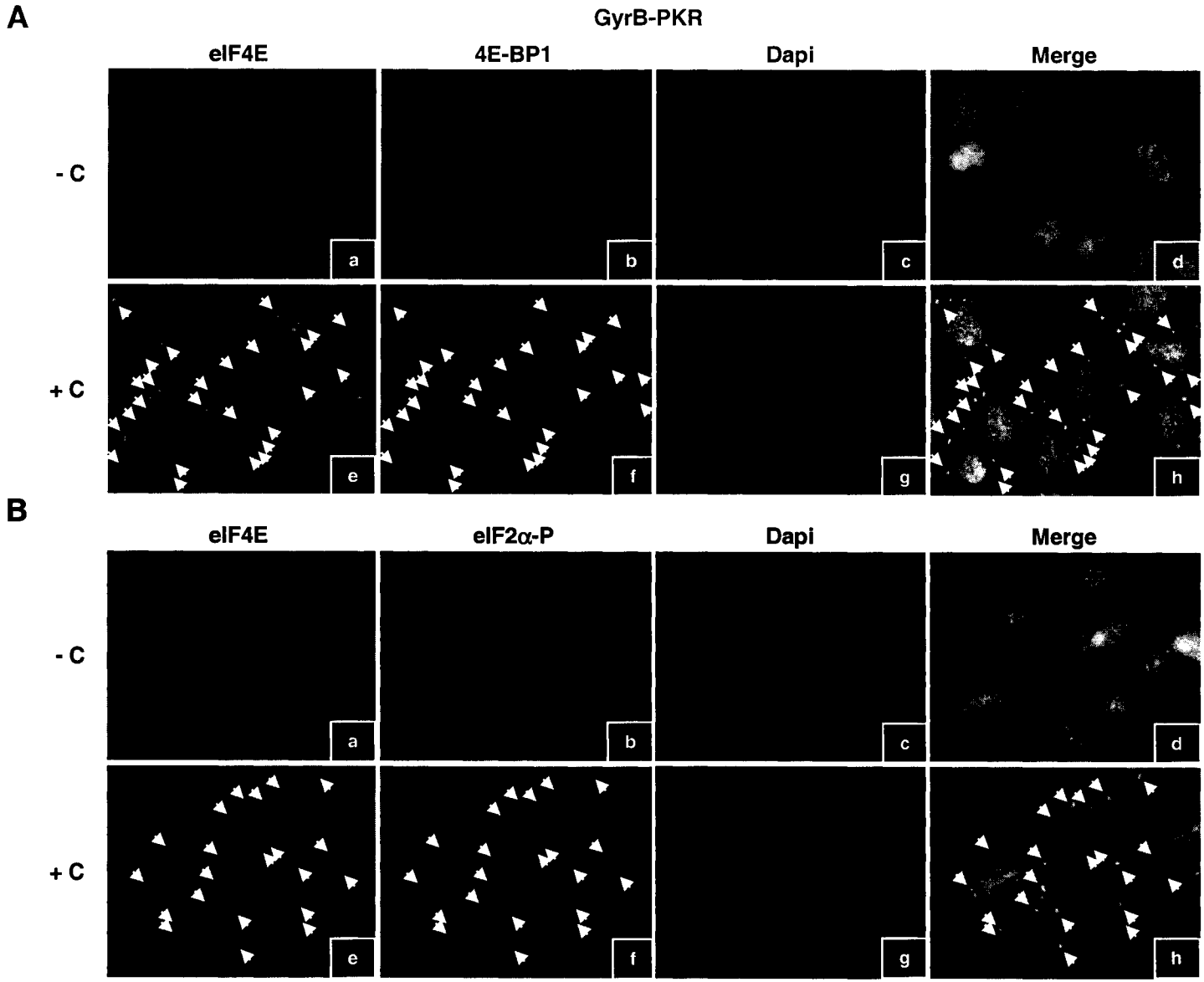

A
GyrB-PKR
eIF4E
4E-BP1
Dapi
Merge
- C
+ C
a
b
c
d
e
f
g
h
B
eIF4E
eIF2α-P
Dapi
Merge
- C
+ C
a
b
c
d
e
f
g
h

Figure 23

Figure 24. eIF4GI co-localizes with eIF4E in stress granules.

HT1080 cells expressing GyrB-PKR were grown on 22-mm coverslips, left untreated or treated with coumermycin for 6 hours and subjected to immunofluorescence analysis with anti-eIF4E and anti-eIF4G antibodies.

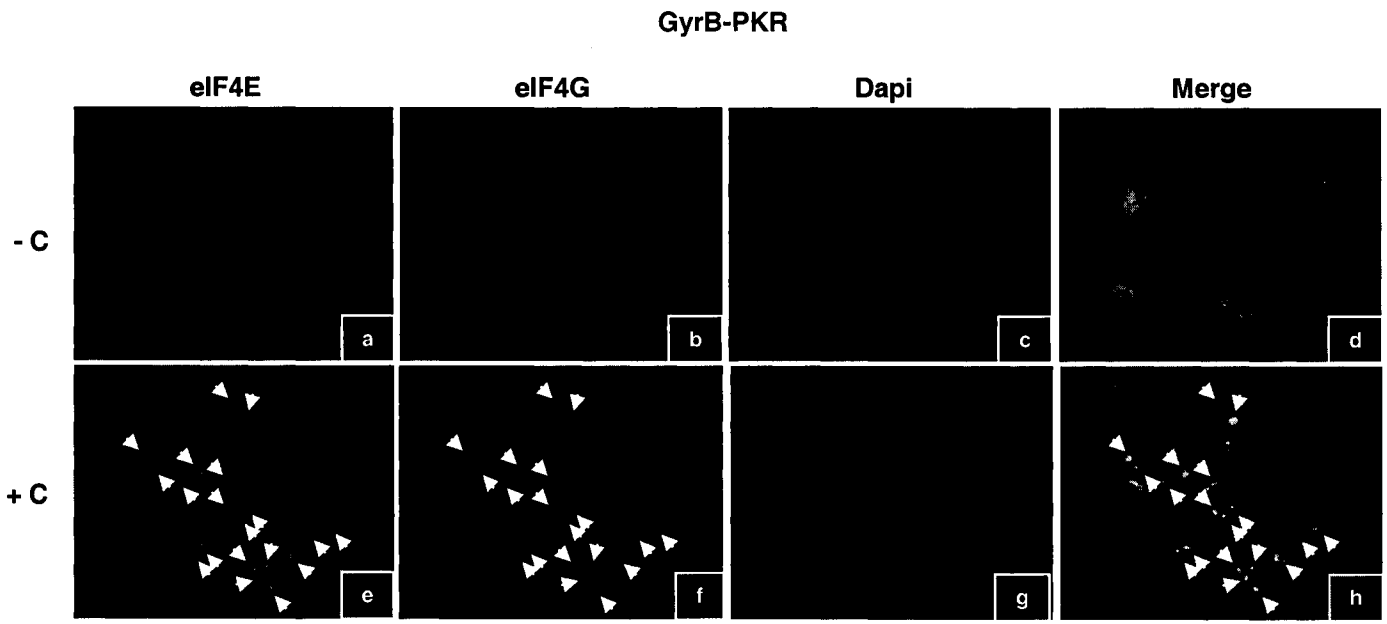


Figure 24

Figure 25. Coumermycin treatment of GyrB-PKRK296H does not induce the formation of stress granules and the colocalization of eIF4E and 4E-BP1.

HT1080/GyrB-PKRK296H cells were grown on 22-mm coverslips, left untreated or treated with coumermycin for 6 hours and subjected to immunofluorescence analysis.

A. Cells were immunostained with anti-eIF4E and anti-4E-BP1 antibodies.

B. Cells were subjected to immunostaining with anti-eIF4E and phosphospecific antibody against eIF2α-pSer51.

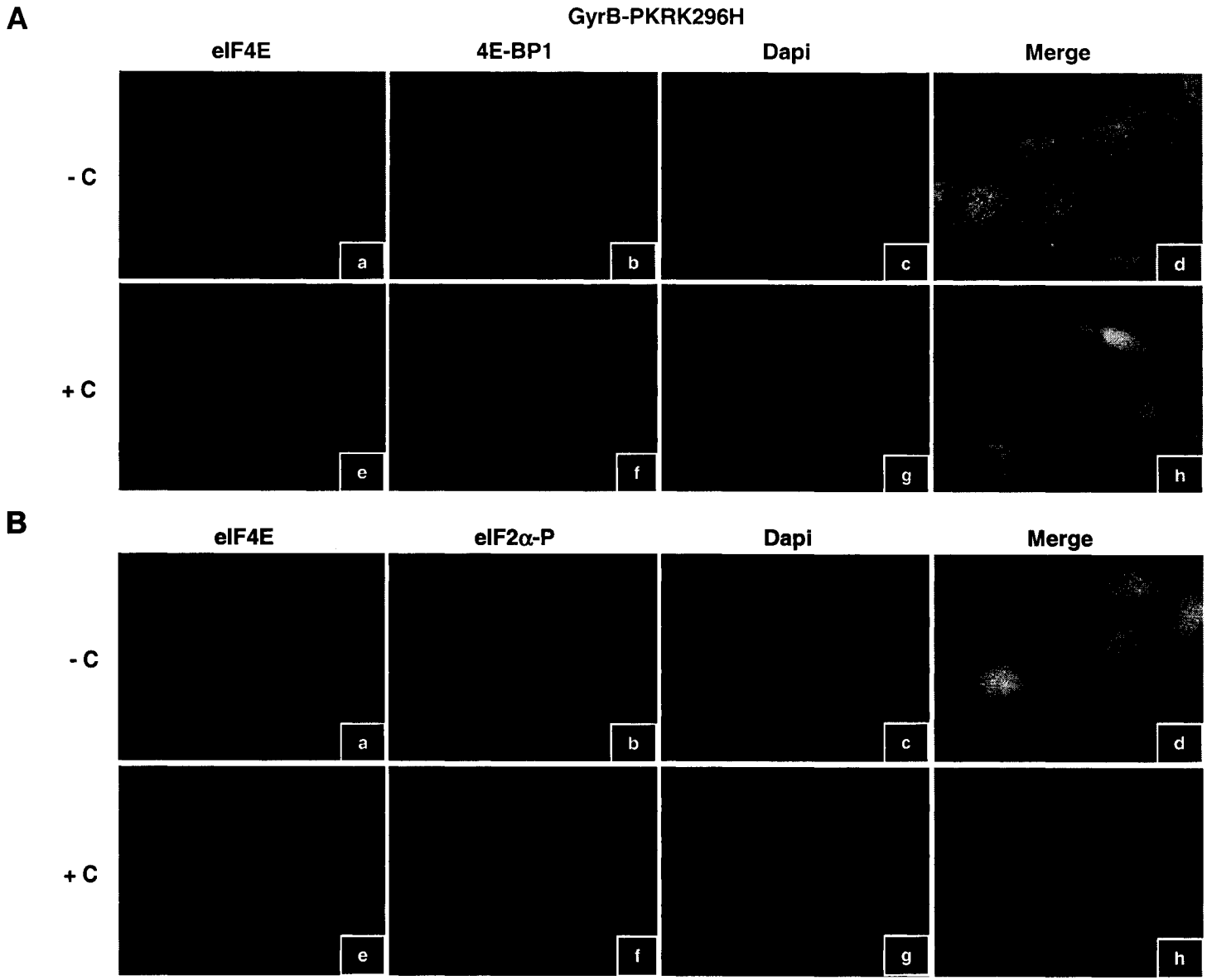

A
GyrB-PKRK296H
eIF4E
4E-BP1
Dapi
Merge
- C
+ C
a
b
c
d
e
f
g
h
B
eIF4E
eIF2α-P
Dapi
Merge
- C
+ C
a
b
c
d
e
f
g
h

Figure 25

2.3.2 Induction of 4E-BP1 phosphorylation upon PKR activation can not bypass the translation inhibitory effects of eIF2α phosphorylation.

We further examined the role of the PI3K pathway in inhibition of protein synthesis by activated GyrB-PKR. The levels of global protein synthesis were assessed by ^{35}S-methionine labeling of GyrB-PKR-expressing cells treated with LY294002 and/or rapamycin in the absence or presence of coumermycin (Fig. 26A). We found that in cells with latent GyrB-PKR (i.e. without coumermycin treatment), inhibition of PI3K by LY294002 resulted in ~40% decrease of global protein synthesis. In cells with activated GyrB-PKR (i.e. coumermycin-treated cells), we measured a ~50% inhibition of cellular protein synthesis, which was further reduced by ~40% upon inhibition of PI3K by LY294002. Treatment with LY294002 resulted in the same degree of protein synthesis inhibition in cells expressing GyrB-PKRK296H either in the absence or presence of coumermycin. Given that PI3K inhibition by LY294002 did not further enhance eIF2α phosphorylation by GyrB-PKR (Fig. 15B) this indicates that the PI3K pathway counterbalances the inhibitory effect of PKR on protein synthesis without interfering with eIF2α phosphorylation. Treatment with rapamycin did not further enhance global protein synthesis inhibition by activated GyrB-PKR (Fig. 26A).

Interestingly, we noticed that rapamycin treatment did not inhibit the overall levels of protein synthesis in cells with latent GyrB-PKR indicating that inhibition of the FRAP/mTOR pathway does not affect global protein synthesis. This is consistent with previous observations (272) and our data that rapamycin induces qualitative rather than quantitative effects on protein synthesis. Since eIF4E interaction with 4E-BP1 is higher in rapamycin-treated than in coumermycin-treated GyrB-PKR cells (Fig. 22,

compare lane 3 with 2), eIF4E/4E-BP1 complex formation can not account for the global inhibition of protein synthesis by activated GyrB-PKR.

2.3.3 Overexpression of eIF4E cannot bypass inhibition of protein synthesis by PKR-mediated phosphorylation of eIF2α.

To further address the role of the eIF4E/4E-BP1 interaction in translational control by eIF2α phosphorylation, eIF4E was overexpressed in cells containing GyrB-PKR and tested the regulation of global protein synthesis in these cells (Fig. 26B, panel a). Activation of GyrB-PKR by coumermycin led to the induction of eIF2α phosphorylation at equal levels in HA-eIF4E-expressing cells and control cells (panel b) indicating that eIF4E does not interfere with eIF2α phosphorylation by the activated kinase. We further observed that the protein levels of both endogenous and HA-eIF4E were resistant to the protein synthesis inhibitory effects of eIF2α phosphorylation (panel a). When global protein synthesis was assessed, we found that HA-eIF4E induced the synthesis of certain polypeptides suggesting that eIF4E overexpression was functional and capable of stimulating cap-dependent translation of specific mRNAs (Fig. 26C, compare panels a and b). However, activation of GyrB-PKR by coumermycin dramatically decreased protein synthesis even of the polypeptides that were induced by the overexpression of HA-eIF4E (compare panels c and d). Thus, protein synthesis inhibition by eIF2α phosphorylation exerts a dominant effect over the stimulation of cap-dependent translation by eIF4E.

