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Regulation of PKR- the Ras connection

by

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ABSTRACT

This investigation, with the help of reovirus, is a study the Ras-dependent regulation of the interferon-induced dsRNA-activated protein kinase, PKR. Ras is a major player in transduction of external signals to the nucleus, while PKR central in control of cellular growth, differentiation and apoptosis.

We confirm a number of earlier observations and shed light on the Ras-PKR connection, showing a level of regulation that has not been determined earlier. Cells with activated Ras and those with low PKR activity exhibit higher susceptibility to reovirus infection than those with normal activity levels. For the first time, this investigation shows that in susceptable cells, instead of preventing PKR autophosphorylation, the activated Ras reverses it by directly or indirectly dephosphorylating the phospho-PKR. In order to show this, a novel assay was developed which showed that the extent of dephosphorylation correlated directly with the susceptibility of the cell line to reovirus infection. The final observation of this investigation involved the phosphorylation of higher molecular weight bands only in those lysates where PKR was being dephosphorylated. This may suggest an intermediate step between Ras and PKR.

This regulation mechanism unites two crucial cellular regulatory pathways in a mutually dependent, sensitively controlled regulatory system involving growth factors, transcriptional and translational regulation as well as cellular differentiation and programmed cell death. Strong evidence supports the involvement of both of the proteins in a large number of human cancers.

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Eternal respect for Emilia and Jeffina (past residents of the animal research facility) in recognition of the sacrifice they made to help produce the antibodies required for the work in our lab, including the work done for this thesis.

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To my mother - my best friend.

Carpe diem.
Horace

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LIST OF ABBREVIATIONS

The following abbr. were used in the text:

10T½	C3H 10T½
Abbr.	abbreviations
ADP	Adenosine-5'-diphosphate
ATCC	American Tissue Culture Collection
ATP	Adenosine-5'-triphosphate
BSA	bovine serum albumin
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DTT	dithiothreitol
EBER	Epstein-Barr XXX RNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eIF-2α	eukaryotic initiation factor 2, α subunit
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FBS	fetal bovine serum
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IFN	interferon
IFN-β-R	interferon-β receptor
IPTG	isopropyl-1-thio-β-D-galactoside
ISG	interferon stimulated genes
iv kinase	*in vitro* kinase
JNK	c-Jun amino-terminal kinase
KSR	kinase suppressor of Ras
LB-broth	
MAP	Mitogen-activated protein
MAPK	MAP kinase
met	methionine
MOI	multiplicity of infection
M_r	relative molecular weight
mRNA	messenger RNA
EGF	epidermal growth factor

NBS	newborn bovine serum
NF-κB	nuclear factor -κB
NIH	National Institute of Health
NP-40	noniped-40
NS	non-structural
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFU	plaque forming units
PKC	protein kinase C
PKR	dsRNA-activated protein kinase
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
PSB	protein sample buffer
Reo3	reovirus serotype 3, Dearing strain
-Reo3	un-infected with reovirus
+Reo3	infected with reovirus
RIKI	Ras induced kinase inhibitor
RIP	radioimmunoprecipitation
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SOC	
Sos	Son of sevenless
TAR	
TE	Tris/EDTA (buffer)
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TENS	Tris/EDTA/
TIK	
TNFα	tumor necrosis factor, the α subunit
tRNA	transfer RNA
UV	ultraviolet
VAI RNA	virus associated RNA I
Y	tyrosine

Chapter 1: Introduction

1.1 General Cancer Research

Over the last century our society has witnessed the development and increased sophistication of cancer research leading to a continuously growing understanding of the processes involved in the origin, advance, and spread of this disease. It has become more than obvious that the better we understand Cancer, the stronger our artillery of weapons against it.

The first half of the century was mostly devoted to the study of the comparative biology of cancer: its definition, histology as well as structural and behavioural studies of tumour growth and development. This period of investigation resulted in establishment of general standards of tumour grading that are in use in clinical oncology as well as patient management to date.

Then, the focus shifted to the more in depth characterization of the mechanisms of carcinogenesis, tumour progression and metastasis. The fundamental concepts of two-stage carcinogenesis, metastatic cascade, oncogenes and tumour suppressors emerged during this second phase of cancer research. Genetic and environmental risk factors also began to be considered.

Finally, over the last two or three decades researchers have focused on the molecular and biochemical events involved in carcinogenesis, metastasis.

Researchers have also realized the importance of fully elucidating the regulation of cell growth, differentiation, and apoptosis in normal cells to the comprehensive understanding of cancer. The 'exponential growth' of the body of knowledge during this last phase has opened the door for a myriad of new, revolutionary therapies of the merciless disease that has touched the life of every person alive today (Sherbet and Lakshmi, 1997).

Molecular virology has played a major role not only in characterizing a large number of human (and non-human) viral pathogens and the events that occur in the host cell during infection, but also served as a pool of new methods and techniques that have been vitally important in the study of normal cellular processes. For instance, studies of reovirus, baculovirus, herpesvirus, adenovirus and countless number of other viruses greatly contributed to defining and characterizing molecular events in every part of normal and abnormal cellular regulation, notably leading to promising viral-based treatments of cancer.

1.2 Reovirus

Mammalian reoviruses are common agents found in water sources and infect a large range of mammalian species. The first three letters of the name reovirus are an acronym for respiratory enteric orphan representing its possible routs of infection as well as its lack of association with any serious human disease (Sabin, 1959). In fact, during the polio scare in the early 1960's, due to some similarity under the electron microscope, symptoms of reovirus infection through inhalation were examined on twenty-seven 'volunteers' in an American prison. Twenty-five of the twenty seven prisoners exhibited no major noticeable symptoms, whereas the remaining two individuals were reported to have mild cold and light flu-like symptoms (Rosen *et al.*, 1963). This ratio is consistent with the fact that over 80% of adults in America have been exposed to reovirus, as determined by the presence of protective anti-reovirus antibody in the bloodstream (Minuk *et al.*, 1985).

This investigation utilizes the Dearing strain of reovirus serotype 3, genus *Orthoreovirus*, family *Reoviridae*. Dearing strain was originally isolated from a child with diarrhea in late 1950's and after years of extensive study its 24kb segmented genome is completely sequenced, and its structure and replication cycle are relatively well characterized (review in Nibert et al., 1996 and references therein).

Briefly, the ten linear segments of the reovirus dsRNA genome code for eleven viral proteins; eight of them are structural (present in the mature virion) and three are non-structural protein that play a role in viral replication. The genomic segments and their products are grouped into three classes according to size L, M, S and λ, μ, σ, respectively. Reovirus dsRNA genome is tightly packed in two concentric non-enveloped icosahedral capsids. Though it has been shown that non-genomic, short, single-stranded oligonucleotides compose roughly a quarter of the total RNA in the virion, their function and exact location is still a topic for debate.