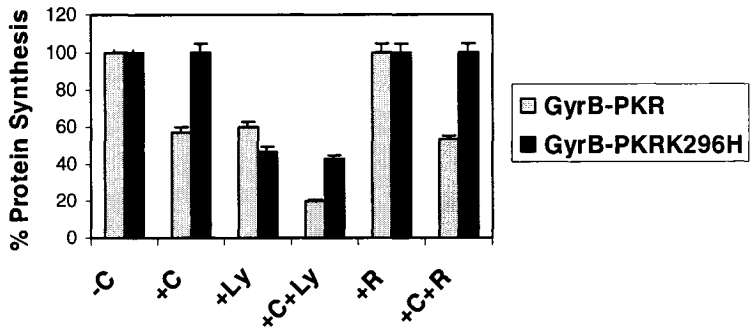
Figure 26. Regulation of cap-dependent translation in GyrB-PKR cells

A. HT1080 cells expressing either GyrB-PKR (open bars) or GyrB-PKRK296H (closed bars) were incubated in media lacking methionine and supplemented with 10% dialyzed serum for 1 hour. Cells were then treated with LY294002 (20 μM) or rapamycin (20 nM) for 1 hour followed by the addition of coumermycin (100 ng/ml) for additional 4 hours. After these incubations, ^{35}S-methionine (100 μCi per 10^6 cells) was added to cells for further 2 hours followed by the quantification of radioactive TCA precipitates. Values represent the average of three separate experiments performed in triplicates. (C: coumermycin, R: rapamycin, LY: LY294002).

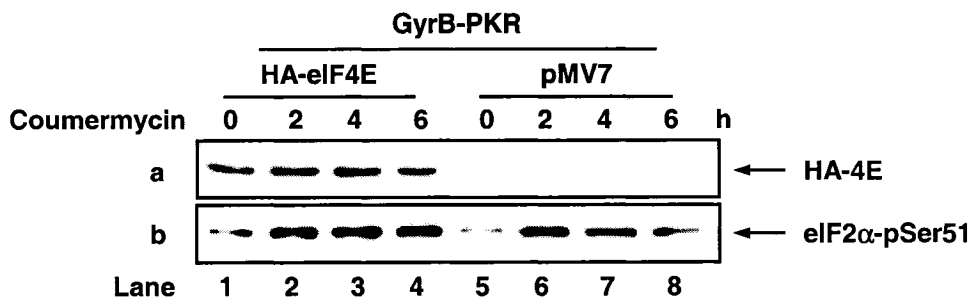
B. HT1080/GyrB-PKR cells stably transfected with either pMV7 vector (control) or pMV7/HA-eIF4E were left untreated or treated with coumermycin (100 ng/ml) for the indicated time points. Protein extracts (50 μg) were subjected to immunoblotting with anti-eIF4E antibody (a) or phosphospecific antibody against eIF2α-pSer51 (b).

C. Control cells or cells overexpressing HA-eIF4E were incubated in methionine-free medium supplemented with 10% dialyzed serum for 2 hours, left untreated or treated with coumermycin (100 ng/ml) for 4 hours and followed by ^{35}S-methionine labeling (100 μCi per 10^6 cells) for additional 2 hours. Protein extracts (80 μg) were subjected to 2D gel electrophoresis and radioactive bands were visualized by autoradiography.

A



B



C

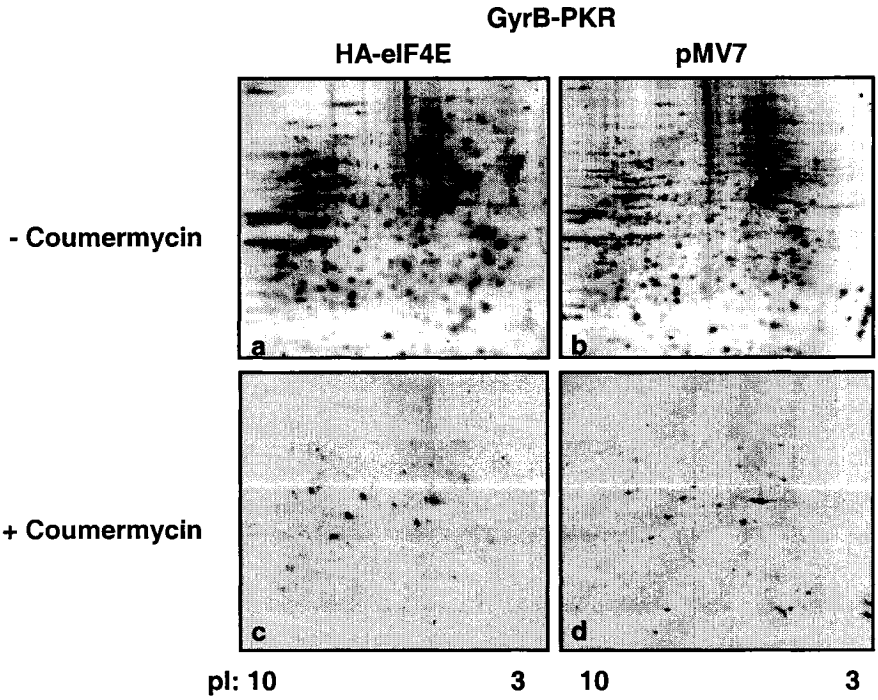


Figure 26

2.3.4 The PI3K pathway antagonizes GyrB-PKR-mediated cell death.

We previously demonstrated that activation of GyrB-PKR leads to the induction of cell death as a result of eIF2α phosphorylation (260). As such, we wished to examine the role of the PI3K pathway in PKR-mediated cell death. To this end, we assessed the apoptotic function of GyrB-PKR in the presence of LY294002 or rapamycin (Fig. 27A). In the absence of coumermycin, treatment of GyrB-PKR cells with either rapamycin or LY294002 did not induce cell death. When cells were treated with coumermycin, activation of GyrB-PKR led to a significant induction of cell death as previously described (260). The presence of rapamycin, however, did not significantly affect cell death induced by GyrB-PKR as opposed to treatment with LY294002, which resulted in a considerable (~50%) increase in GyrB-PKR-mediated cell death. These results demonstrated that activation of PI3K pathway antagonizes cell death induced by activated PKR.

Given that PKR possesses antiproliferative activities, cells devoid of PKR activity (PKR knockout: $PKR^{-/-}$) are expected to exhibit a higher rate of growth, if not the same, than their wild-type counterparts ($PKR^{+/+}$). However, our observations indicate that mouse embryonic fibroblasts (MEFs) from a catalytic knockout of PKR (198) grow slower than $PKR^{+/+}$ MEFs in culture. To further investigate these observations, the proliferation rate of these cells was assessed under normal conditions (Figure 27B). The higher rate of proliferation in $PKR^{+/+}$ MEFs, correlates well with the ability of PKR to activate the PI3K pathway, which exerts a positive effect on cell growth and proliferation. This indicates that PKR may possess dual activity; i.e. under physiological conditions PKR may exhibit a stimulatory effect on growth and

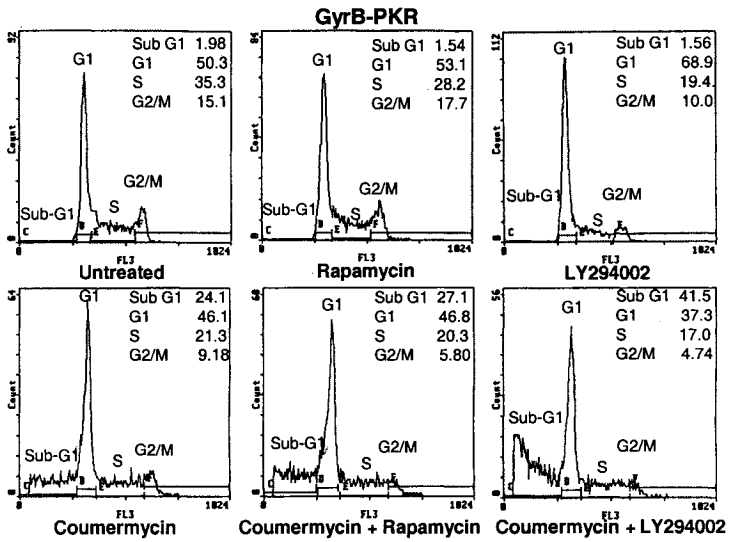
proliferation, whereas under stress conditions it serves as a negative regulator by inhibiting protein synthesis and inducing apoptosis.

Figure 27. Control of PKR-mediated apoptosis by PI3K pathway

A. HT1080 cells expressing GyrB-PKR were treated with coumermycin (100 ng/ml) in the absence or presence of either LY294002 (20 μM) or rapamycin (20 nM) for 24 hours. Cells were harvested, fixed in ethanol, stained with propidium iodide and subjected to flow cytometry analysis. The percentage of apoptotic cells or cells in various phases of the cell cycle is indicated. Data represent one of four reproducible experiments.

B. $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were grown for 5 days and the proliferation rate was assessed every day by adding the CellTiter 96® AQ_{ueous} One Solution Reagent directly to cultured cells and reading the absorbance at 490 nm.

A


GyrB-PKR
Sub G1 1.98
G1 50.3
S 35.3
G2/M 15.1
Untreated
Sub G1 1.54
G1 53.1
S 28.2
G2/M 17.7
Rapamycin
Sub G1 1.56
G1 68.9
S 19.4
G2/M 10.0
LY294002
Sub G1 24.1
G1 46.1
S 21.3
G2/M 9.18
Coumermycin
Sub G1 27.1
G1 46.8
S 20.3
G2/M 5.80
Coumermycin + Rapamycin
Sub G1 41.5
G1 37.3
S 17.0
G2/M 4.74
Coumermycin + LY294002

B

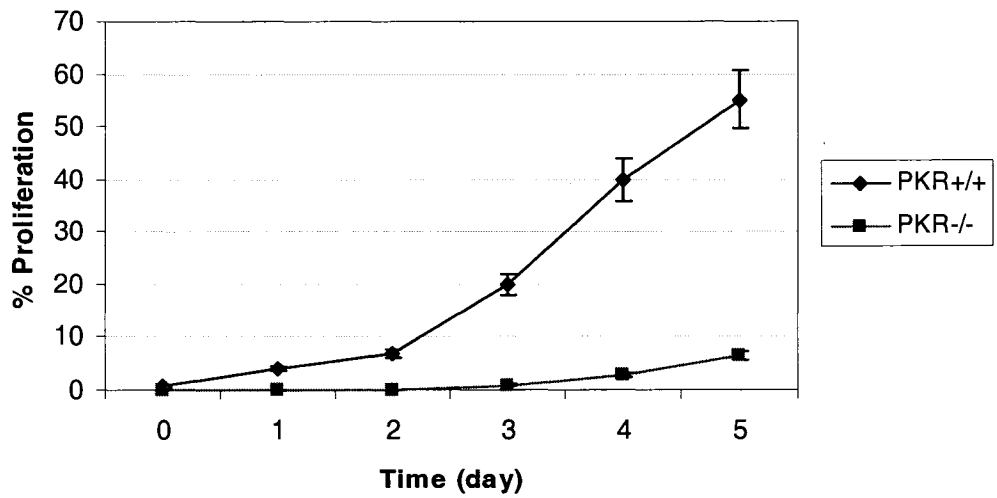

% Proliferation
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (day)
PKR+/+
PKR-/-

Figure 27

2.4 Biological relevance of PI3K activation by PKR

To better address the physiological relevance of these findings, the role of endogenous PKR in the induction of 4E-BP1 phosphorylation was examined in response cytokines. Recent findings have provided evidence for the ability of interferons (IFNs) to induce the phosphorylation of 4E-BP1 through the activation of PKB/Akt (273). To address the possible role of PKR in this process, we assessed 4E-BP1 phosphorylation in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (198). These $PKR^{-/-}$ MEFs are devoid of the C-terminus of PKR and its kinase activity. They only express the N-terminal domain of PKR which still retain functions attributed to this region. However, in this experiment we would like to study the role of kinase activity of PKR and thus these cells would serve the purpose. We found that IFNγ treatment resulted in a higher induction of 4E-BP1 phosphorylation at Thr37/46, Ser 65 and Thr70 in $PKR^{+/+}$ than in $PKR^{-/-}$ MEFs (Fig. 28, compare lanes 1-4 to 5-8).

These findings further demonstrate the role of PKR in 4E-BP1 phosphorylation through the activation of the PI3K pathway and support the notion that PKR may play a role in signaling pathways in response to cytokines.

Figure 28. PKR is involved in activation of the PI3K pathway in response to cytokines.

$PKR^{+/+}$ and $PKR^{-/-}$ MEFs were maintained in the absence of serum for 16 hours followed by 100 IU/ml of mouse IFNγ treatment for the indicated time points. Protein extracts (50 μg) were subjected to immunoblotting with phosphospecific antibodies against 4E-BP1-pThr37/46 (a), -pSer65 (b), -pThr70 (c), anti-4E-BP1 rabbit polyclonal antibody (d).

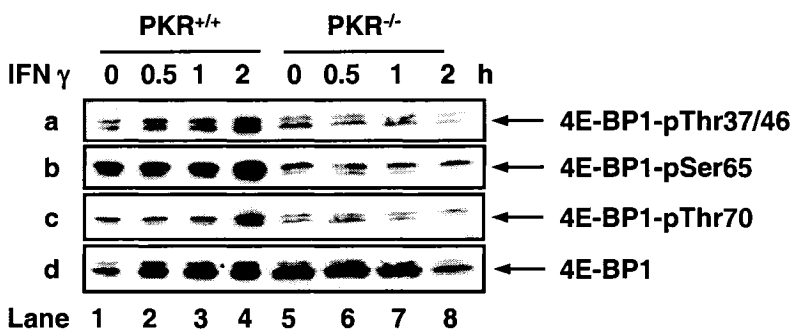

PKR+/+
PKR-/-
IFN γ 0 0.5 1 2 0 0.5 1 2 h
a
b
c
d
4E-BP1-pThr37/46
4E-BP1-pSer65
4E-BP1-pThr70
4E-BP1
Lane 1 2 3 4 5 6 7 8

Figure 28

2.5 Role of other eIF2α kinases in activation of PI3K pathway

While all eIF2α kinases share a homologous kinase domain, GyrB-PKR not only represents the kinase activity of PKR, but also it mimics the activation of other eIF2α kinases. Therefore, it is highly possible that activation of other eIF2α kinases also result in activation of PI3K. Accordingly, induction of ER stress in $PERK^{+/+}$ MEFs resulted in induction of PKB/Akt phosphorylation which was not observed in $PERK^{-/-}$ MEFs (Fig. 29). Treatment of cells with thapsigargin induces ER stress through depletion of lumenal calcium stores. Activation of PERK in wild-type cells resulted in induction of PKB/Akt and eIF2α phosphorylation (panels a and c), whereas in cells devoid of PERK this induction was abolished.

These data indicate a possible role of all eIF2α kinases in induction of the PI3K pathway. Further studies, however, is required to address this issue. On the other hand, since a cross-talk between PERK and PKR in response to viral infection has been demonstrated (274), it is reasonable to speculate that the two kinases also cooperate in induction of the PI3K pathway.

Figure 29. Activation of PERK in response to ER stress results in activation of the PI3K pathway.

$PERK^{+/+}$ and $PERK^{-/-}$ MEFs were treated with thapsigargin (1 μM) in the presence of serum for the indicated time points. Protein extracts (50 μg) were subjected to immunoblotting with phosphospecific antibody against PKB/Akt-pSer473 (a), anti-PKB/Akt antibody (b), phosphospecific antibody against eIF2α-pSer51 (c) or anti-eIF2α antibody (d).

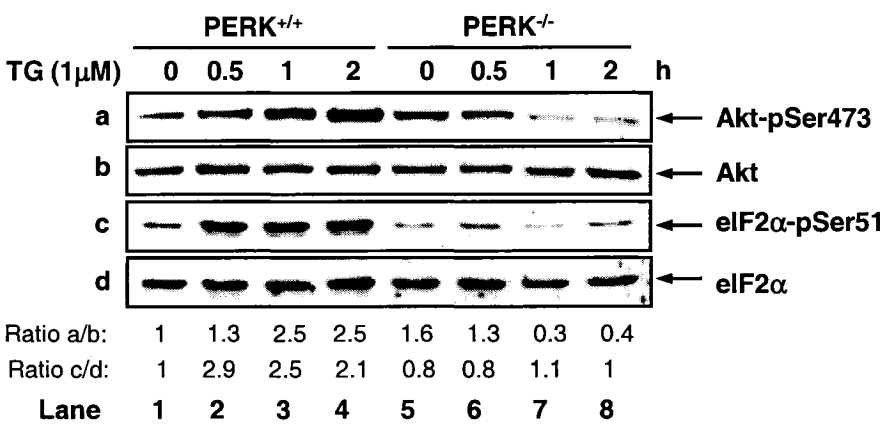

PERK+/+
PERK-/-
TG (1μM) 0 0.5 1 2 0 0.5 1 2 h
a
b
c
d
Akt-pSer473
Akt
eIF2α-pSer51
eIF2α
Ratio a/b: 1 1.3 2.5 2.5 1.6 1.3 0.3 0.4
Ratio c/d: 1 2.9 2.5 2.1 0.8 0.8 1.1 1
Lane 1 2 3 4 5 6 7 8

Figure 29

3. Human Papillomaviruses and PKR

Development of cervical cancer has been demonstrated to be strongly associated with HPV infection. Therefore, the use of IFNs as antiviral agents for therapy against cervical cancer has been under investigation. The efficiency of this method, however, depends on the HPV type and the immune system of the patients. The molecular basis of this resistance is not fully understood; therefore a better knowledge of the molecular mechanism of IFNs and their inducible genes in HPV infected cells may contribute to the development of strategies to combat HPV-associated diseases including cervical cancer. In this regard, our laboratory has focused on studying HPV oncoproteins and their functional interaction with interferon signaling pathway.

The HPV E6 oncoprotein contributes in virus-induced pathogenicity through multiple mechanisms including the inhibition of apoptosis and the blockade of IFN action. Therefore, it was of interest to investigate functional interaction of HPV E6 with PKR which is a mediator of anti-viral and anti-proliferative activities of type 1 IFNs.

3.1 Regulation of HPV-18 E6 protein synthesis by PKR

Activation of PKR results in inhibition of global protein synthesis through phosphorylation of eIF2α. This leads to decreased expression of cellular and viral proteins. In this regard we investigated the regulation of HPV E6 oncoprotein by PKR.

3.1.1 Inhibition of HPV-18 E6 protein synthesis by IFNα

Human epithelial-like fibrosarcoma HT1080 cell line carrying a FLAG-tagged form of the high-risk HPV-18 E6 were established (246). When these cells were treated with IFNα, we noticed a decrease in FLAG-18 E6 expression levels compared to

untreated cells (Fig. 30A, panel a, compare lane 3 with 1). Inhibition of FLAG-18 E6 expression, however, was not observed in cells treated with IFNγ (lane 5). Since E6 protein stability is controlled by the 26S proteasome (275), we tested whether inhibition of FLAG-18 E6 by IFNα involved the proteasome-dependent degradation of the viral protein. Incubation of HT1080 cells with the proteasome inhibitor MG-132 equally increased FLAG-18 E6 levels in untreated as well as in IFNα or IFNγ-treated cells (panel a, lanes 2, 4 and 6). MG-132, however, failed to completely recover FLAG-18 E6 levels in IFNα-treated cells (lane 4) as opposed to untreated (lane 2) or IFNγ-treated cells (lane 6), suggesting that the inhibitory effect by IFNα is not exclusively based on the proteasome-mediated degradation of the viral protein. Since FLAG-18 E6 expression in HT1080 cells was mediated by a heterologous promoter, whose activity is not affected by IFNs (246), our findings implied a translational regulation of FLAG-18 E6 by IFNα. This notion was supported by our observation that downregulation of FLAG-18 E6 was associated with an induction of the eIF2α kinase PKR protein in IFNα treated cells (panel b, lanes 3 and 4).

3.1.2 E6 protein synthesis is controlled by eIF2α phosphorylation.

To establish a direct link between E6 protein synthesis and PKR and to study their functional interaction, we utilized the GyrB-PKR system. Given that the GyrB-PKR system was functional in HT1080 cells, we further established cells expressing FLAG-18 E6 together with either GyrB-PKR or GyrB-PKRK296H. GyrB-PKR/FLAG-11 E6 expressing cells were also established to serve as a negative control for oncogenic properties of 18 E6. Cells were stably transfected with GyrB-PKR or GyrB-PKRK296H cDNA in pSG5 vector together with either FLAG-18 E6 or FLAG-11 E6 in

pcDNA3.1/Zeo vector at a ratio 5:1. Monoclonal populations with equal levels of GyrB-PKR and GyrB-PKRK296H, as well as 18 and 11 E6 were selected.

Treatment of these cells with coumermycin resulted in a rapid repression of FLAG-18 E6 levels in GyrB-PKR cells but not in GyrB-PKRK296H cells (Fig. 30B, panel a, compare lanes 1-4 with 5-8). Northern blot analysis showed that 18 E6 mRNA levels were not diminished by coumermycin treatment of GyrB-PKR or GyrB-PKRK296H cells (Fig. 30C). Collectively, these data suggested that E6 expression is translationally suppressed by PKR-mediated eIF2α phosphorylation.

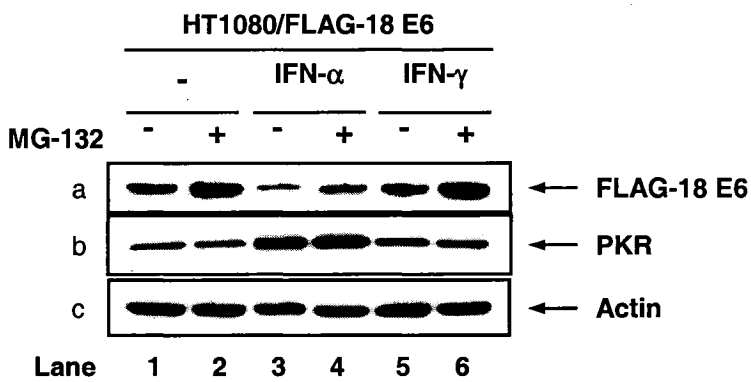
Figure 30. E6 protein levels are down regulated upon treatment with IFN-α.

A. HT1080 cells expressing FLAG-tagged HPV 18 E6 were left untreated or treated with either IFNα or IFNγ (100 IU/ml) for 18 hours followed by MG-132 treatment (40 μM) for additional 2 hours. Protein extracts (50 μg) were subjected to immunoblot analysis with anti-FLAG antibody (a), anti-human PKR antibody (clone F9) (b) or anti-actin antibody (c).

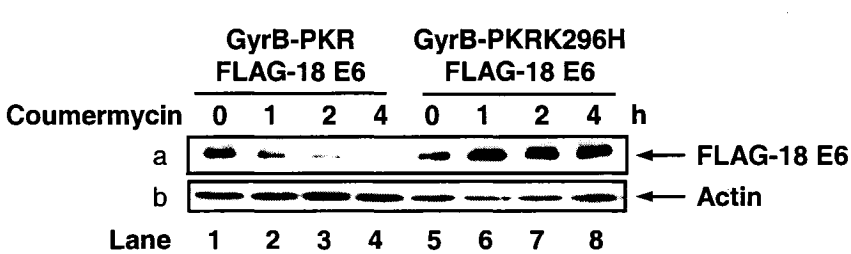
B. HT1080 cells expressing FLAG-18 E6 together with either GyrB-PKR or GyrB-PKRK296H were incubated with 100 ng/ml coumermycin for up to 4 hours. Protein extracts (50 μg) were subjected to immunoblot analysis with anti-FLAG antibody (a) or anti-actin antibody (b).

C. HT1080 cells expressing FLAG-18 E6 and either GyrB-PKR or GyrB-PKRK296H treated with coumermycin (100 ng/ml) were subjected to Northern blot analysis using 10 μg of total RNA followed by hybridization with either [^{32}P]-labeled 18 E6 cDNA (a) or [^{32}P]-labeled glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA (b). The radioactive bands were quantified by densitometry and the ratio of FLAG-18 E6 to GAPDH is shown.

A



B



C

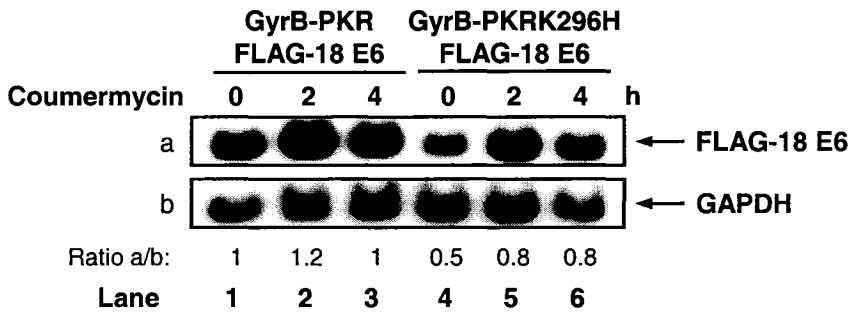


Figure 30

3.2 Modulation of PKR functions by HPV-18 E6

Over the course of time, viruses have evolved in such a way to bypass the cellular antiviral response pathway including PKR. The vaccinia virus E3L and K3L proteins, NS1 protein of influenza virus, HSV $\gamma_1 34.5$ gene product and adenovirus VA_I RNA are examples of viral products that antagonize PKR function. In this regard, it was intriguing to determine the effect of HPV encoded proteins such as E6 oncoprotein on PKR functions.

3.2.1 HPV-18 E6 decreases phosphorylation levels of eIF2α.

To address the mechanism of translational control by E6, we assessed the phosphorylation levels of eIF2α in GyrB-PKR cells lacking or expressing FLAG-18 E6 (Fig. 31A). Immunoblot analysis with phosphospecific antibodies against serine 51 of eIF2α showed that phosphorylation was significantly reduced in coumermycin-treated cells expressing GyrB-PKR and FLAG-18 E6 compared to cells expressing GyrB-PKR only (panel b). We further verified this finding by testing eIF2α phosphorylation by isoelectric focusing (IEF), 2D gel electrophoresis and immunoblotting with phosphospecific antibody against eIF2α-pSer51 (Fig. 31B). We found that eIF2α phosphorylation was highly induced in GyrB-PKR cells after coumermycin treatment. The levels of phosphorylated eIF2α induced in coumermycin treated GyrB-PKR cells expressing FLAG-18 E6 were; however, lower than in GyrB-PKR cells lacking the viral oncoprotein. We also noticed that several species of phosphorylated eIF2α were recognized by the phosphospecific antibodies based on their migration to acidic pH upon coumermycin treatment. This data indicated that activation of GyrB-PKR leads to hyperphosphorylation of eIF2α at multiple sites including serine 51. It is noteworthy

that, although serine 51 is the only residue directly phosphorylated by PKR (276), hyperphosphorylation of eIF2α is indirect most probably due to the ability of GyrB-PKR to activate pathways leading to multiple phosphorylation of eIF2α. Nevertheless, the above data clearly demonstrated the inhibitory effect of FLAG-18 E6 on GyrB-PKR-mediated eIF2α phosphorylation. We also compared eIF2α phosphorylation in GyrB-PKR cells expressing either FLAG-18 E6 or FLAG-11 E6 (Fig. 31C). We found that the induction of eIF2α phosphorylation by activated GyrB-PKR was higher in cells expressing FLAG-11 E6 than in FLAG-18 E6 expressing cells (panel b, compare lanes 1-4 with 5-8) indicating a higher capacity of FLAG-18 E6 to inhibit eIF2α phosphorylation than FLAG-11 E6.

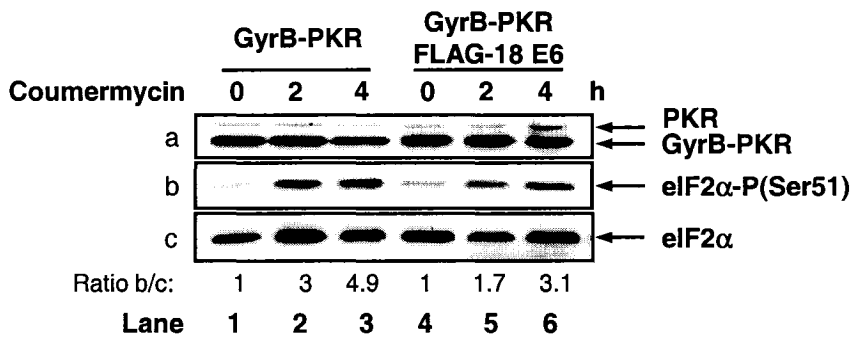
Figure 31. E6 impairs eIF2α phosphorylation in response to GyrB-PKR activation.

A. HT1080 cells expressing GyrB-PKR alone or in the presence of FLAG-18 E6 were induced by 100 ng/ml coumermycin for 2 or 4 hours. Protein extracts (50 μg) were subjected to immunoblot analyses with anti-human PKR antibody (clone E8) (a), phosphospecific antibody against eIF2α-pSer51 (b) or anti-eIF2α antibody (c). The slower migrating band recognized by the anti-PKR antibody (a) is the fusion GyrB-PKR protein, which is slightly smaller in size than endogenous PKR.

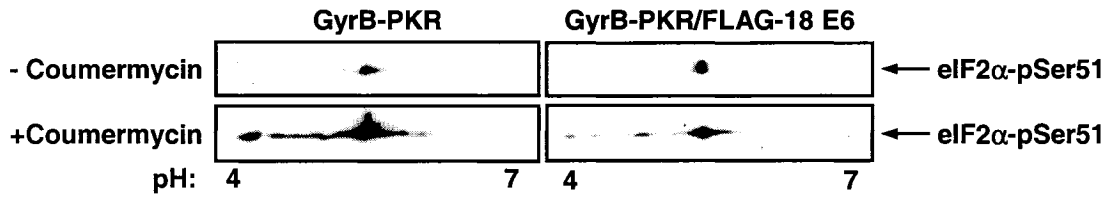
B. Detection of eIF2α phosphorylation by isoelectric focusing (IEF) and 2D gel electrophoresis. Protein extracts (100 μg) of untreated or coumermycin treated (4 hours; 100 ng/ml) HT1080 cells expressing GyrB-PKR in the absence or presence of FLAG-18 E6 were subjected to IEF and 2D gel electrophoresis. The phosphorylated forms of eIF2α were detected by immunoblotting with phosphospecific Ser51 eIF2α specific Ab.

C. HT1080 cells containing GyrB-PKR in the presence of either FLAG-18 E6 or FLAG-11 E6 were treated with 100 ng/ml coumermycin for the indicated times and subjected to immunoblotting analysis with anti-human PKR antibody (clone E8) (a), phosphospecific antibody against eIF2α-pSer51 (b) or anti-eIF2α antibody (c).