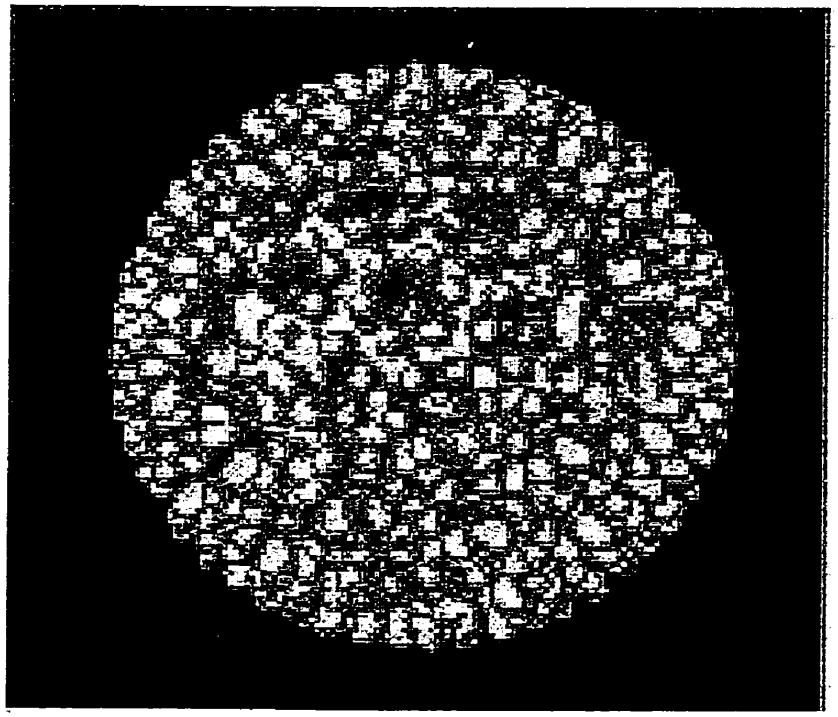
The inner capsid is thought to serve as a safety barrier between the genomic dsRNA and external or cellular factors. This protective, relatively impermeable protein shell consists of over 120 copies of each of the dsRNA binding proteins σ2 and λ1. Minor structural proteins, λ3 and μ2 are localized near each of the five-fold axes of the inner capsid, totaling only about 12 copies each (Figure 1B).

A much more conformationally flexible and penetrable outer capsid is composed of 60 copies of λ2, 600 copies of μ1 and, more peripherally, 36-48 copies of σ1 and 600 copies of σ3 (Figure 1A). Most of the μ1 is found around large axial solvent channels and are stabilized by cross-binding σ3 molecules. λ2 assembles into the spikes that protrude from the surface of the core to the

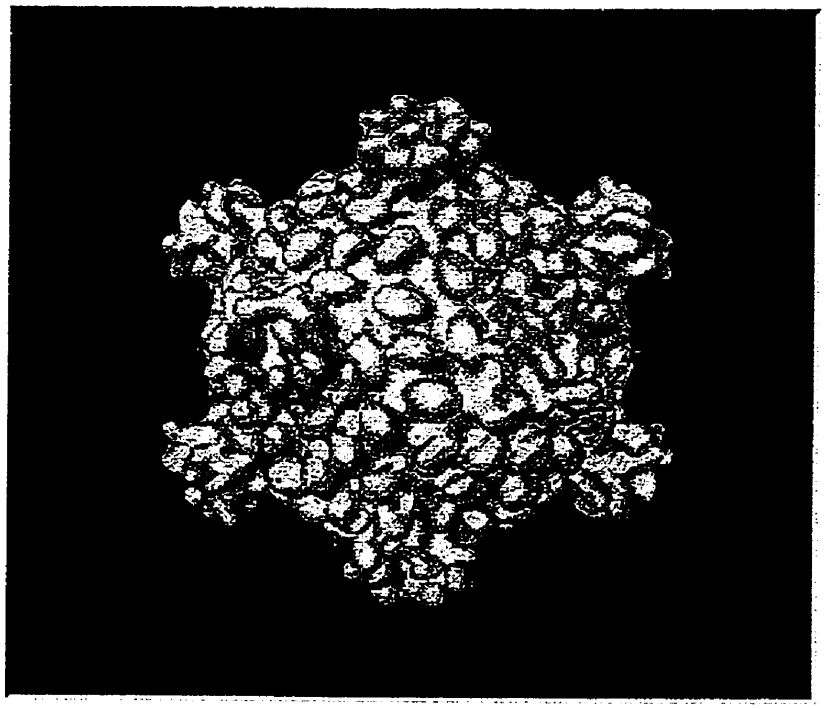
Figure 1:

Computer image of the proposed reovirus structure. A. Outer capsid, B. Core of the reovirus virion.

A



B



periphery. Each of these spikes encompasses an 8nm axial channel and are closely associated with the 40nm long fibers that project from the virion surface. The fibers have a head-and-tail morphology, consist of σ1 trimers and act as the cell attachment proteins, not seen in Figure 1.

Reovirus virion is stable for extended periods of time, in aerosols, in the presence of organic solvents and detergents, at pH values between 2 and 9, extreme ionic strengths as well as temperatures approaching 55ºC. Infectivity decreases with prolonged exposure to ultraviolet radiation, however.

The events involved in the productive infection cycle of reovirus were also described in detail in Nibert et al., 1996 and are illustrated in Figure 2 taken from that publication. In brief, the virion attaches to the receptor molecules on the cell surface through its σ1 proteins and is taken into the cellular vacuoles by receptor-mediated endocytosis. Within these endo/lysosome-like vacuoles, acid dependent proteolysis cleaves the outer capsid proteins, leaving the inner capsid in tact. Membrane interaction and penetration into the cytoplasm occur simultaneously with initiation of primary transcription of the genomic RNA, by 2 hours after infection. The resulting transcripts are capped and serve as templates for the translation of viral proteins by the host translational machinery. Between 2 and 10 hours post-infection, primary transcripts assemble with the newly made proteins to form "RNA assortment complexes"

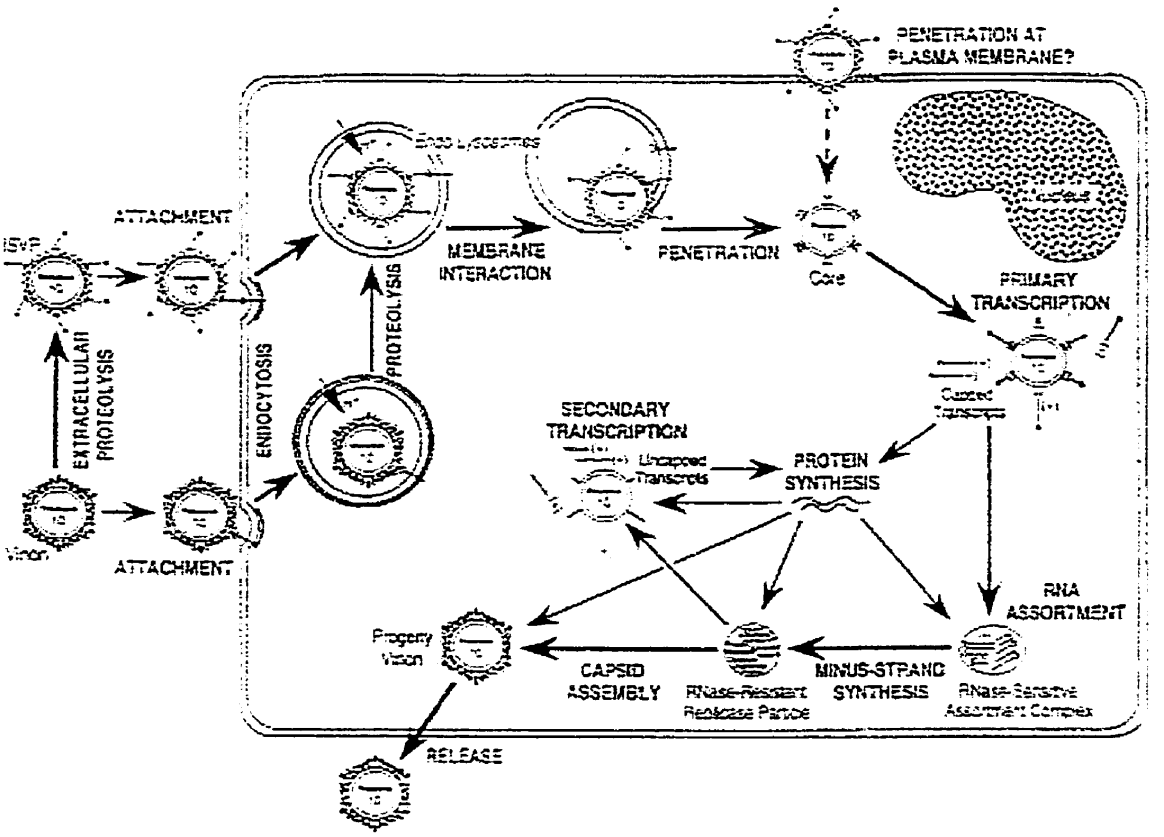


Figure 2: Reovirus replication cycle. (Nibert *et al.*, 1996)