A



B



C

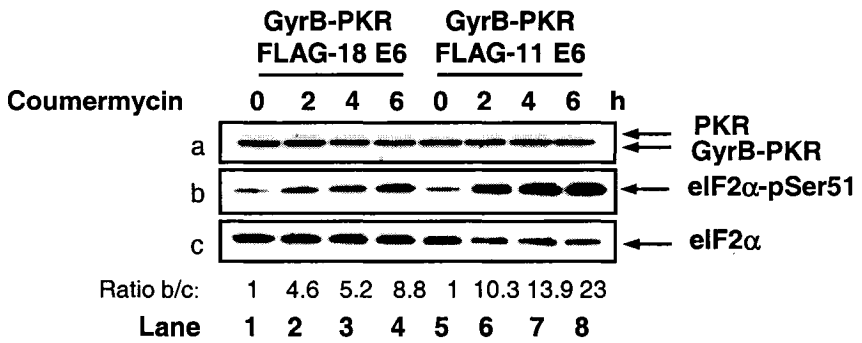


Figure 31

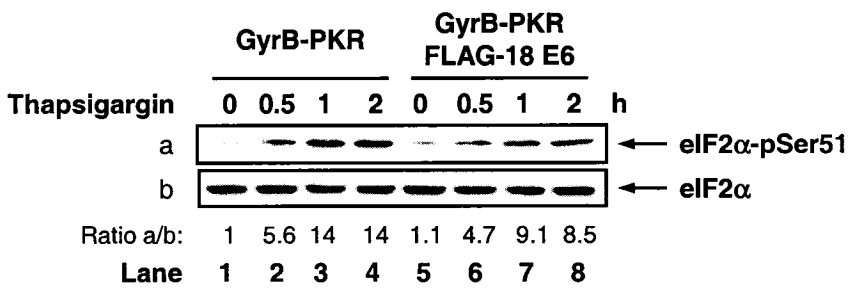
We next addressed the specificity of FLAG-18 E6 to inhibit eIF2α phosphorylation. If eIF2α phosphorylation was generally inhibited by E6, this should also take place in cells subjected to endoplasmic reticulum (ER) stress, which induces eIF2α phosphorylation through the activation of the PKR-like ER-resident kinase PERK (277). When cells expressing either GyrB-PKR alone or GyrB-PKR and FLAG-18 E6 were treated with the ER stress-inducer thapsigargin in the absence of coumermycin, induction of eIF2α phosphorylation was reduced in cells expressing the viral oncoprotein compared to cells lacking it (Fig. 32A, panel a, compare lane 2 with 6 and lane 3 with 7). It has been well established that induction of eIF2α phosphorylation in ER stressed cells leads to the expression of C/EBP homologous protein (CHOP), which is also known as growth arrest and DNA damage gene 153 (GADD153) (278,279). When CHOP/GADD153 was used as a marker for responses to eIF2α phosphorylation in thapsigargin-treated cells, we found that CHOP/GADD153 protein levels were more highly induced in cells with GyrB-PKR alone than in cells with GyrB-PKR and the viral oncoprotein (Fig. 32B, panel c, compare lane 2 with 6 and lane 3 with 7).

Taken together, these findings demonstrated that the ability of FLAG-18 E6 to impair eIF2α phosphorylation is not specific for the PKR pathway but can be seen in other eIF2α kinase pathways such as ER stress, which induces eIF2α phosphorylation through the activation of PERK.

Figure 32. E6 impairs eIF2α phosphorylation in response to ER stress.

HT1080 cells expressing GyrB-PKR alone or together with FLAG-18 E6 were treated with 1 μM thapsigargin for short (a and b) or long time periods (c and d). Protein extracts (50 μg) were used for immunoblot analysis with phosphospecific antibody against eIF2α-pSer51 (a), anti-eIF2α antibody (b), anti-CHOP antibody (c) or anti-actin antibody (d).

A


GyrB-PKR
GyrB-PKR FLAG-18 E6
Thapsigargin 0 0.5 1 2 0 0.5 1 2 h
a
b
eIF2α-pSer51
eIF2α
Ratio a/b: 1 5.6 14 14 1.1 4.7 9.1 8.5
Lane 1 2 3 4 5 6 7 8

B

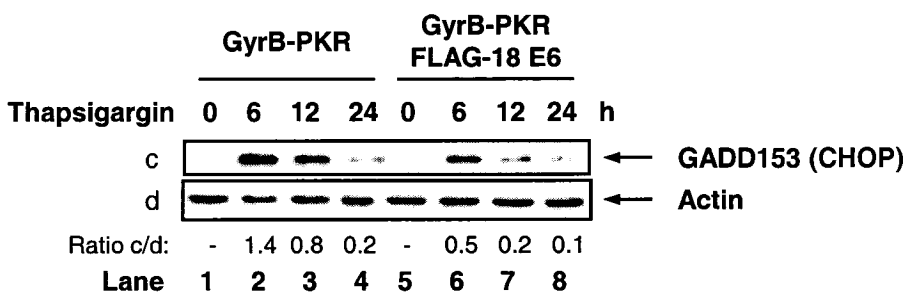

GyrB-PKR
GyrB-PKR FLAG-18 E6
Thapsigargin 0 6 12 24 0 6 12 24 h
c
d
GADD153 (CHOP)
Actin
Ratio c/d: - 1.4 0.8 0.2 - 0.5 0.2 0.1
Lane 1 2 3 4 5 6 7 8

Figure 32

3.2.2 HPV 18 E6 promotes eIF2α dephosphorylation by recruiting GADD34 and PP1.

The growth arrest and DNA damage gene product 34 (GADD34) is a stress-inducible regulatory subunit of a holophosphatase complex, which contains the catalytic subunit of protein phosphatase 1 (PP1c) and specifically promotes the dephosphorylation of eIF2α in cells subjected to ER stress (245,280,281). Because eIF2α phosphorylation is reduced in ER stressed cells expressing FLAG-18 E6, we hypothesized that E6 might play a role in eIF2α dephosphorylation via GADD34/PP1. To test this hypothesis, we first tested for a possible interaction between E6 and GADD34 or PP1 (Fig. 33A). To this end, we used either FLAG-GADD34 or different mutants of FLAG-GADD34 with a deletion of the last 121 aa in the C-terminus (1-553), deletion of the first 179 aa in N-terminus (180-674) or substitutions of the highly conserved KVRF sequence involved in PP1-binding (KARA mutant) (245). FLAG-GADD34 proteins were transiently expressed in HeLa cells, and binding to E6 was assessed in pull down assays with a GST-18 E6 fusion protein (246). Immunoblot analysis with anti-FLAG antibody revealed the interaction between E6 and the FLAG-GADD34 proteins independently of the type of mutation (panel a). The interaction of E6 with GADD34 was further tested in transient transfection assays in HeLa cells. That is, co-expressed FLAG-GADD34 and FLAG-E6 proteins were subjected to immunoprecipitation with anti-GADD34 antibody followed by immunoblotting with anti-FLAG antibody (Fig. 33B). We found that both E6 subtypes were co-immunoprecipitated with GADD34 (lanes 1 and 2), thus confirming the interaction.

We also examined the ability of E6 to interact with PP1. To this end, FLAG-E6 proteins transiently expressed in HeLa cells were immunoprecipitated with anti-FLAG antibody followed by immunoblotting with anti-PP1 antibody (Fig. 33C). We observed that a higher amount of endogenous PP1 was bound to FLAG-18 E6 than FLAG-11 E6 (compare lanes 2 and 3). It is noteworthy that in this experiment the amount of transfected FLAG-18 E6 DNA was 5 fold higher than FLAG-11 E6 DNA in order to achieve equal levels of expression of the two viral proteins. This data suggested that PP1 interacts more efficiently with FLAG-18 E6 than FLAG-11 E6. To verify the significance of the interaction, we performed an eIF2α-dephosphorylation assay by incubating FLAG-E6 immunoprecipitates with ^{32}P-labeled eIF2α in vitro (280). We found that a higher amount of ^{32}P-eIF2α was dephosphorylated by immunoprecipitated FLAG-18 E6 than FLAG-11 E6 (Fig. 33D, lane 2 and 3) suggesting that 18 E6 promotes eIF2α dephosphorylation.

Figure 33. E6 recruits GADD34/PP1 complex to induce eIF2α dephosphorylation.

A. HeLa cells were transiently transfected with FLAG-GADD34 or FLAG-GADD34 mutants bearing either deletions or the KARA mutation in the PP1 binding site. Protein extracts (500 μg) were used in pull-down assays with 1 mg of purified GST alone or GST-18 E6. Protein extracts (25 μg) from each transfection was used as positive control. GADD34 proteins were detected by immunoblotting with anti-FLAG antibody (a). GST proteins were visualized by Coomassie blue staining of SDS-PAGE (b and c).

B. HeLa cells were transfected with 1 μg of either FLAG-18 E6 DNA or FLAG-11 E6 in the presence of 1 μg pcDNA3 vector DNA or 1 μg of FLAG-GADD34 cDNA. Protein extracts (500 μg) were then subjected to immunoprecipitation with anti-GADD34 antibody followed by immunoblotting with anti-FLAG antibody to detect the levels of the viral proteins (a) or GADD34 (b).

C. HeLa cells were transiently transfected with 2 μg of pcDNA3 vector DNA, 2 μg of FLAG-18 E6 DNA or 0.4 μg of FLAG-11 E6 DNA and 1.6 μg pcDNA3 vector. Protein extracts (500 μg) were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were immunoblotted with anti-PP1 antibody (a) or anti-FLAG antibody for detection of viral protein levels (b).

D. Protein extracts (500 μg) from HeLa cells transfected as in C were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were then subjected to dephosphorylation of ^{32}P-labelled histidine-tagged eIF2α. Phosphorylated eIF2α was detected by autoradiogrpahy (a) whereas E6 protein levels were detected immunoblotting with anti-FLAG antibody (b).

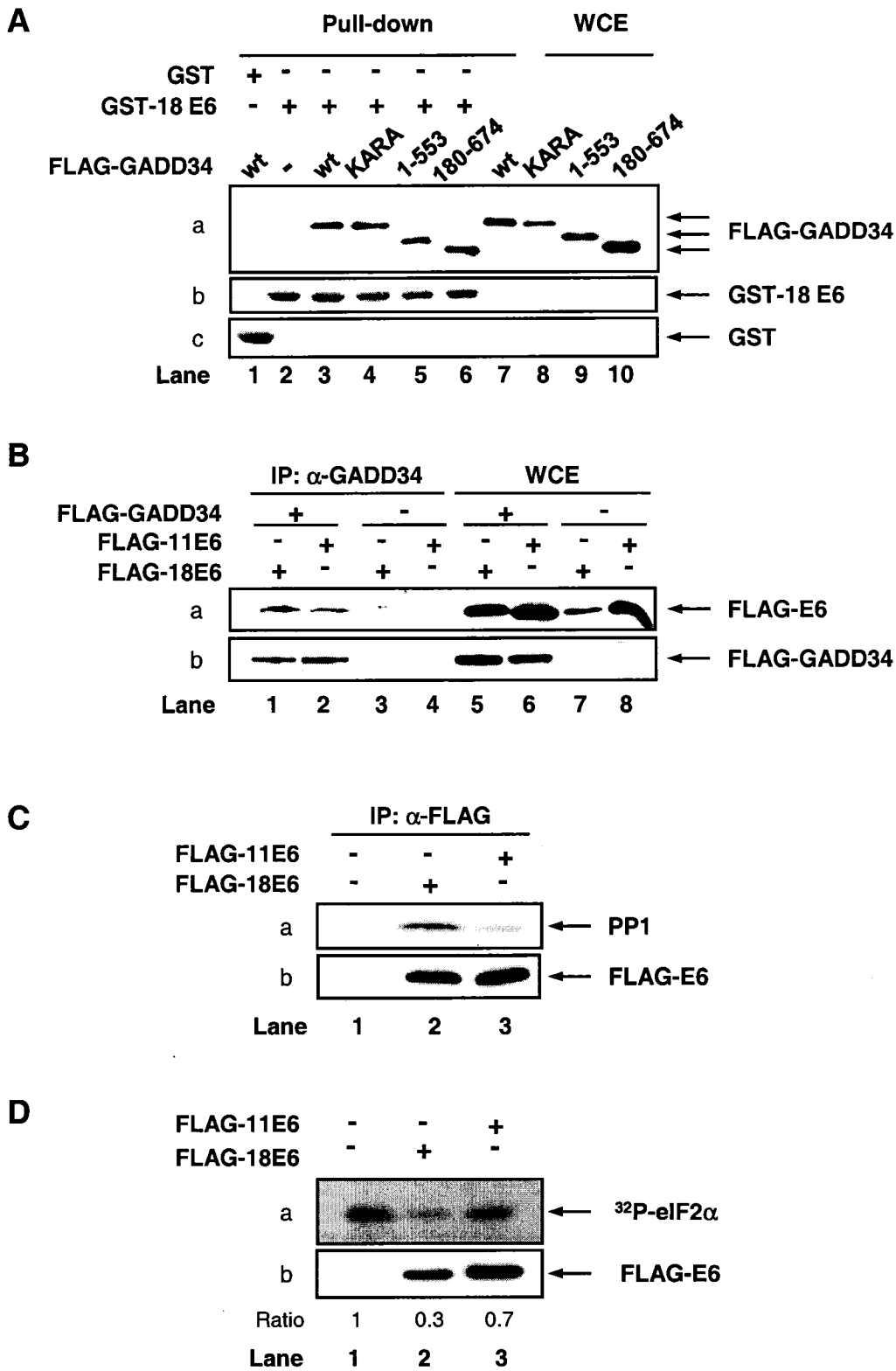

A
Pull-down
WCE
GST
GST-18 E6
FLAG-GADD34
wt
-
wt
KARA
1-553
180-674
wt
KARA
1-553
180-674
a
b
c
FLAG-GADD34
GST-18 E6
GST
Lane 1 2 3 4 5 6 7 8 9 10
B
IP: α-GADD34
WCE
FLAG-GADD34
FLAG-11E6
FLAG-18E6
a
b
FLAG-E6
FLAG-GADD34
Lane 1 2 3 4 5 6 7 8
C
IP: α-FLAG
FLAG-11E6
FLAG-18E6
a
b
PP1
FLAG-E6
Lane 1 2 3
D
FLAG-11E6
FLAG-18E6
a
b
32P-eIF2α
FLAG-E6
Ratio 1 0.3 0.7
Lane 1 2 3

Figure 33

3.2.3 High-risk 18 E6 impairs translational control by PKR.

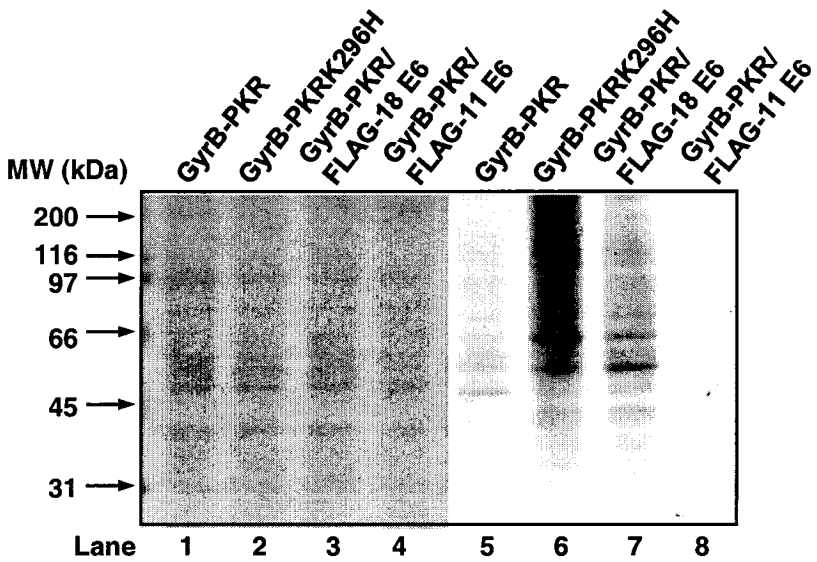
Since PKR-mediated cell death is tightly associated with protein synthesis inhibition (282), we next sought to examine the regulation of protein synthesis in HT1080 cells expressing GyrB-PKR in the absence or presence of the E6 proteins. Specifically, coumermycin-treated cells were labeled with ^{35}S-methionine, and protein extracts were subjected to SDS-PAGE and autoradiography (Fig. 34A, lanes 5-8). We observed that, although total protein load measured by Coomassie blue staining was equal in all cells (lanes 1-4), treatment with coumermycin drastically inhibited protein synthesis in cells with GyrB-PKR (lane 5) as opposed to cells bearing the catalytic mutant GyrB-PKRK296H (lane 6). Interestingly, the presence of FLAG-18 E6 relieved the inhibition of protein synthesis by GyrB-PKR significantly (compare lane 7 with lane 5). Contrary to FLAG-18 E6, expression of FLAG-11 E6 did not affect inhibition of protein synthesis by GyrB-PKR (compare lane 8 with lane 5). To better assess the differences in protein synthesis, cells were labeled with ^{35}S-methionine and radioactive proteins were extracted and quantified (Fig. 34B). We observed that protein synthesis was inhibited after 6 hours or 12 hours of coumermycin treatment of GyrB-PKR cells or GyrB-PKR cells expressing FLAG-11 E6. On the other hand, protein synthesis was still reduced in GyrB-PKR cells expressing FLAG-18 E6 but to a lesser extent than GyrB-PKR cells or GyrB-PKR cells containing FLAG-11 E6 particularly at 6h after coumermycin treatment. These data showed the ability of FLAG-18 E6 to relieve the translational blockade induced by PKR-mediated eIF2α phosphorylation.

Figure 34. Regulation of GyrB-PKR-mediated inhibition of protein synthesis by E6

A. HT1080 cells expressing GyrB-PKR, GyrB-PKRK296H, or GyrB-PKR together with either FLAG-18 E6 or FLAG-11 E6 were treated with 100 ng/ml coumermycin for 10 hours followed by labeling in vivo with ^{35}S-methionine for additional 2 hours. Protein extracts (50 μg) were subjected to SDS-PAGE. Total protein was visualized by Coomassie blue staining whereas radioactive proteins were detected by autoradiography.

B. HT1080 cells were left untreated or treated with 100 ng/ml coumermycin for 4 or 10 hours followed by [^{35}S]-methionine labeling for additional 2 hours. The radioactive proteins were quantified in 10 μg of total protein extract after trichloroacetic acid (TCA) precipitation and counting (66). Values represent the average percentage of protein synthesis (i.e.^{35}S-methionine incorporation) calculated from 2 independent experiments performed in triplicates.

A


GyrB-PKR
GyrB-PKRK296H
GyrB-PKR/ FLAG-18 E6
GyrB-PKR/ FLAG-11 E6
GyrB-PKR
GyrB-PKRK296H
GyrB-PKR/ FLAG-18 E6
GyrB-PKR/ FLAG-11 E6
MW (kDa)
200
116
97
66
45
31
Lane 1 2 3 4 5 6 7 8

B

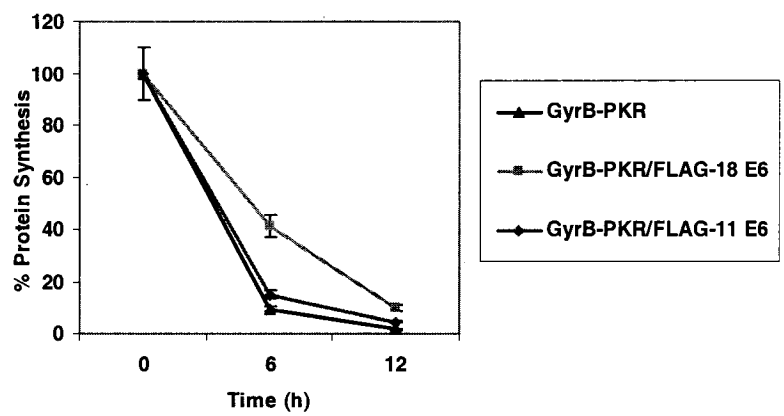

120
100
80
60
40
20
0
% Protein Synthesis
0
6
12
Time (h)
GyrB-PKR
GyrB-PKR/FLAG-18 E6
GyrB-PKR/FLAG-11 E6

Figure 34

3.2.4 HPV-18 E6 inhibits PKR-mediated apoptosis.

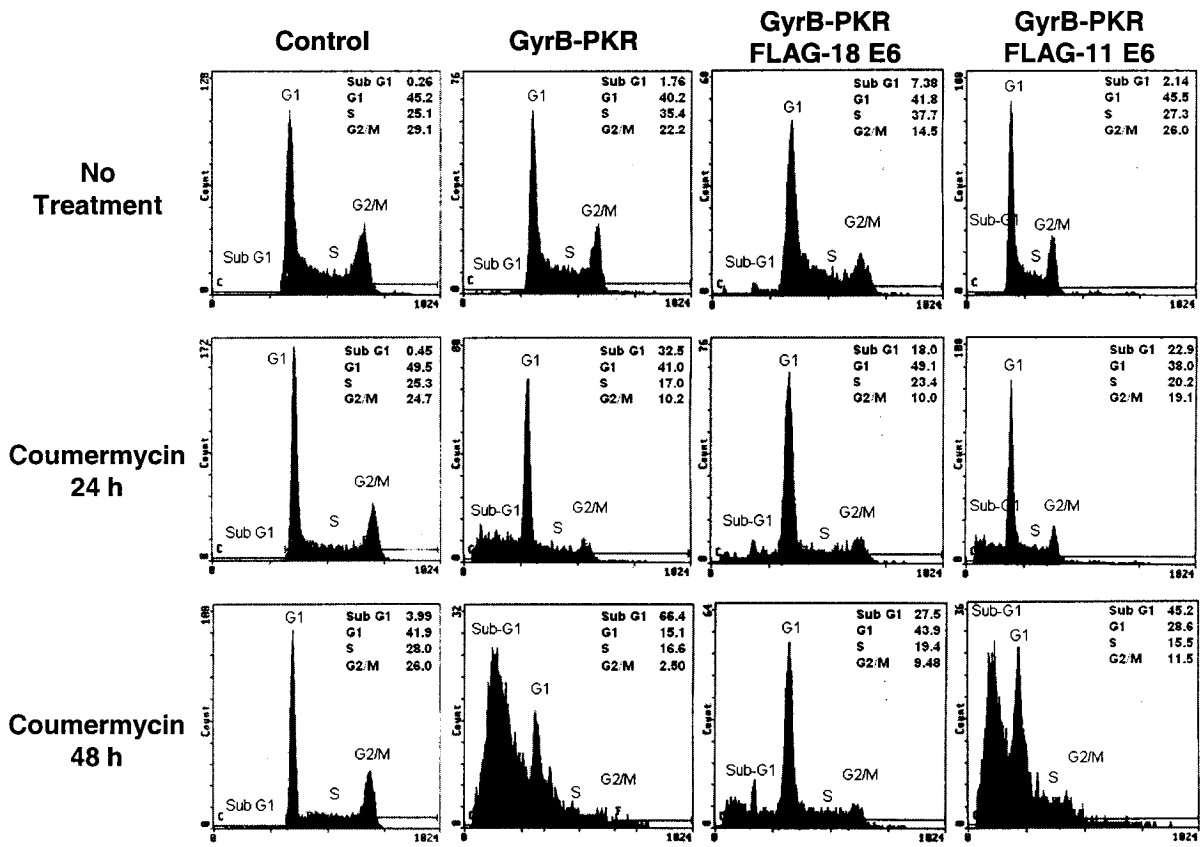
The inhibition of E6 protein synthesis by GyrB-PKR prompted us to examine a possible role of the viral protein in the biological effects of PKR activation. Considering that activation of PKR promotes apoptosis (282), we assessed the induction of death in HT1080 cells expressing GyrB-PKR in the absence or presence of either FLAG-18 E6 or FLAG-11 E6 (Fig. 35A). When control HT1080 cells (i.e. cells transfected with the expression vector bearing the zeocin resistant gene only) were treated with coumermycin, we observed that neither the growth nor the viability of the cells was affected by the presence of the antibiotic (left panels). On the other hand, HT1080 cells expressing GyrB-PKR underwent massive death (see increased sub-G_1 population) after treatment with coumermycin (panels second from the left). Interestingly, death was significantly lower in coumermycin-treated cells expressing GyrB-PKR and FLAG-18 E6 (panels third from the left). Contrary to this, the percentage of dead cells was not diminished in GyB-PKR cells expressing FLAG-11 E6 compared to GyrB-PKR cells after treatment with coumermycin (right panels). Quantitative analysis of cell death induced by GyrB-PKR activation in the absence or presence of E6 proteins is shown in Fig. 35B. Collectively, these findings clearly demonstrated the killing potential of activated GyrB-PKR and the ability of the high-risk 18 E6 only to rescue cells from PKR-mediated death.

Figure 35. Control of GyrB-PKR-mediated cell death by E6

A. HT1080 control cells (i.e. expressing the zeocin-resistant plasmid only) and cells expressing either GyrB-PKR alone or GyrB-PKR in the presence of either FLAG-18 E6 or FLAG-11 E6 were treated with 100 ng/ml coumermycin for 24 or 48 hours. Cells were harvested, fixed in ethanol, stained with propidium iodide and subjected to flow cytometry analysis. The percentage of apoptotic cells or cells in various phases of the cell cycle is indicated. Data represent one of four reproducible experiments.

B. Quantification of cell death. The values represent the average percentage of cell death (sub-G1 population) for each cell line treated with coumermycin from three independent experiments.

A


Control
GyrB-PKR
GyrB-PKR FLAG-18 E6
GyrB-PKR FLAG-11 E6
No Treatment
Coumermycin 24 h
Coumermycin 48 h
Sub G1
G1
S
G2/M
Sub-G1

B

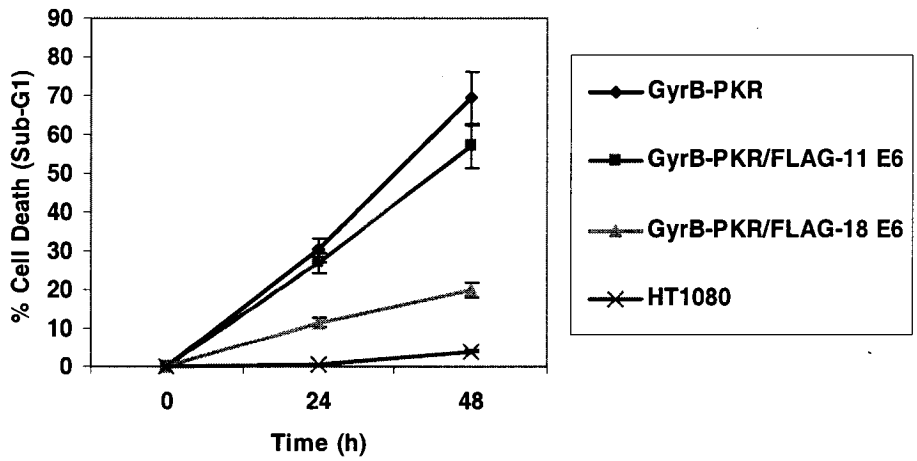

% Cell Death (Sub-G1)
Time (h)
0
10
20
30
40
50
60
70
80
90
0
24
48
GyrB-PKR
GyrB-PKR/FLAG-11 E6
GyrB-PKR/FLAG-18 E6
HT1080

Figure 35

3.2.5 HPV 18 E6 inhibits Bax induction by activated PKR.

Considering the anti-apoptotic role of FLAG-18 E6 in coumermycin-treated GyrB-PKR cells, we next sought to identify proteins implicated in the anti-apoptotic function of E6 (Fig. 36). Oncogenic forms of E6 were shown to activate Bcl2 and inactivate p53, Bak or Bax (222), whereas induction of apoptosis by PKR was found to be associated with an increase in Bax and Fas protein synthesis (141,195,283). Immunoblot analysis showed that expression of the anti-apoptotic Bcl2 was resistant to GyrB-PKR activation (panel a, lanes 1-4), although its overall protein levels were elevated in cells expressing FLAG-18 E6 (lanes 5-8). On the other hand, expression of the pro-apoptotic Bak (panel b) was not affected by the induction of GyrB-PKR (lanes 1-4) nor was its expression impaired by the presence of FLAG-18 E6 (lanes 5-8). Contrary to the above proteins, the pro-apoptotic Bax (panel c) was highly induced upon GyrB-PKR activation (lanes 1-4). Significantly, Bax induction did not take place in cells expressing FLAG-18 E6 (lanes 5-8) suggesting a specific regulation of this protein in GyrB-PKR-mediated apoptosis. Unlike Bax, the pro-apoptotic Fas protein (panel e) was not affected significantly by GyrB-PKR either in the absence or presence of FLAG-18 E6. When we probed for p53, we found that its protein levels were reduced by 80% in cells expressing FLAG-18 E6 prior to GyrB-PKR activation (panel f, compare lane 1 with 5). This effect was most likely due to the proteasome-dependent degradation of the tumor suppressor protein by E6 in HT1080 cells (284). We also observed that p53 protein levels were upregulated in coumermycin treated GyrB-PKR cells containing FLAG-18 E6 (lanes 5-8). Since FLAG-18 E6 protein synthesis is rapidly repressed by activated GyrB-PKR (Fig. 30B); the most conceivable explanation

is that downregulation of FLAG-18 E6 contributes to stabilization of p53. From the above data, we concluded that FLAG-18 E6 specifically targets Bax protein in cells with activated PKR.

Figure 36. Expression of anti- or pro-apoptotic proteins in response to eIF2α phosphorylation

HT1080 cells expressing GyrB-PKR alone or together with FLAG-18 E6 were induced with 100 ng/ml coumermycin for up to 24 hours. Protein extracts (50 μg) were subjected to immunoblot analysis with antibodies against the indicated proteins.