and serve as templates for the synthesis of the minus strands. These subviral particles have been shown to initiate secondary transcription producing uncapped mRNA, which may be used for translation later in the infection (at about 12 hours). The exact events of virion assembly (assembly of the outer capsid) are not yet completely elucidated. Cell lysis inefficiently releases the mature virions from the infected cell.

Having described the viral events that occur during infection, we now turn our attention to the concurrent cellular events. However, when discussing intercellular events that are induced by reovirus entry into the cell, we must be very careful in first establishing whether this cell can support productive viral infection. This differentiation occurs at the level of translation, since Matt Coffey (in our lab) has shown that transcription occurs equally regardless of the final outcome of the infection (Strong *et al.*, 1998).

First, let us focus on the cellular events in permissive cells, newly synthesized viral proteins can be detected a few hours after infection, and at late stages the infected cell translates viral proteins almost exclusively (Zweerink and Joklik, 1970). Reovirus replication is completely cytoplasmic and normally induces inhibition of DNA, RNA and protein synthesis (Cox and Shaw, 1974), alterations of cytoskeleton, cell membrane integrity disruption, perturbation of signal transduction pathways as well as apoptosis (reviewed in Yue and Shatkin, 1998). During infection process, cells develop non-membrane bound inclusions

(viral factories) that are originally scattered in the cytoplasm and then increase and migrate towards the nucleus. The viral factories are composed of viral dsRNA, viral proteins and viral particles at different stages of assembly. The mechanism of the migration of the viral inclusions is not clear, however, rearrangement of intermediate filaments occurs during infection. This may contribute to cellular injury and thus the cytopathic effect observed in susceptible cells (reviewed in Nibert *et al.*, 1996).

Proportional to the multiplicity of infection is the selective inhibition of host DNA, RNA and protein synthesis, all of which can be detected at about 8 – 12 hours after infection. Ongoing studies are attempting to clarify the roles of σ1 and σ3 in host DNA and RNA synthesis inhibition, respectively. The basis of differential rates of translation are also being heavily investigated. There are suggestions that this effect is due to compartmentalization of infection or to the increase in viral RNAs which dramatically dilute the host mRNA in the cytosol and thus monopolize host translational apparatus. Some labs have shown that late in infection host uncapped RNAs are translated more efficiently, attributing this switch to σ3. Opposing results exist showing no difference between the translational efficiency of capped and uncapped RNAs in reovirus infected cells. (Reviewed in Nibert *et al.*, 1996 & references therein) A large amount of work is still required before these processes are fully elucidated.

As with many other viruses, reoviruses induce expression of interferon which inhibits the spread and efficiency of reovirus infection. Interferon has long been shown to activate three branches of anti-viral defense mechanism: proteins Mx, 2',5'-oligoadenylate synthetase (and its downstream effector RNAse L) and the dsRNA-activated eIF-2α protein kinase (PKR).

Since Mx proteins are only involved in anti-viral response to influenza and vesicular stomatitis viruses and 2',5'-oligoadenylate synthetase to picornavirus, and have no effect on reovirus infection, they will not be discussed further in this thesis. The third branch of interferon-induced anti-viral response plays a major role in the fate of infection of a wide range of viruses: adenovirus, HIV, influenza, polio, vaccinia and reovirus to name just a few. (Samuel, 1988). PKR is discussed in much more detail in the following section.

1.3 PKR

The interferon-induced RNA-dependent serine/threonine protein kinase (PKR) has recently been named so, eliminating a vast collection of previous names such as p68/p65 kinase (for human and murine analogues, respectively), DAI, dsI, dsRNA-PK, PK_{ds}, P1 kinase, P1/eIF-2α kinase , PRKR (Clemens *et al.*, 1993). The GenBank accession number of murine PKR is M35663 (Barber *et al.*, 1993).

PKR is found in most cell types in latent cytosolic and nuclear form (Wu and Kaufman, 1996). It was originally characterized as part of a major branch of the cellular antiviral defense. In the last few years, however, its involvement has been demonstrated not only in antiviral pathways, but also in cell cycle progression and cell growth, differentiation, stress-induced apoptosis, and tumour suppression, regulation of transcription, mRNA splicing, translation and cytokine signaling (Bevilacqua *et al.*, 1998, Srivastava *et al.*, 1998). This is in part supported by the fact that over-expression of wild-type PKR inhibits protein synthesis and cell growth (Dever, *et al.*, 1993), increases the murine cell sensitivity to apoptosis upon influenza infection (Balachandran *et al.*, 2000), enhances splicing efficiency of transcripts containing 2-APRE (Osman *et al.*, 1999). Over-expression of catalytically inactive PKR mutants transforms NIH-3T3 cells to induce tumours in nude mice (Barber *et al.*, 1995), interferes with the differentiation of myogenic cells (Salzberg *et al.*, 2000). PKR kinase activity

levels vary cyclically throughout the cell cycle of human glioblastoma cells, reaching two peaks in early G1 and at the G1/S boundary, and decline drastically in early S phase (Zamanian-Daryoush *et al.*, 1999). Clinically, low activity levels of PKR correlate with increased proliferation of several human tumours (Savinova *et al.*, 1999).

The gene that codes for PKR is found on chromosome 2p21-22 in humans and 17 E2 in mice (Barber *et al.*, 1993). Its promoter contains a large number of potential regulatory elements including ISRE (interferon α/β stimulated response element), as well as the GAS (γ-interferon activation site), NF-IL6 (IL6 sensitive), KCS (a novel 15-bp DNA kinase conserved sequence), NRD (40-bp negative regulatory domain) (Kuhen and Samuel, 1999), APRF and NF-κB elements (Kuhen and Samuel, 1997). PKR mRNA is 2.5 kb long and is strongly induced by IFN-β. It is detectable as early as 2 hours post stimulation and is maximal at 16 hours. Other factors that have been shown to regulate *Pkr* transcription are IRF-1 (activator), TNF-α and TGF-β (inhibitors) (Nguyen *et al.*, 1997; Yeung *et al.*, 1996; Salzberg *et al.*, 1995).

As a protein, PKR is a ubiquitously expressed protein in mammalian cells under normal growth conditions, with the nuclear to cytoplasmic PKR ratio of 1:5. Its half life is about 3-4 hours. Upon IFN-β treatment, PKR in the cytoplasm increases 3-10 fold peaking between 16 and 24 hours post induction, but

Figure 3: Domain structure of the PKR protein

remains constant in the nucleus (Meurs *et al.*, 1990). The predicted molecular weight, based on PKR length of 551 amino acids is 62,016 Da (Meurs *et al.*, 1990) however, due to clusters of charged amino acids throughout the protein, the apparent molecular weight (M_r) of the ^{35}S-methionine labelled PKR is 68,000 daltons, and that of the more acidic phosphorylated form is 70,000 daltons (Laurent *et al.*, 1985).

PKR contains neither leucine zipper domains, nor zinc finger motif that characterize nucleic acid binding proteins (Meurs *et al.*, 1990). Instead, the N-terminus of PKR binds dsRNA through a pair of tandem copies of the 65-68 residue double-stranded RNA-binding domains (dsRBD) which are conserved in a diverse family of dsRNA-binding proteins (Kharrat *et al.*, 1995). This dsRNA binding is sequence-independent and occurs through specific recognition of the 2'-OH functional groups on the two strands of the dsRNA helix and their binding to the mainly basic amino acids of the dsRBD (Bevilacqua and Cech, 1996).

The C-terminus contains the catalytic kinase domain composed of 11 kinase subdomains that are highly conserved among the proteins of the serine/threonine protein kinase subfamily (Hanks and Hunter, 1995). This region contains the ATP - binding site and the invariable lysine 296 which is imperative for phosphate transfer (Meurs *et al.*, 1990) (Figure 3).

The initial activation event is dimerization of PKR which occurs when two PKR molecules bind adjacently on one stretch of dsRNA. RNA binding may induce a conformational change alleviating the negative regulatory effect of the N-terminal domain, as well as bringing two PKR molecules into proximity promoting its activation through trans-phosphorylation (Thomis and Samuel, 1993).

It is not surprising that viruses with dsRNA genomes are likely to activate PKR upon entering the potential host cell. In addition to dsRNA viruses, a large number of ssRNA and DNA viruses go through the stage of complementary RNA synthesis during genome replication or overlapping transcription of genes that are encoded on opposite DNA strands, respectively. Although it is apparent why these viruses are generally very sensitive to the PKR dependent anti-viral response, we must also realize that 'life must go on'. Viruses have thus developed a wide range of strategies to overcome this defense system. Some have evolved to utilize cellular proteins to down-regulate PKR, others code for their own proteins to play that vital role. What follows is a discussion of a number of levels of cellular regulatory mechanisms of PKR and their exploitation by viruses.

A variety of dsRNAs, single stranded RNAs with extensive double stranded regions and other agents like heparin, can activate PKR. It has recently

been found that dsRNAs containing non-Watson-Crick domains such as A-G mismatches can also regulate activity of PKR (Bevilacqua *et al.*, 1998).

The efficiency of the dimerization step is dependent on the concentration as well as the length of the activating dsRNA. There exists an optimal dsRNA length and concentration, deviations from which decrease the efficiency of dimerization of PKR (Figure 4). As discussed above, binding to a single stretch of dsRNA is thought to induce a conformational change and bring two molecules of PKR into proximity *in vitro*. If the segment of dsRNA is too short, it can accommodate the binding site of only one molecule and if it is too long, the two molecules can bind anywhere along the length of the dsRNA. In neither case does the dsRNA region contribute to the proximity of the two PKR molecules. Similarly, if the concentration of dsRNA is too low, only a small proportion of PKR dimerizes, and if it is too high, each PKR molecule can bind its own dsRNA without creating any dimers (Wu and Kaufman, 1997). It follows that from the point of view of the cell (or virus) the fastest and most energy efficient way to inhibit PKR dimerization is to produce high amounts of short dsRNA. Evolution seems to have made it so. Both the cell and the viruses take advantage of this situation.

Concentration of a number of host dsRNA species has been shown to be regulated by extracellular conditions. For instance, cell stress and translational inhibition increase the level of RNA polymerase III genes, SINE and *Alu* RNA (Liu

et al., 1995). Considering that both of these RNA species contain short regions of extensive secondary structure, it is not unexpected that they inhibit PKR activation *in vivo* (Chu *et al.*, 1998). Elegantly, adenovirus (Panning and Smiley, 1993), HIV (Jang *et al.*, 1992) and HSV (Jang and Latchman, 1992) increases host *Alu* and SINE RNA expression upon infection by activating cellular TFIIIC (Jang *et al.*, 1992).

Adenovirus and Epstein-Barr virus also adopt a much more forward strategy of increasing dsRNA levels to inhibitory concentrations. These viruses code for their own species of RNA with extensive secondary structure: adenovirus VA RNAs (Katze *et al.*, 1987) and Epstein-Barr EBER RNAs (Sharp *et al.*, 1993). Extremely high levels of these dsRNAs are synthesized early during infection. These short dsRNAs bind and inhibit PKR, and hypothetically remove it from the vicinity of translational apparatus by displacing it from the ribosomes (Kaufman, 1999). During infection with mutant viruses lacking the genes encoding these viral RNAs both host and viral protein synthesis is inhibited and PKR is highly phosphorylated (Mathews and Shenk, 1991). It is intriguing that adenovirus VA RNAs and Epstein-Barr EBER RNAs are also transcribed by host RNA polymerase III as are SINE and *Alu* RNAs. HIV also regulates PKR at this step, however instead of quantity of binding it uses the fact that Tar (transactivator responsive) RNA region interacts strongly with PKR preventing its activation by other dsRNAs (Brand *et al.*, 1997).