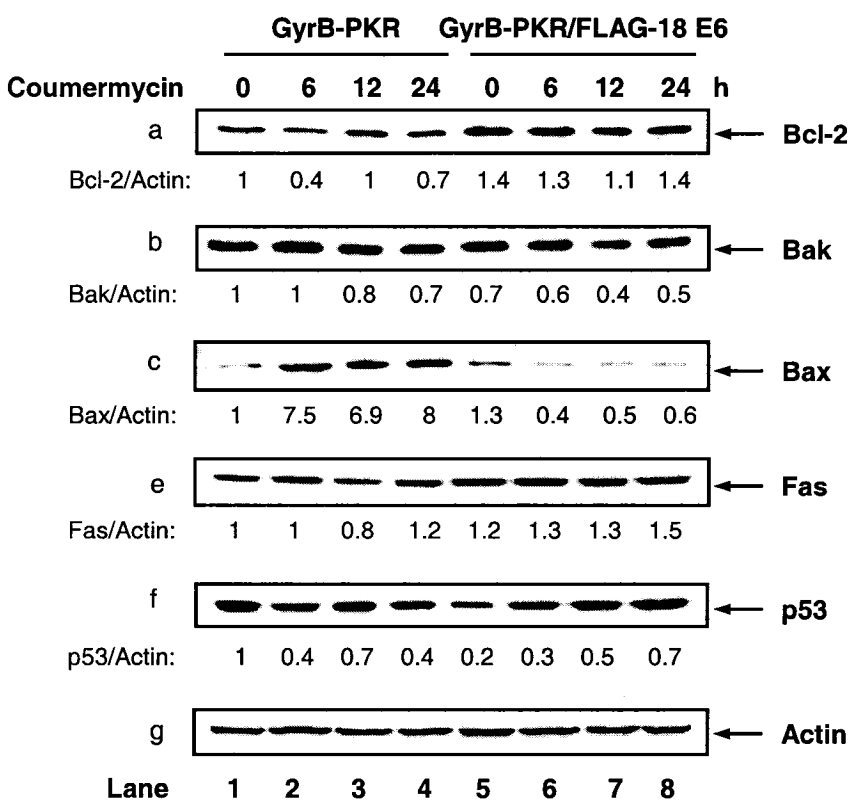

GyrB-PKR
GyrB-PKR/FLAG-18 E6
Coumermycin 0 6 12 24 0 6 12 24 h
a
Bcl-2
Bcl-2/Actin: 1 0.4 1 0.7 1.4 1.3 1.1 1.4
b
Bak
Bak/Actin: 1 1 0.8 0.7 0.7 0.6 0.4 0.5
c
Bax
Bax/Actin: 1 7.5 6.9 8 1.3 0.4 0.5 0.6
e
Fas
Fas/Actin: 1 1 0.8 1.2 1.2 1.3 1.3 1.5
f
p53
p53/Actin: 1 0.4 0.7 0.4 0.2 0.3 0.5 0.7
g
Actin
Lane 1 2 3 4 5 6 7 8

Figure 36

3.2.6 Transcriptional responses induced by eIF2α phosphorylation are mitigated by 18 E6.

In addition to protein synthesis, induction of eIF2α phosphorylation can control gene transcription in response to diverse stressful conditions (277,285). Based on this, we sought to identify genes that are transcriptionally regulated by GyrB-PKR in the absence or presence of E6. To this end, GyrB-PKR cells were subjected to cDNA microarray analysis using the human U133A DNA chip from Affymetrix that contains 22,000 genes (257). Genes that were either induced or suppressed more than 5-fold in coumermycin-treated GyrB-PKR cells are shown in Table I. Among the 9 genes that were suppressed by GyrB-PKR, some have been clearly implicated in cell cycle progression, such as the cyclin E1 and E2 (286) and the centromere-associated protein E (CENPE) (287), in DNA repair, for instance the radiation sensitivity RAD54L (288), or cell signaling, such as the Rho GDP dissociation inhibitor beta (RhoGDIβ) (289), the regulator of G-protein signaling 4 (RSG4) (290) and peroxiredoxin 1 (PRDX1) (291). On the other hand, among the 22 genes induced by GyrB-PKR, some encode for proteins involved in apoptosis, such as the growth arrest and DNA damage gene (GADD45) A and B (292), the natural born killer and BH3-only Bcl-2 homologous protein (NBK/BIK) (293), the interferon regulatory factor 1 (IRF1) (294) as well as the forkhead transcription factor FOXO3A/FKHRL1 (7). Interestingly, regulation of expression of these genes by GyrB-PKR was significantly mitigated by FLAG-18 E6 and to a much lesser extent by FLAG-11 E6 (Table 3). This data provided strong evidence for a role of E6 in gene transcription induced by the activation of PKR/eIF2α phosphorylation pathway.

Table 3. HPV-18 E6 modulates transcriptional function of PKR.

HT1080 cells expressing GyrB-PKR, GyrB-PKRK296H, GyrB-PKR/18 E6 or GyrB-PKR /11 E6 cells were treated with coumermycin for 12 hours and subjected to cDNA microarray analysis using the human U133A DNA chip from Affymetrix containing 22,000 genes. Genes that were either induced or suppressed more than 5-fold in coumermycin-treated cells are shown in the table.

FOLD REGULATION BY COUMERMYCIN				GENE	FUNCTION
GyrB-PKR	GyrB-PKRK296H	GyrB-PKR/18 E6	GyrB-PKR/11 E6		
-29.3	-1.5	-1.9	-10.1	Human homologue of yeast YA22 (HYA22)	Putative tumor suppressor
-20.0	1.2	-2.2	-1.9	Human homologue of rat pituitary tumor transforming gene (PTTG3)	Proto-oncogene, regulator of cell proliferation and survival
-13.5	-1.4	-2.5	-2.6	Cyclin E2 (CCNE2)	Cell cycle, overexpressed in human cancers
-12.8	-1.8	-1.2	-3.3	Radiation sensitivity (RAD) 54L	DsDNA-dependent ATPase, DNA repair, putative tumour suppressor
-12.0	1.0	-1.3	-3.4	Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB)	Extracellular signalling, cytoskeleton organization, cell mobility
-6.6	1.3	-1.7	-1.2	Regulator of G-protein signalling (RGS) 4	Regulation of G-protein function
-5.6	-2.0	-1.4	-1.3	Cyclin E1 (CCNE1)	Cell cycle, G/S transition
-5.3	-1.0	-1.1	-1.9	Peroxiredoxin 1 (PRDX1)	Redox-regulation, cell signalling and apoptosis
-5.1	1.0	-1.1	-1.8	Centromere-associated protein E (CENPE)	Spindle microtubule organization, mitotic checkpoint signalling
5.4	1.4	-1.9	-1.4	Lymphotoxin beta (LT-beta; TNF superfamily, member 3)	Apoptosis, Inflammatory responses
5.5	1.4	1.4	2.9	RNA polymerase III subunit 62 (RPC62)	Transcription of short mRNAs, involved in tRNA splicing
5.6	-1.4	-1.1	13.6	Methylthioadenosine phosphorylase (MTAP)	Putative tumor suppressor
5.7	1.1	1.1	1.5	UV radiation resistance associated gene (UVRAG)	Complement to UV sensitivity of xeroderma pigmentosum complementation group C
5.8	-1.3	1.5	2.2	Protein kinase AMP-activated, beta-1 non catalytic subunit (PRKAB1)	Adaptation to metabolic stress
6.2	-1.1	1.6	1.9	Keratin 7 (KRT7)	Marker of epithelial differentiation
6.3	1.6	-1.1	1.5	Thierodoxin reductase (TR)	Cellular defence against oxygen damage
6.4	1.2	-2.7	4.1	Melanoma differentiation-associated gene-7 (mda-7)/Interleukin (IL)-24	Tumor suppressor
7.0	1.1	1.1	1.7	Retinoic acid receptor alpha (RARA)	Disruption of tumor progression
7.8	1.2	1.2	2.6	Interferon regulatory factor (IRF) 1	Transcription factor, cell cycle and apoptosis
8.0	1.4	1.5	1.6	Transciption factor RelB (RELB)	Regulation of development, cell cycle, cell survival
8.4	1.5	2.7	2.6	Growth arrest and DNA-damage inducible (GADD) 45A	Regulator of DNA damage, Apoptosis
9.7	1.9	4.7	10.8	Growth arrest and DNA damage gene (GADD) 34	Promoter of eIF-2alpha dephosphorylation
10.2	1.5	-2.1	7.3	Growth arrest and DNA-damage inducible (GADD) 45B	Regulator of DNA damage, Apoptosis
10.5	2.0	1.5	1.4	Transcription factor JunB (JUNB)	Regulation of cell differentiation, proliferation and apoptosis
11.9	1.2	1.7	2.6	Tumor necrosis factor alpha receptor associated factor (TRAF) 1	Adaptor protein, negative regulator of TNF signaling
13.0	-1.4	6.6	-1.1	BH3-only Bcl-2 homologous protein BIK	Inducer of apoptosis
15.9	2.0	-1.3	1.9	Tumor necrosis factor alpha induced protein (TNFAIP) 3 or A20	Transcription/growth regulatory factor, involved in NFkappaB pathway
23.0	-1.2	1.8	6.2	Winged helix/forkhead transcription factor (FOXO3A)	Transcription factor, cell cycle, apoptosis, cell metabolism and oxidative stress
26.9	1.1	3.3	3.6	Interferon stimulatory gene (ISG) 20	Interferon induced RNase, 3' to 5' exonuclease, antiviral activity
27.8	1.7	3.7	3.5	Vitamin D3-up-regulated protein (VDUP1/TXNIP)	Major regulator of cellular redox state, inhibtor of thioredoxin and tumor metastasis
42.8	2.8	2.0	5.2	Early growth response (EGR) 1 transcription factor	Regulator of tumorigenesis

Chapter IV

Discussion

Chapter IV - Discussion

1. Functional interaction between PKR and PI3K pathway

1.1 Activation of PI3K pathway by PKR

Originally, PKR has been implicated in mediating the antiviral and anti-proliferative effects of IFNs in response to virus infection (123,182,295). The best characterized role of PKR is to inhibit translation and induce apoptosis, through which it is believed to exhibit tumor suppressor activity in vitro (141,282,296,297). Activation of PKR results in inhibition of cell growth in yeast (133) and induction of apoptosis in mammalian cells (195,283,298-300). Compelling evidence for a role of PKR in tumor suppression is provided by experiments where the expression of dominant negative catalytically inactive or dsRNA-binding defective mutants of PKR in immortalized NIH 3T3 cells resulted in inactivation of PKR and induction of tumors upon injection to nude mice (136,301,302).

Although PKR is best known for its role in host viral defense and under stress conditions, there is growing evidence for the role of PKR in other cellular processes, such as transcription and signal transduction under normal conditions. In this regard, PKR targets several cellular proteins such as IRF1(303), p53 (179,180), NF-κB (261,304), c-Fos (183), Stat1 (305) and Stat3 (306) which are involved in transcription, and mediates signaling pathways in response to PDGF, IL-3 and IFNs (184,187,306). Since PKR possesses growth inhibitory and apoptotic effects, it is reasonable to speculate that PKR-deficient mice ($PKR^{-/-}$) would exhibit higher rates of growth and proliferation comparing to their wild-type counterparts. However, both available $PKR^{-/-}$

models did not show any growth abnormalities or tumorigenic properties (307). On the other hand, PKR has been demonstrated to be necessary for proper progression of the cell cycle and inhibition of PKR leads to cell cycle arrest in G1/S phase (307). PKR also functions as a mediator of several signal transduction pathways with growth promoting effects, such as PDGF (187,306).

Moreover, despite its growth suppressive activities, the role of PKR as a tumor suppressor has not been established in vivo. This is partly due to the fact that mutational inactivation or deletion of *pkr* gene does not induce tumor formation in animals or humans. Interestingly, several studies show that PKR is overexpressed and overactive in some human cancers such as breast cancer (308,309), melanoma and colon cancer (310). Elevated expression of the *pkr* gene, and higher activity of the kinase, correlates with neoplastic progression and is suggested to play a role in promotion of neoplasticity of breast cancer cells (308).

These findings suggest a requirement for PKR in cell growth and proliferation. In this regard, we have investigated the possible role of PKR in the regulation of signaling pathways downstream to growth factors and cytokines. One such signaling pathway is the well characterized PI3K pathway, which is induced upon stimuli such as hormones, mitogens, cytokines, and growth factors and mediates their proliferative effects. Our data provide strong evidence for a novel function of PKR, which is its ability to induce the PI3K pathway. Utilizing a conditionally active form of PKR, we observed that GyrB-PKR acts upstream of PI3K.

These data demonstrate that indeed PKR is capable of inducing the lipid and protein kinase activity of the PI3K. In proliferating cells, PI3K activity is induced upon

binding of the growth factors and cytokines to their cognate receptors. There is also evidence for activation of PI3K through a direct interaction of the catalytic subunit with Ras (31,32). Our observations indicate that PKR-mediated PI3K activation is independent of Ras, since the Ras activity is not subjected to regulation by PKR in this system. Moreover, the possibility of an autocrine effect through secretion of a growth factor was ruled out by using the conditional media from cells with activated PKR to treat the HT1080 parental cells.

Induction of PI3K activity by PKR leads to phosphorylation of the downstream target PKB/Akt on two residues which are necessary for its activation; Thr308 and Ser473. Activation of PKB/Akt, results in the induction of proliferative and anti-apoptotic responses. One downstream target of PKB/Akt is GSK3, whose phosphorylation by PKB/Akt results in its inactivation and relief of its inhibitory effect on its downstream pathways such as glycogen synthesis (44). Our data indicate an induction of phosphorylation of both GSK3 isoforms upon activation of PKR.

Another pathway regulated by PI3K-PKB/Akt is FRAP/mTOR (43). FRAP/mTOR is an indirect target of PKB/Akt, which plays a role in cell growth by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy in response to nutrient availability (311). Our data demonstrate that activation of PKR also results in upregulation of mTOR activity as assessed by phosphorylation of 4E-BP1 and S6 ribosomal protein.

Induction of FRAP/mTOR activation and GSK3 phosphorylation suggests that phosphorylation of PKB/Akt by PKR, indeed stimulates the pathways lying downstream PKB/Akt.

1.2 Phosphorylation of 4E-BP1 upon PKR activation

Upon activation of GyrB-PKR an induction of 4E-BP1 phosphorylation on several residues was observed. This induction is abolished in the presence of PI3K and FRAP/mTOR inhibitors indicating that induction of 4E-BP1 phosphorylation is an indirect effect of PKR and is mediated through the PI3K signaling pathway.

Based on the ability of GyrB-PKRK296H mutant to partially induce 4E-BP1 phosphorylation compared to catalytically active GyrB-PKR, it appears that both the dimerization and kinase activity of PKR are required for the efficient induction of the PI3K pathway. Induction of PI3K activation and 4E-BP1 phosphorylation declined after prolonged treatment with coumermycin in GyrB-PKR-expressing cells. This negative regulation of the PI3K pathway may be mediated by the inhibition of protein synthesis from the prolonged induction of eIF2α phosphorylation. That is, activation of GyrB-PKR may turn off the synthesis of a protein(s) required for maintaining PI3K activity. Part of the downregulation of the PI3K pathway may proceed independently of the kinase activity of PKR based on the data with the GyrB-PKRK296H cells (Fig. 1A). Thus, dimerization of PKR may also be capable of switching on a pathway that antagonizes PI3K.

There are reports indicating the activation of PI3K in response to IFN signaling. This include both type I and II of IFNs and involves IRS1 (Insulin Receptor Subsrate 1) and possibly c-CBL, respectively (252,312). Thus, it is logical to speculate that as a downstream target of IFN signaling PKR is likely to mediate IFN-dependent PI3K activation.

1.3 The role of PKR-mediated 4E-BP1 phosphorylation in translation

Activation of GyrB-PKR led to the induction of 4E-BP1 phosphorylation at Thr37/46, Thr70 and Ser65, which were previously shown to be involved in the dissociation of 4E-BP1 from eIF4E (16). Interestingly, despite the phosphorylation of 4E-BP1 at these residues, its interaction with eIF4E was not blocked by activated GyrB-PKR. These data reveal that 4E-BP1 phosphorylation is necessary but is not always sufficient for its dissociation from eIF4E. It is possible that dissociation of the eIF4E/4E-BP1 complex is facilitated by a factor(s), whose expression is sensitive to the inhibition of protein synthesis by activated PKR. Another possibility may be the ability of PKR to phosphorylate and modify the function of a protein(s) required for the dissociation of 4E-BP1/eIF4E complex.