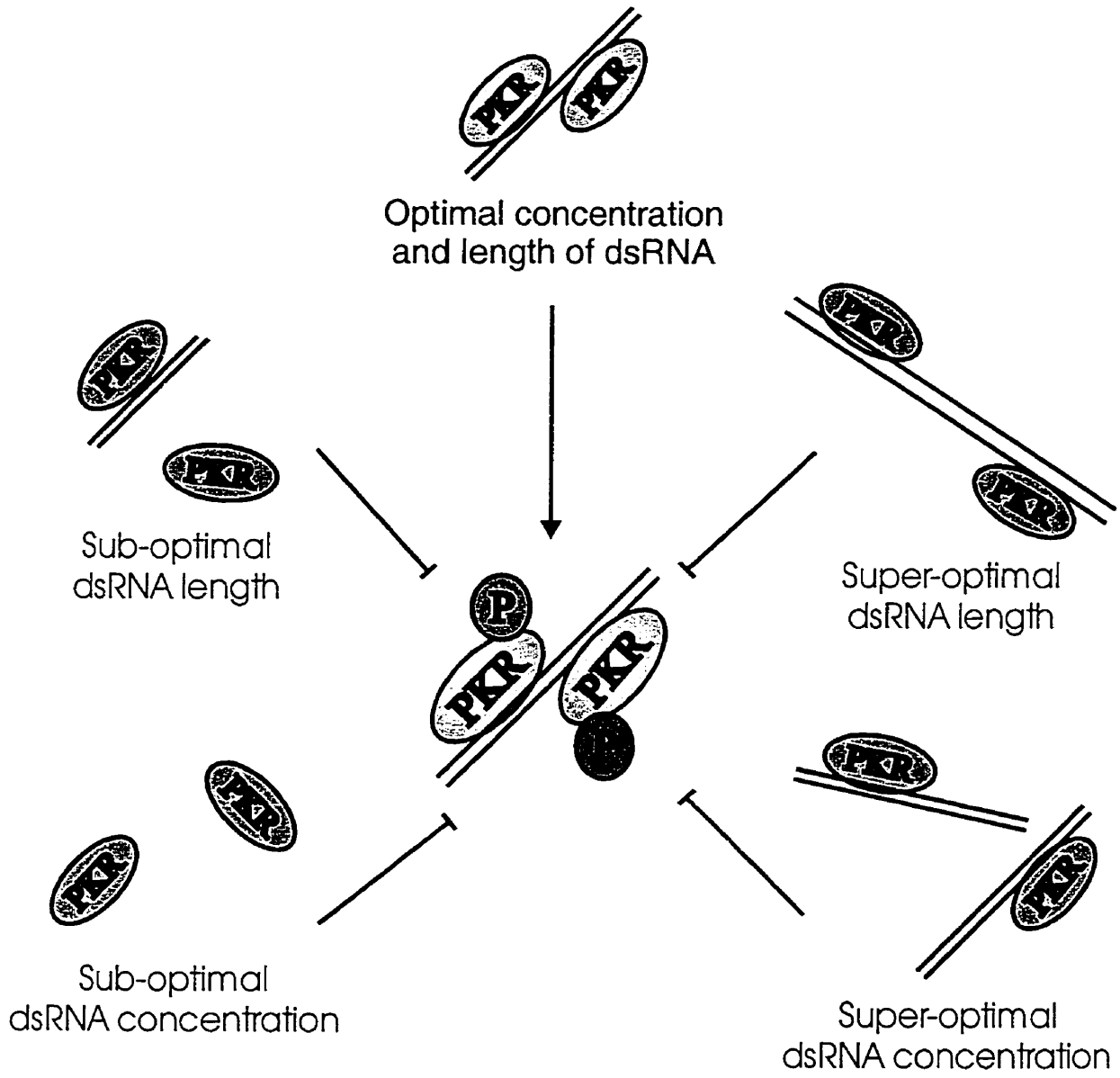


Figure 4: Regulation of PKR by dsRNA

Ribosomes abrogate dsRNA binding by competing with the dsRNA for its binding site on PKR. Ribosomes bind PKR at the dsRBDs and thus localize it to the translational machinery in the cytosol in the absence of dsRNA (Zhu *et al.*, 1997). Upon introduction of activating RNA, with higher affinity for dsRBD than ribosomes, PKR binds presumably dsRNA and becomes activated. Since it is already in the proximity of the translational machinery the response time is much shorter than if it was randomly distributed throughout the cytosol.

The final method of inhibiting PKR dimerization by dsRNA is to eliminate or sequester the activating dsRNA. Any dsRNA binding protein will sequester the limiting dsRNA and thus act as an antagonist of dsRNA-activated proteins, including that of PKR. For instance, a functionally unrelated La antigen inhibits PKR activation when expressed in cells just by the virtue of binding up dsRNA (James *et al.*, 1999). A number of viruses utilize this particular strategy to help during infection. For instance, influenza NS1, reovirus σ3 and vaccinia virus E3L proteins are dsRNA binding proteins that may play roles in inhibiting PKR during infection (Imani and Jacobs, 1988; Kaufman, 1999).

Upon successful dimerization, PKR autophosphorylates in a transmolecular fashion. Autophosphorylation sites that are absolutely required for kinase activity of PKR are Thr-446 and Thr-451 (Romano *et al.*, 1998). Some of the residues whose phosphorylation is much less critical to PKR function are at

Ser-342, Thr-255 and Thr-258 (Taylor *et al.*, 1996) and phosphorylation of these sites does not significantly alter PKR activity.

This step of PKR activation can be inhibited by proteins with homology to the PKR phosphorylation sites as well as phosphatases that can immediately dephosphorylate PKR. Hepatitis C virus envelope protein E2 contains regions that are identical to the PKR phosphorylation sites (Taylor *et al.*, 1999) whereas Herpes Simplex Virus redirects the specificity of the cellular phosphatase PP1 to dephosphorylate PKR (Pain, 1996).

Other modes of activation of PKR may also exist such as phosphorylation of PKR by other proteins, or generation of a catalytically active fragmented kinase domain of PKR. For instance, trimerization of the TNFα receptor may activate the TNFα receptor-interacting protein RIP, which may go on to phosphorylate and activate PKR (Stranger *et al.*, 1995). Alternatively, activation of a protease (such as the interleukin-1 converting enzyme-like protease) in response to cellular stress inducers may cleave the dsRNA binding regulatory domain of PKR, generating the constitutively active kinase domain (Wu and Kaufman, 1996)

Once activated, PKR phosphorylates a number of substrates (Pavon and Esteban, 1999) including the α subunit of the protein synthesis initiation factor 2 (eIF-2α), inhibitor of NF-κB (IκB) (Yang *et al.*, 1995), two newly identified dsRNA

binding nuclear protein (DRBP76 and NF90) (Patel *et al.*, 1999; Langland *et al.*, 1999), the tumor suppressor p53 (Cuddihy *et al.*, 1999) and histones (for review see Tan and Katze, 1999).

Perhaps the best characterized role of PKR, is in control of translation. PKR dependent inhibition of translation initiation is mediated by phosphorylation of the alpha subunit of the eukaryotic translations initiation factor 2. eIF-2α can be phosphorylated on residue 51 whether it is a wild type serine, or substituted threonine or tyrosine (Lu *et al.*, 1999). There are three other eIF-2α kinases besides PKR. Heme-regulated inhibitor, HRI phosphorylates eIF-2α in reticulocytes under heme-deficient and heat shock. Pancreatic eIF-2α kinase, PEK, is predominantly expressed in pancreas cells. The youngest addition to the family is the PKR-like ER kinase, PERK which is activated in response to ER stress (reviewed in Harding *et al.*, 1999) (Figure 5). Considering that this investigation is not concerned with any of the cell types expressing the other members of the eIF-2α kinase family, they will not be discussed further.

Normally, translation initiates by the formation of the ternary complex: heterotrimeric eIF-2, GTP, and initiator met-tRNA. The ternary complex binds the 40S ribosomal subunit, then the RNA binds, followed by the 60S subunit and hydrolysis of GTP. The GDP bound to the eIF-2α must then be exchanged for GTP for the next round of initiation to occur. This is catalyzed by the empirically

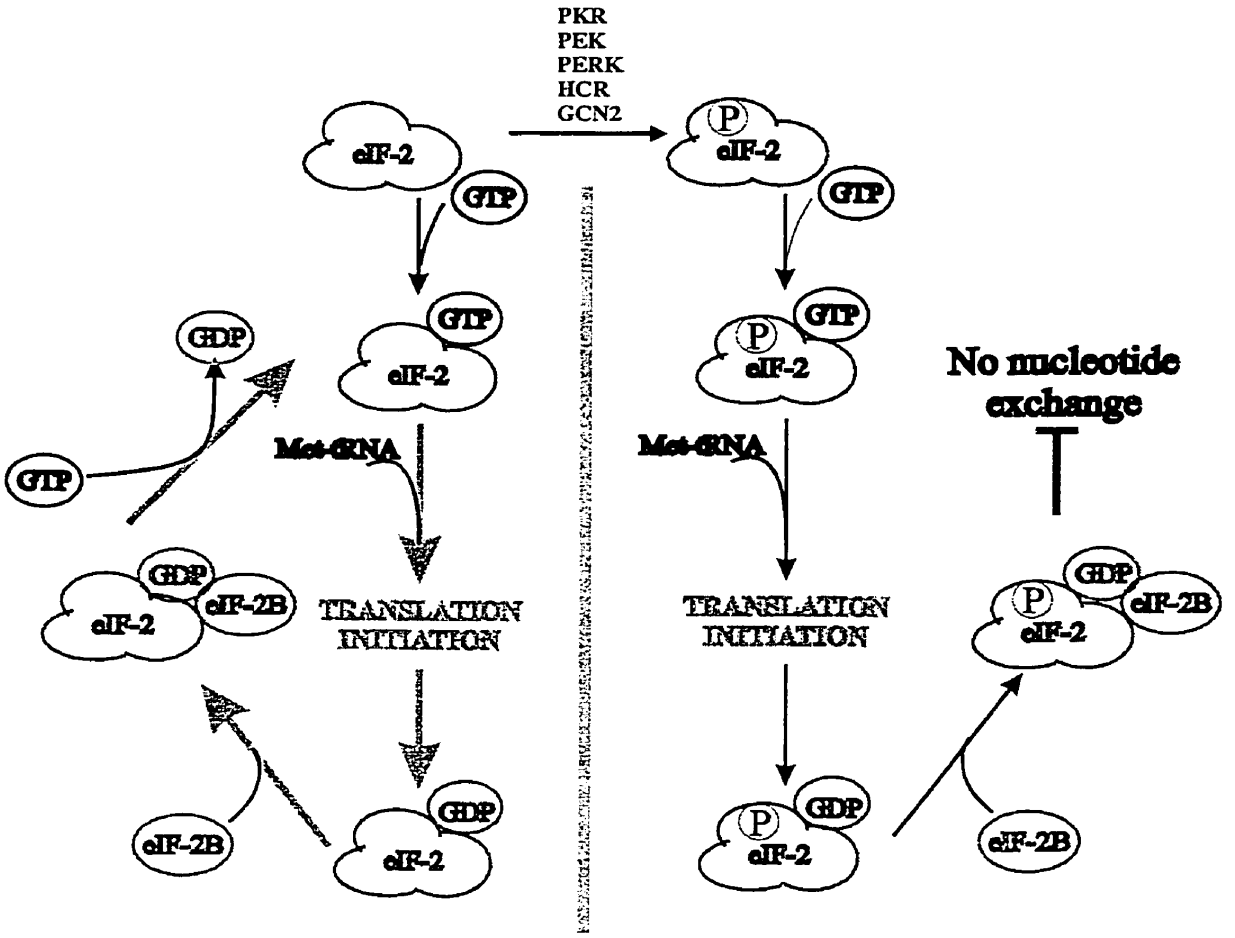


Figure 5: Schematic of eIF-2α phosphorylation dependent regulation of protein synthesis initiation. (adapted from Clemens and Bommer, 1999)

limiting eIF-2B, guanine nucleotide exchange factor. Phosphorylation of eIF-2α stabilizes its complex with GDP and eIF-2B, transforming it from a substrate to a potent competitive inhibitor of eIF-2B and thus preventing the next round of translation initiation. Since the molecular amount of eIF-2B in the cell is much smaller than eIF-2α, only 10-20% of the eIF-2α needs to be phosphorylated to sequester all of eIF-2B and shut down protein synthesis (Safer, 1983) (Figure 5).

Thus occurs the best studied cellular anti-viral response. Not surprisingly, there are also examples of viral inhibition of PKR at this step of regulation. For example, vaccinia virus K3L possesses homology to the phosphorylation site of eIF-2α and this acts as a competitor for the PKR (Katze, 1995) whereas herpes simplex virus $\gamma_1$34.5 protein interacts with protein phosphatase 1 and redirects its activity to dephosphorylate eIF-2α (Cassady *et al.*, 1998).

There are also other methods of inhibiting PKR activation that some more 'inventive' viruses have developed through evolution. Infection with the poliovirus for instance induces degradation of PKR (Black *et al.*, 1989), whereas related encephalomyocarditis virus alters the sub-cellular localization of PKR allegedly minimizing its effect on viral protein synthesis (Dubois and Hovanessian, 1990).

Reovirus infection also induces production of cytokines such as interferons followed by activation of cellular antiviral defense mechanisms (Lloyd and Shatkin, 1992). Although it codes for some transcriptional activities, Reo3 is

completely dependent on cellular translational machinery and is, therefore, extremely sensitive to these host antiviral strategies. Strong *et al.* (1998) observed that in all of the cell lines tested, exposure of non-permissive cells to reovirus was always accompanied by phosphorylation of the 68 kDa protein, which remained unphosphorylated during infection of susceptible cells. This phosphoprotein was shown to be immunoprecipitated by anti-PKR antibodies as well as Poly(I)-poly(C) agarose beads, suggesting that it is in fact PKR (Strong *et al.*, 1998). Thus PKR is activated by reovirus infection and plays a pivotal role in rendering some cells non-susceptible to infection. It has also been shown that PKR -/- cells are much more susceptible to reovirus infection than are normal PKR +/+ cells (Strong *et al.*, 1998). General events occurring during exposure of non-susceptible cells to live reovirus is visually depicted in Figure 6.

Briefly, reovirus is internalized, uncoated and undergoes primary transcription which expels viral transcripts into the cytoplasm of the host cell. S1 mRNA contains significant secondary structure which is sufficient to activate PKR and block initiation of translation (reviewed in Nibert, 1996). This is the level at which the infectious process is halted in non-susceptible cells. A different scenario occurs in cells with an activated Ras pathway. This is further discussed in the next section.

1.4 Ras Pathway

Ras is a central component of signal transduction pathway between extracellular stimuli that induce proliferation and differentiation and the nucleus. *Ras* is a highly conserved proto-oncogene in all eukaryotes examined to date (Bollag and McCormick, 1991) and activating mutations of Ras have been attributed to over 30% of human cancers: carcinoma of the colon, pancreas, lung, thyroid, lymphoma, leukemia, melanoma, adenocarcinoma, seminoma and myeloproliferative disorders (Sakorafas *et al.*, 2000). A recent study of over 2600 patients reported a correlation of *ras* gene mutations with higher tumour recurrence after treatment as well as poor prognosis (Rosen and Sepp-Lorenzino, 1997). An even larger group of cancers exhibit enhanced Ras activity (without direct mutations in the genes) induced by activating mutations in its upstream regulators: ovarian, breast, cervical, bladder, stomach and esophageal cancers, glioblastomas, and neuroblastomas. Activated Ras induces expression of the surface glycoprotein CD44 (Hoffman *et al.*, 1994; Aunoble *et al.*, 2000; Rabbani and Cordon-Cardo, 2000), which has been associated with increased metastatic potential. Ras has been suggested to not only stimulate cellular proliferation in cancers, but also contribute to genetic instability (Oliff and Friend, 1997) and in some cases cause radioresistance leading to inefficacy of traditional cancer treatments (Gibbs and Oliff, 1997).

By the virtue of its apparent importance in cancers, Ras has been the target for a number of developing cancer therapies (reviewed in Pincus *et al.*, 2000). Some therapeutics involve small inhibitory molecules that interfere with Ras binding to the effectors and are at the basic stages of research. Better developed treatments that are being clinically tested are based on introducing *ras* anti-sense (Cowsert, 1997) and interfering with posttranslational modifications of Ras (Gibbs *et al.*, 1996).

Ras proteins are a superfamily of 50 to 60 small guanine-nucleotide binding proteins that are active in a GTP-bound state. There are six main subfamilies in the Ras superfamily that are defined according to their sequence and functional homology: Rac/Rho, Rab/Ypt, Ran, Arf, Rad, and Ras subfamilies (Wittinghofer and Herrmann, 1995). Ras proteins function to transduce signals from the receptor tyrosine kinases to a cascade of serine/threonine kinases that control growth, cellular proliferation and differentiation. N-*ras*, K-*ras*, and H-*ras* are closely related, and code for protein products ~21 kDa and known collectively as $p21^{ras}$. The H-*ras* and K-*ras* genes have constitutively active v-*ras* counterparts in Harvey and Kirsten strains of murine sarcoma virus, respectively. v-H-*ras* has a mutation at position 12 which leads to its constitutive activity.