We also show that the interaction of eIF4E with 4E-BP1 takes place in distinct cytoplasmic foci induced by activated PKR. These foci are reminiscent of stress granules, which are formed upon conditions that favor the phosphorylation of eIF2α including endoplasmic reticulum (ER) stress and oxidative stress by arsenite (313,314). Interestingly, eIF4E is recruited to the cytoplasmic granules in a complex with either 4E-BP1 or eIF4GI in cells with activated GyrB-PKR. This may indicate the presence of a pool of eIF4E in equilibrium between its eIF4GI and 4E-BP1-bound forms. Since stress granules consist of components of the 48S pre-initiation complex, the presence of eIF4E, 4E-BP1 and eIF4GI in them could be explained by a situation in which the eIF4F complex is unstable thus allowing an interchange between the 4E-BP1-bound and eIF4GI-bound eIF4E. Another possibility is that activation of PKR favors the formation of the 48S pre-initiation complex independently of the eIF4F activity. This is in line

with other findings suggesting that inhibition of eIF4F formation by Maskin and Cup proteins through their interactions with eIF4E may not prevent the recruitment of the 48S pre-initiation complex (2). Inhibition of PI3K and/or FRAP/mTOR activity is not sufficient to induce the cytoplasmic granules unless is accompanied by the induction of eIF2α phosphorylation. This indicates that eIF4E/4E-BP1 interaction by itself is not sufficient for the formation of the cytoplasmic granules. The biological significance of the cytoplasmic granules induced by various forms of stress remains speculative (315). Since formation of stress granules is dependent on eIF2α phosphorylation, they might play a role in regulation of PKR-dependent apoptosis.

Given the important role of eIF4E in cap-dependent translation, induction of eIF4E/4E-BP1 interaction could serve as a mechanism of translation inhibition that acts synergistically with eIF2α phosphorylation. We obtained evidence; however, that eIF2α phosphorylation exerts a dominant inhibitory effect on global protein synthesis regardless of the level of eIF4E and its interaction with 4E-BP1. Specifically, induction of eIF4E/4E-BP1 interaction by rapamycin treatment did not further decrease translation inhibition by activated GyrB-PKR. Conversely, overexpression of eIF4E was not capable of overcoming the translational inhibitory effects of activated PKR. Therefore, it appears that eIF2α phosphorylation is the rate limiting step for the inhibition of cap-dependent translation. Unlike rapamycin, LY294002 treatment decreased the overall protein synthesis either in the absence or presence of activated GyrB-PKR. This indicated that the PI3K facilitates protein synthesis without interfering with the eIF2α phosphorylation pathway. In fact, inhibition of the PI3K pathway by LY294002 treatment did not affect eIF2α phosphorylation by activated GyrB-PKR

providing further evidence that the translational effects exerted by each pathway proceed independently.

1.4 The biological effects of PI3K activation by PKR

Activation of the PI3K pathway serves as a negative feedback mechanism against GyrB-PKR-mediated apoptosis. This anti-apoptotic function of PI3K may not be exerted at the translational level through 4E-BP1 and its interaction with eIF4E since treatment with rapamycin, which further induced the interaction between the two proteins, did not increase PKR-mediated apoptosis. It is interesting that overexpression of eIF4E was not able to rescue GyrB-PKR-dependent apoptosis consistent with its incapability to relieve cells from the translational inhibitory effects of GyrB-PKR. Given that eIF4E is an important anti-apoptotic component of the PI3K pathway (316), inhibition of PKR-dependent apoptosis by the PI3K is most probably mediated at the post-translational level and may involve the inactivation of apoptotic proteins by phosphorylation as a result of PKB/Akt activation (317,318).

Our findings provide evidence for a role of PKR in PI3K activation and 4E-BP1 phosphorylation in response to mitogenic signaling. In the first instance, this was an unexpected finding given the well-established anti-proliferative and apoptotic properties of the kinase. PKR-dependent apoptosis, however, is dependent on eIF2α phosphorylation (260,283,319), and conditions that lead to PKR activation without inducing the phosphorylation of eIF2α may turn PKR from an inhibitor to an inducer of cell proliferation. Serum induces eIF2α dephosphorylation (320) and stimulates eIF2B activity (321), both of which are capable of neutralizing the anti-proliferative effects of PKR at the translational level. The induction of the PI3K pathway by PKR may have

important implications in tumor biology. For instance, it was reported that both PKR protein and activity are induced in breast cancer cells (308,309) in which PI3K pathway is constitutively active (268). It is conceivable that in tumors with inactivated eIF2α, activation of PKR might contribute in the induction of cell proliferation through the activation of the PI3K pathway and/or the induction of the anti-apoptotic NF-κB pathway (322).

The PI3K pathway is induced by IFNs and utilizes at least two distinct pathways for the regulation of mRNA translation (273). One involves the activation of p70 S6 kinase and phosphorylation of ribosomal protein S6, and the other involves phosphorylation and inactivation of 4E-BP1 (252,312). Our data show that PKR is required for the induction of 4E-BP1 phosphorylation in response to IFN-γ thus providing evidence for a potential role of the kinase in regulating antiviral responses through eIF4E. Although the PI3K-PKB/Akt–FRAP/mTOR pathway is activated by growth factors and other mitogenic stimuli to transduce pro-survival and growth-promoting signals, its activation by IFNs, which suppress growth and can mediate pro-apoptotic effects, indicates that differential regulation of the cascade by various stimuli can lead to divergent biological responses (273).

Activation of the PI3K pathway by PKR is a novel finding that reveals a dual but opposing biological function of the kinase. That is, PKR may have the capacity to inhibit or promote cell proliferation depending on a functional interplay between the PI3K and eIF2α phosphorylation pathways. Under conditions that eIF2α phosphorylation is blocked activation of PKR may facilitate cell proliferation through the induction of the PI3K pathway. Conversely, under conditions that eIF2α

phosphorylation is induced, the apoptotic function of PKR prevails over the induction of the survival PI3K pathway.

1.5 The role of PI3K activation by PKR in Virus infection

Activation of PKR by viral dsRNA is an important defense against viral infection, through inhibition of protein synthesis and induction of apoptosis. The significance of regulating PKR activity is appreciated by the distinct mechanisms evolved by DNA tumor viruses to circumvent PKR activation and eIF2α phosphorylation. During the course of evolution, viruses have developed mechanisms to modulate a variety of host cell signaling pathways, most importantly, those regulating apoptosis and cell survival (323). During acute infection, inhibition of apoptosis results in short-term cell viability, which creates a favorable condition for viral replication. Persistent inhibition of apoptosis, however, contributes to latency and chronic infection, which in the case of certain viruses leads to cellular transformation. Inhibition of apoptosis can be achieved through induction of anti-apoptotic or inhibition of pro-apoptotic proteins. Given the important role of the PI3K pathway in cell survival, virus modulation of this pathway provides an alternative to the expression of viral oncogenes or the direct inhibition of pro-apoptotic proteins. It has become evident that many viruses require up-regulation of this pathway to sustain long-term infections and it is modulated, in some cases, by specific viral products to create an environment favorable for cellular transformation. In other cases, PI3K-PKB/Akt signaling simply helps to create an environment favorable for virus replication and virion assembly. Thus, PKR-mediated activation of PI3K may be utilized by the virus to facilitate its replication. There is actually a pre-incidence of PKR acting as a molecular clock through

chronological regulation of cell survival and death through activation of NF-κB and phosphorylation of eIF2α, respectively and this is mediated by catalytic-dependent and -independent activities of PKR (324). Thus, the same mechanism could apply to upregulation of survival pathways through activation of PKB/Akt.

1.6 PKR and PKB/AKT in aging and neurodegenerative diseases

Recently, PKB/Akt has been implicated in senescence (325), an irreversible growth arrest which occurs at the end of replicative lifespan of cells and plays an important role in human aging and age-associated diseases (326). Signals other than extended proliferation have also been shown to induce premature senescence. These signals include mitogenic signaling, DNA damage or oxidative stress (327-329). Activation of p53 and telomerase deficiency also cause premature aging (330,331). A recent study has shown that PKB/Akt activity increases with cellular senescence and its inhibition extends the lifespan of human primary endothelial cells. Constitutive activation of PKB/Akt results in phosphorylation and inactivation of the forkhead transcription factors, which leads to accumulation of oxygen reactive species (ROS), which in turn induces p53 activation and premature senescence (332).

On the other hand, PKR has been implicated in aging and neurodegenerative diseases such as Huntington disease (HD), Parkinson's disease (PD), Alzheimer disease (AD), and Amyotrophic Lateral Sclerosis (333). The study of PKR in aging has been restricted to mice. In all tissues examined which included kidney, liver, colon, brain, testes, pancreas, lung, and heart, the expression of PKR was negligible in 2-month-old mice and significantly increased in mice 20 months of age. The function of PKR, and in particular active PKR, in the normal adult brain is unclear; however, its increase in

normal aging could reflect an increased vulnerability to cellular and/or environmental stressors that occurs concomitant with disease processes.

In regard to neurodegenerative diseases, nuclear aggregation of phosphorylated PKR in the hippocampus of brain tissues has been detected. The expression of phosphorylated PKR in cytoplasm was not associated with neuronal cell death indicating that translocation of phosphorylated PKR into the nucleus might play a pivotal role in neurodegenerative pathology (334). Taken together, the PKB/Akt-dependent regulation of forkhead transcription factor in the nucleus resulting in senescence, accumulation of PKR in the nucleus in HD, PD and AD and our data indicating activation of PKB/Akt by PKR, there may be a link between PKB/Akt activation, induction of senescence and generation of neurodegenerative diseases by PKR. Based on the data collected in this part of our studies we propose a model which is presented in Figure 37.

Figure 37. A model representing activation of PI3K pathway by PKR

Activation of PKR in response to stress condition results in phosphorylation of eIF2α and induction of PI3K pathway. In addition PKR is involved in activation of PI3K upon stimulation with growth factors and cytokines. Induction of PI3K activity results in activation of the downstream effectors, such as PKB/Akt and FRAP/mTOR leading to phosphorylation of 4E-BP1 and S6 ribosomal protein. PKR-mediated phosphorylation of 4E-BP1, however, does not result in its dissociation from eIF4E under stress conditions.

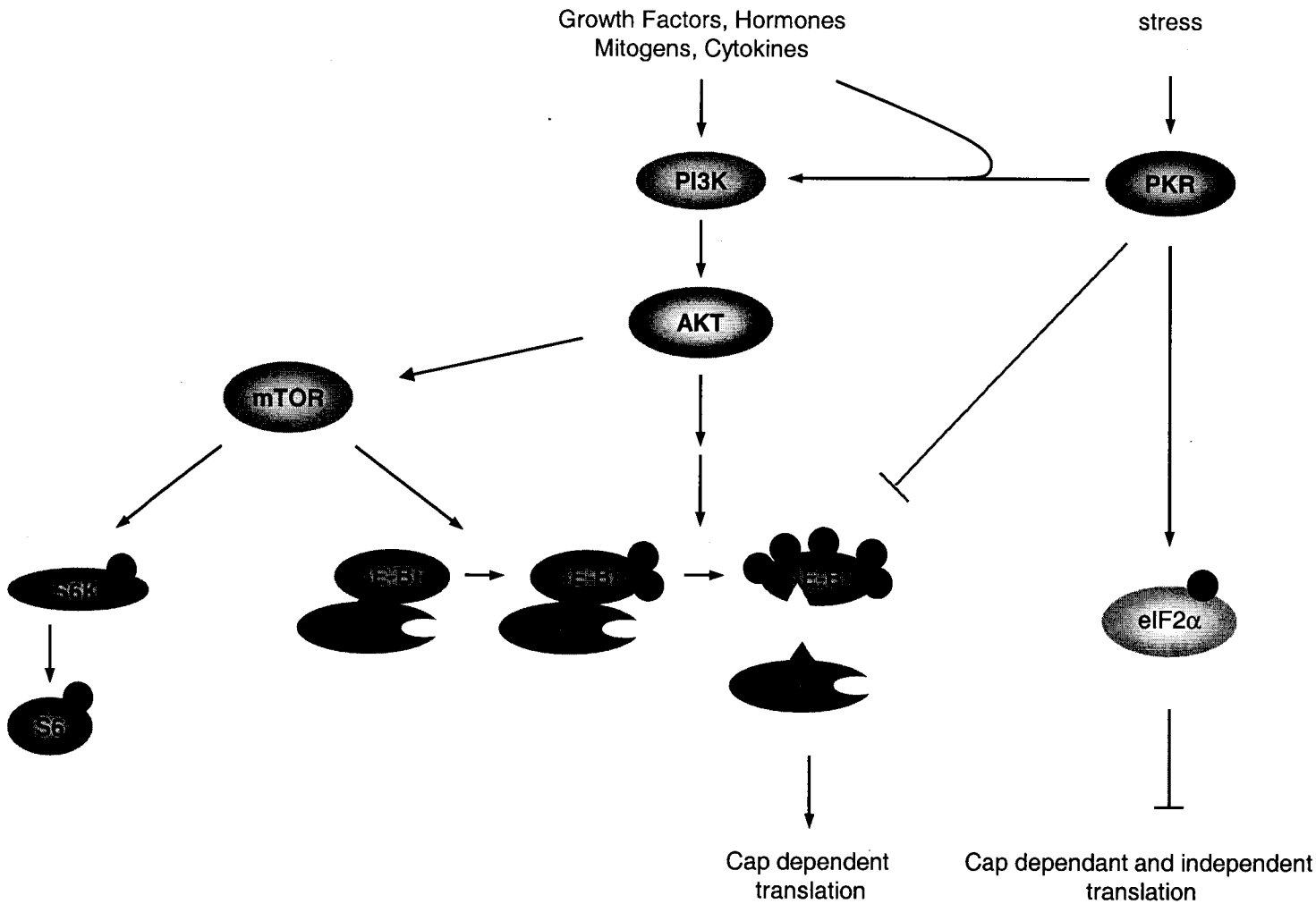

Growth Factors, Hormones
Mitogens, Cytokines
stress
PI3K
PKR
AKT
mTOR
S6K
S6
4EBP1
eIF4E
eIF2α
Cap dependent
translation
Cap dependant and independent
translation

Figure 37

2. Functional interaction of HPV-18 E6 and PKR

Regulation of protein synthesis by eIF2α phosphorylation plays an important role in host cell defense against viral infection (154,155,285). Therefore, it is not surprising that several RNA and DNA viruses have evolved mechanisms to target PKR. Our findings further extend these observations and demonstrate a functional relationship between E6 and eIF2α pathway.

2.1 Regulation of E6 protein synthesis by PKR

Specifically, E6 protein level is tightly regulated through post-transcriptional modifications. The E6 protein is maintained at low levels possibly to escape the immune surveillance of the host cell. Our data demonstrate that E6 protein from both high-risk and low-risk HPVs is subjected to proteasome-dependent degradation. In this regard, our data reveal a potentially important common feature, which is the first identified biochemical process shared between low- and high-risk HPV E6 proteins, with respect to both being targeted for ubiquitination and proteasome-mediated degradation. In addition to acting as a target for degradation, it is possible that ubiqutination may enhance biological function(s) of the E6 proteins. Recent reports have shown roles for ubiquitination in cellular trafficking, kinase activation and transcriptional regulation (335), and may be relevant with respect to HPV E6 modification by ubiquitin; however, since both low-risk and high-risk HPV E6 proteins became polyubiquitinated this argues that ubiquitination does not contribute to the oncogenic properties of high-risk HPV E6.