One of the very well characterized signaling pathways upstream of Ras is dependent on the epidermal growth factor (EGF). The EGF bind its receptors (EGFR) which then dimerizes. The two molecules trans-phosphorylate each

other on tyrosine residues creating a docking site for the SH2-domain containing Grb2. This localizes Grb2 at the membrane along with Sos, a guanine nucleotide exchange factor which is normally bound to Grb2 (Cox and Der, 1997).

Without external signal, Ras is found in the inactive form, tightly bound to GDP. However, guanine-nucleotide exchange factors such as Sos, CDC25 and C3G accelerate the release of GDP, and allow binding of the more abundant cellular GTP (Cox and Der, 1997). Nine amino acids in the N-terminus of Ras are identical in all of the *ras* genes sequenced to date. This region, called the effector loop, undergoes major conformational changes upon binding GTP (Polakis and McCormick, 1993).

Activated Ras interacts with a wide range of targets in many different organisms, as well as within a single cell (White *et al.*, 1995). The best studied candidate for an effector of Ras is the serine/threonine kinase Raf-1. Interaction of Raf-1 with post-translationally modified and activated Ras localizes Raf-1 to the membrane. The exact identity of the kinase that phosphorylates Raf-1 is still unclear though it is known that phosphorylation of Raf-1 on Y340 and Y341 can increase its catalytic activity (for review see Morrison and Cutter Jr., 1997). Activated Raf-1 phosphorylates MEK (MAPK or ERK kinase) which in turn phosphorylates MAPK. This phosphorylation cascade transduces the growth signal from the cell surface to the nucleus (Cowley *et al.*, 1994).

In our lab, it was observed that cell lines with elevated Ras activity do not exhibit PKR phosphorylation and are much more susceptible to reoviral infection (Strong *et al.*, 1998). This lead to an important finding that though normal tissue is not susceptible, a large number of tumour types support reoviral infection (Coffey *et al.*, 1998, as well as work done by Kara Norman in our lab). At this point, one of our goals is to characterize the biochemical nature of *Ras* dependent control of PKR activity and the intermediate proteins involved in this Ras-dependent susceptibility to reovirus infection.

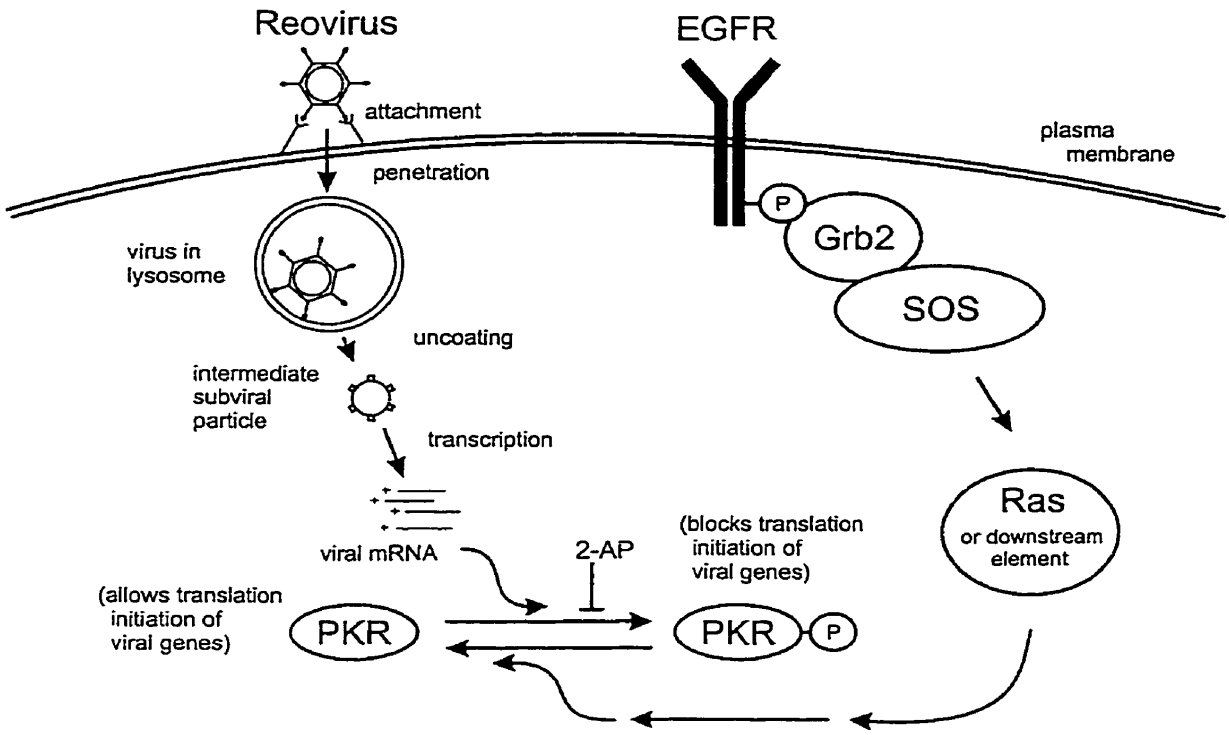


Figure 6: Reovirus infection of non-susceptible cell

Chapter 2. Methods and Materials

Cells, virus and plasmids

The following cell lines were used in this investigation;

NIH-3T3 parental cells were obtained from ATCC

H-ras cells are NIH-3T3 cells transfected with the Harvey-*ras* (*H-ras*) oncogene were a generous gift from Dr. Douglas Faller (Boston University School of Medicine) (Mundschau and Faller, 1992)

EJ-ras cells are NIH-3T3 cells transfected with the *EJ-ras* oncogene (EJ-ras has greater affinity for GTP.) were also a generous gift from Dr. Douglas Faller (Boston University School of Medicine) (Cai *et al.*, 1990)

THC-11 cells are NIH-3T3 cells transfected with the v-*erbB* oncogene which were obtained from Dr. H.-J. Kung (Case Western Reserve University)

TNIH-#5 cells are NIH-3T3 cells transfected with *Sos* were a generous gift from Dr. Michael Karin (University of California, San Diego) (Aronheim *et al.*, 1994).

22W1-4 cells are NIH-3T3 cells transfected with the *raf-1* oncogene were donated by Dr. Jonathan Cooper (Fred Hutchinson Cancer Research Center) (Fabian *et al.*, 1994)

C3H 10T½ cells are a mouse fibroblast line

2H1 cells are derivatives of the C3H 10T½ line transfected with c-Harvey-*ras* gene (from human bladder carcinoma cell line) under transcriptional regulation of the mouse metallothionein-I promoter. The cells thus express the H-ras in the presence of 50μM $ZnSO_4$. Both the parental C3H 10T½ and 2H1 cell lines were obtained from Dr. Nobumichi Hozumi (Mount Sinai Hospital Research Institute) (Trimble *et al.*, 1986).

Reovirus serotype 3, Dearing strain was propagated in L-929 cells (obtained from ATCC) grown in suspension and isolated as per Smith *et al.* 1969, with the omission of β-mercaptoethanol from the extraction buffer. Upon purification the particle/PFU ratio was normally 100/1. In order to UV inactivate reovirus, the suspension was exposed to ultraviolet radiation for 30 minutes.The ^{35}S-methionine labelled reovirus that was used as the marker for radiolabelled SDS-PAGE gels was purified according to the protocol of McRae and Joklik (1978).