E6 protein is also subjected to translational control. Specifically, E6 protein synthesis is rapidly decreased in cells with activated PKR. The levels of 18 E6 mRNA

is not decreased upon coumermycin treatment and inhibition of the proteasome pathway does not rescue this downregulation, which together suggest that decrease of E6 protein is indeed at the translational level.

2.2 Inhibition of PKR-mediated eIF2α phosphorylation by 18 E6

Despite the significant downregulation of the viral protein, the remainder of E6 is able to partially rescue cells from the translational blockade posed by eIF2α phosphorylation. This is possibly mediated, partly, by the ability of E6 to promote the dephosphorylation of eIF2α through the GADD34/PP1 holophosphatase complex. This notion is based on our observations that high-risk E6 interacts with both GADD34 and PP1 and promotes the dephosphorylation of eIF2α in vitro. In fact, a higher amount of PP1 was bound to 18 E6 than 11 E6 consistent with the higher degree of eIF2α dephosphorylation by the high-risk viral protein. Although the precise mechanism utilized by E6 to promote eIF2α dephosphorylation through GADD34/PP1 is currently not known, this may be facilitated, partly, by the ability of high-risk E6 protein to be localized both in the nucleus and cytoplasm as opposed to low-risk viral protein, which exhibits predominantly nuclear localization (336). Mapping of the interaction between E6 and GADD34 showed that the domain of GADD34 required for binding to the viral protein is within the region between residues 180 and 553. The interaction is not mediated by the KVRF PP1-binding sequence of GADD34 since the KARA mutation, which abolishes PP1 binding to GADD34 (245), did not affect the interaction between GADD34 and 18 E6. These findings also indicate that binding of 18 E6 to GADD34 does not interfere with GADD34/PP1 complex formation. Interestingly, PP1 was previously found to directly bind and nullify PKR activity through the

dephosphorylation of the activated kinase (337). GyrB-PKR; however, is unlikely to be affected by PP1 since the N-terminus regulatory domain of the kinase, which is required for PP1 binding (337), is missing from the fusion protein. In addition, PKR autophosphorylation is not affected in E6 expressing cells further supporting the notion that the viral protein exerts its effects downstream of the activated kinase. A role of the GADD34/PP1 complex in eIF2α dephosphorylation is further supported by the cDNA microarray analysis data showing the regulation of *GADD34* gene expression by GyrB-PKR. Specifically, the mRNA level of *GADD34* is induced almost 10-fold in coumermycin-treated GyrB-PKR cells and only 2-fold in GyrB-PKRK296H cells (Table I). Interestingly, GADD34 mRNA expression was inhibited by 50% in GyrB-PKR cells expressing FLAG-18 E6 and remained unaffected in GyrB-PKR cells with FLAG-11 E6 (Table I). Since induction of *GADD34* mRNA levels was previously shown to be dependent on eIF2α phosphorylation (281), its inhibition by the HPV-18 E6 further supports the inhibitory role of the viral oncoprotein in eIF2α phosphorylation.

2.3 The biological effects of PKR-E6 functional interaction

The biological consequences of eIF2α phosphorylation and induction of apoptosis can be best explained in the context of virus infection. The ability of viruses to exert total control over the apoptotic response in infected cells is critical to their replication and induction of pathogenicity (338). For example, inhibition of early apoptosis is a necessary step to ensure efficient viral replication and facilitate virus spread by supporting replication in a broad range of cells and tissues. As such, viruses have evolved sophisticated means to inhibit apoptosis in infected cells. For example,

adenoviruses and herpesviruses contain homologues of the Bcl-2 family and anti-apoptotic proteins, whereas other viruses encode inhibitors of caspases (339). In case of HPV, it has become clear that E6 plays a prominent role in prevention of apoptosis through the proteolytic inactivation of the pro-apoptotic p53, Bak or Bax (222). Our findings further substantiate the anti-apoptotic function of the oncogenic E6 and provide strong evidence that it can be mediated through the regulation of eIF2α phosphorylation. Our data support the notion that the anti-apoptotic activities of E6 are mediated by its ability to attenuate both transcriptional and translational responses induced by the PKR/eIF2α phosphorylation pathway. At the translational level, we show that inhibition of apoptosis is likely to be mediated, at least in part, by downregulating the pro-apoptotic Bax. Interestingly, a role of Bax in PKR-dependent apoptosis was previously described in mouse cells expressing a tetracycline (Tet)-inducible PKR (195). Mechanistically, it was proposed that increased Bax protein synthesis resembles the translational regulation of yeast GCN4 mRNA (141). That is, translation of GCN4 is controlled by the presence of 4 upstream open reading frames (uORFs) within the 5' untranslated region (UTR) of its mRNA (7). Induction of eIF2α phosphorylation facilitates the correct initiation at the GCN4 AUG codon leading to increased GCN4 protein synthesis (7). In analogy to GCN4, Bax mRNA possesses 3 upstream AUGs, all in frame with the authentic initiation codon, the first and third of which are followed by a termination codon (141). This striking similarity between GCN4 and Bax mRNAs has led to the hypothesis that the unusual 5' UTR of Bax plays a role in its translational induction upon eIF2α phosphorylation (141). It is also possible

that translation of anti-apoptotic genes is facilitated in FLAG-18 E6 cells and this may play a role in the inhibition of cell death by PKR activation.

2.4 E6, PKR and transcription

In addition to translation, we provide evidence for a role of E6 in PKR-mediated gene transcription and apoptosis. For example, transcription of several pro-apoptotic genes is induced in cells with activated GyrB-PKR (Table I). Expression of these genes is strongly suppressed by HPV-18 E6 and to a lesser degree by HPV-11 E6. These genes include GADD45, whose dependency on eIF2α phosphorylation was previously demonstrated in mouse cells containing a homozygous mutation at serine 51 phosphorylation site of eIF2α (eIF2αS51A) (279). Specifically, GADD45 transcription was induced 15-fold in ER-stressed cells from wild type mice but was completely abolished in knock-in cells with the eIF2αS51A mutation (279). Among the genes induced by GyrB-PKR, the melanoma differentiation-associated gene 7 (Mda-7) is an interesting target because of its strong apoptotic functions (340). It was recently shown that Mda-7 induces and activates PKR in lung cancer cells leading to the destruction of the tumor cells by apoptosis (341). The interferon regulatory factor 1 (*IRF1*) is another gene induced by GyrB-PKR activation (Table I). IRF1 is a protein with antiviral and tumor suppressor activities (294). Previous data provided evidence that transcription of *IRF1* is defective in a PKR null mouse (303) whereas the antiviral and anti-proliferative effects of IRF1 are mediated, at least in part, by the activation of PKR (190,342). It should be emphasized, however, that we do not as yet know whether transcription of all the genes in Table I is solely dependent on eIF2α phosphorylation. Inasmuch as PKR has been implicated in signaling to gene transcription through its functional interaction

with transcriptional factors (285), it is conceivable that transcriptional control of some of the above genes could be exerted independently of eIF2α phosphorylation. Based on the data presented in this section, we propose a model for functional interaction of HPV E6 oncoprotein and PKR (Fig. 38).

In addition to the data presented here, we have preliminary observations indicating antagonizing effect of HPV-18 E6 on PKR-mediated PI3K activation. Further analysis; however, is required to address the mechanism and the significance of this property of E6 oncoprotein.

Collectively, our findings provide strong evidence for a role of the oncogenic 18 E6 in gene translation and transcription modulated by the eIF2α phosphorylation pathway. Our data reveal a novel mechanism utilized by HPVs to bypass the translational blockade of eIF2α phosphorylation and the induction of an antiviral response by activated PKR. Although the role of eIF2α phosphorylation in virus-mediated tumorigenesis has already been established (285), the possibility that E6 affects various levels of translation in addition to eIF2α phosphorylation cannot be ruled out. Further understanding of the molecular functions of HPV oncoproteins in translational control and the identification of genes that are translationally regulated in HPV infected cells may prove helpful in the design of strategies to combat HPV infection and associated disease.

Figure 38. A model for regulation of PKR/eIF2α phosphorylation pathway by E6

During infection, HPV gene expression produces transcripts containing dsRNA structures able to activate PKR by autophosphorylation (step 1). Activated PKR then catalyzes the phosphorylation of eIF2α at serine 51 (step 2), an event that leads to the translational inhibition and induction of apoptosis (step 3). Although most of the genes are translationally repressed by eIF2α phosphorylation, translation of specific mRNAs is likely to escape from this general translational blockade. These mRNAs may encode for proteins that are involved in the inhibition of cell proliferation and induction of apoptosis. Translational inhibition by PKR rapidly downregulates 18 E6 protein synthesis (step 4). However, the remainder of 18 E6 is capable of counteracting this translational blockade by facilitating the dephosphorylation of eIF2α through the recruitment of GADD34/PP1 holophosphatase complex (step 5). This results in a translational relief that permits the expression of proteins with anti-apoptotic properties. This may represent an important mechanism utilized by the high-risk HPVs to counteract the antiviral properties of PKR activation and promote virus-mediated oncogenesis.

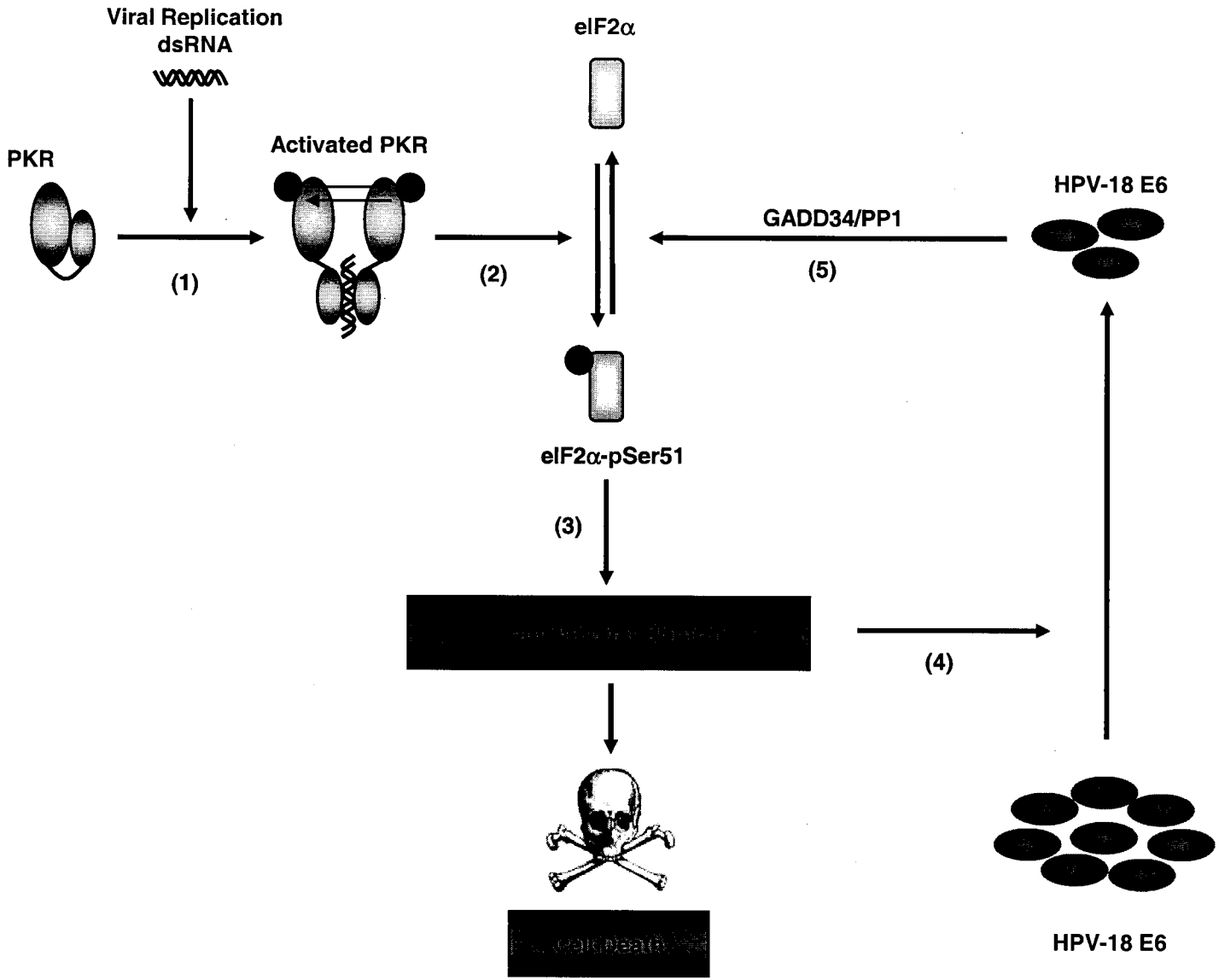

Viral Replication
dsRNA
PKR
Activated PKR
(1)
(2)
eIF2α
eIF2α-pSer51
(3)
(4)
(5)
GADD34/PP1
HPV-18 E6
HPV-18 E6

Figure 38

Chapter V

Contribution to the original knowledge

Chapter V - Contribution to the original knowledge

Research presented within this document has provided evidence for a novel function of PKR in activating the PI3K pathway. These studies are some of the first ones to demonstrate a role of PKR in growth stimulatory pathways. Moreover, it provides new insights into molecular mechanisms of HPV E6 to counteract the anti-proliferative and anti-viral functions of PKR. The candidate's major contributions are summarized as follows:

1. The candidate generated inducible cell lines, expressing GyrB-PKR fusion protein. This inducible cell line is provides an excellent tool for analyzing kinase-dependent functions of PKR, and since all eIF2α kinases share homologous kinase domain, this system could represent all eIF2α kinases. Presently, these cell lines are used in different projects in our laboratory and our collaborator's.

2. The candidate demonstrated for the first time, the activation of PI3K pathway by PKR in response to stress conditions and growth factors. These experiments demonstrated that PKR induces PI3K lipid and protein kinase activity and induces the downstream targets involved in cell growth and survival. These studies provide further evidence for dual activity of PKR in regulating cell growth and apoptosis.

3. Studies conducted by the candidate shows, for the first time, colocalization of 4E-BP1 with other initiation factors to the stress granules formed in cytoplasm upon PKR activation. These data explains the association of 4E-BP1 with eIF4E despite 4E-

BP1 phosphorylation and dominant inhibitory effect of eIF2α phosphorylation on translation.

4. In order to address its mechanism of evasion of the host immune system and antiviral defense, the candidate has contributed to the studies of functional interaction between HPV E6 oncoprotein and the components of IFN signaling pathway, and the regulation of E6 expression and its stability. The contents of these studies are beyond the scope of this document.

5. The candidate was first to investigate molecular mechanisms of HPV to combat translational inhibitory and apoptotic functions of PKR and eIF2α phosphorylation. Moreover, microrray analysis performed by the candidate demonstrates that HPV mitigates transcriptional functions of PKR.

6. Experiments performed by the candidate demonstrated that HPV E6 oncoprotein exerts antagonizing effect on eIF2α phosphorylation pathway by recruiting GADD34/PP1 phosphatase and promoting eIF2α dephosphorylation.

Chapter VI

References

Chapter VI - References

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