Cell-culture

All cells were cultured at 37°C with 10% CO_2 in Dulbecco's modified essential medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS) (Gibco), with the exception of NIH-3T3 cells which were grown with 10% newborn bovine serum (NBS) (Gibco). All cells were plated in multi-well flat-bottomed plates before drug treatment or infection.

2-aminopurine treatment

The cells were grown in a monolayer to 60-70% confluency. The growth medium was removed and replaced with fresh DMEM supplemented with 10% FBS and 7.5mM 2-aminopurine. Appropriate amount of virus was added at the same time. Cells were treated as required by the specific experiment.

IFN-β treatment

The cells were grown in a monolayer to 60-70% confluency. The growth medium was removed and replaced with fresh DMEM supplemented with 10% FBS and 100U/mL IFN-β . Appropriate amount of virus was added 18-24 hours later. Cells were treated as required by the specific experiment.

Zinc Treatment

The cells were grown in a monolayer to 60-70% confluency. The growth medium was removed and replaced with fresh DMEM supplemented with

10% FBS and 50 μM $ZnSO_4$ (Sigma). Appropriate amount of virus was added 24 hours later. Cells were treated as required by the specific experiment

Infection of a monolayer

The cells were grown in 96-well flat-bottom plates in DMEM medium supplemented with 10% FBS until ~80% confluent. The medium was suctioned off and replaced with 100 μL of appropriate reovirus dilution in fresh medium. If the infection was to be synchronized, the cells were incubated at 4ºC for 1 hr to allow virus attachment and then transferred to 37ºC. If no synchronization was required, the cells were returned to the 37ºC immediately after infection.

^{35}S-methionine labelling of cells in monolayer

The cells were grown in 96-well flat-bottom plates in DMEM medium supplemented with 10% FBS until ~80% confluent. The growth medium was suctioned off, the cells were washed twice with pre-warmed PBS and pre-warmed methionine and cysteine deficient DMEM supplemented with 10% dialyzed FBS and 25 μCi/mL ^{35}S-methionine (Amersham) was replaced. After incubation for the required time in 37°C incubator, the medium was removed, the monolayer washed twice with cold sterile PBS and lysed in 100 □l of cold sonication buffer [20mM HEPES pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% Triton X-100]. The cells were scraped off the plate with a pipette tip, pipetted

up and down a few times to disrupt the membrane. Lysates were collected in 1.5 mL eppendorf tubes, centrifuged at 4°C at 14,000Xg for 10 minutes to pellet the nuclei. The supernatant was flash frozen in a fresh tube in liquid nitrogen. The samples were stored at –70°C for up to 6 months.

lysate preparation

Monolayers were washed 3 times with ice cold PBS, then scraped off into *in vitro* kinase lysis buffer [20 mM HEPES pH 7.5, 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40, 2 µg/mL leupeptin, 50 µg/mL aprotinin]. The lysates were centrifuged at 14000 X g at 4ºC for 1 minute and flash frozen in fresh eppendorf tubes. The lysates were stored for upto 6 months at –70ºC.

In vitro kinase ^{32}P- or ^{33}P-phosphate incorporation assay

20 µL of cellular extract was incubated with 7.5 µL the reaction buffer [20 mM HEPES pH 7.5, 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 µCi ^{32}P- or ^{33}P-phosphate–γATP per reaction] for 30 minutes at 37ºC. Immediately after incubations the reactions were either immunoprecipitated or boiled in PSB and analyzed on 10% SDS-PAGE.

Radioimmunoprecipitation of radiolabelled lysates

Appropriate antibody was added to the labelled lysate in 1:50 dilution. Binding was allowed for 1 hour at 4ºC. 20 µL of IgSorb (The Enzyme Center)

(washed in lysis buffer and pre-bound to 5 mg/mL BSA) was added and the mix was incubated for an additional hour at 4ºC. The reaction was centrifuged for 3 minutes at 10 000Xg and washed 4 times with ice cold sarcosyl wash buffer [50 mM sodium phosphate pH 7.5, 100 mM KCl, 0.02% NaN_3, 0.5% N-lauroylsarcosine (Sigma)]. After the last wash, the pellet was resuspended in PSB, boiled for 5 minutes and run on 10% SDS- PAGE.

SDS-PAGE of radiolabelled lysates

All SDS-PAGE gels that were used were prepared as follows:

Resolving gel mixture [9.9mL ddH_2O, 8.3 mL 30% bis-acrylamide mix, 6.3 mL 1.5M Tris (pH 8.8), 0.25 mL 10%SDS, 0.25 mL 10% ammonium persulfate, 0.01 mL TEMED] was poured into sealed 1mm thick and about 10 cm high space between clean glass plates and allowed to polymerize. Stacking gel mixture [5.5 mL ddH_2O, 1.3 mL 30% bis-acrylamide mix, 1.0 mL 1.0M Tris (pH 6.8), 0.08 mL 10% SDS, 0.08 mL 10% ammonium persulfate, 0.008 mL TEMED] was poured on top of the resolving gel and the comb was immediately inserted. The polymerized gel was clamped into a lucite boat, running buffer [25mM Tris base, 190 mM glycine, 0.3% SDS pH 8.5] was added, the samples were loaded into the wells and a current of 35 mA (3.5 hours) or 8 mA (for 16 hours) was applied. The protein amounts were standardized by conserving the number of cells that were harvested in a constant volume across all of the cell lines.

Processing of the SDS-PAGE gel

The gel was carefully removed from the glass plates and placed in fix [15% methanol, 10% acetic acid v/v in ddH_2O] for 30 minutes at room temperature. The fix was removed, and the gel rinsed in 7% acetic acid and then 1X used DMSO before being placed into fresh DMSO for 45 minutes. The DMSO was replaced with 20% PPO in DMSO and incubation continued for 30 - 45 minutes. The gel was then soaked in water for 30 minutes, dried on gel dryer and exposed to film for 12-48 hours at –70ºC.

Western blot

Western blot was performed according to the protocol provided with the ECL detection kit.

Preparation of electro-competent cells

XL1-Blue E. coli were inoculated into 1L LB-broth. The cells were incubated overnight at 37°C with vigorous shaking until ABS_{600} approximately 0.5-0.7. The cell culture was centrifuged in Nalgene buckets at 4000Xg and 4°C for 15 min. LB broth was carefully discarded and the pellet resuspended in 1L of pre-chilled freshly prepared 10% glycerol. Centrifugation was repeated and the pellet was resuspended in 0.5L of pre-chilled 10% glycerol. Similarly, the volume was reduced to 250 mL, and then to 3-4 mL equivalent to about 1-3 X 10^{10} cells/mL. The suspension was aliquoted into 80 µL aliquots, flash frozen in liquid nitrogen and kept at –70°C for upto 4 months.

Electroporation of E.coli.

Electro-competent cells were thawed on ice. 40 μL of cells and 1 – 2 μL of DNA (in TE) were mixed in a pre-chilled 1.5 mL eppendorf tube for 1 minute. E. coli Pulser apparatus was set at 200Ω and 25 μFD. DNA cell mixture was transferred to pre-chilled 0.2 cm cuvette, shaken to the bottom and pulsed once. The pulse parameters were recorded. 1mL of pre-warmed SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was immediately added to the cells. The cells were transferred to a 1.5 mL tube and incubated at 37°C for 1 hour. 250 μL of the mixture was plated on and LB plate with appropriate antibiotic and incubated overnight at 37°C.

Mini-prep

Six single colonies of transformed E. coli were picked off the LB-plate and grown overnight in 5 mL LB cultures at 37°C with vigorous shaking. 1mL of the overnight culture was centrifuged at 14000xg for 1 minute, the supernatant was removed, and the pellet resuspended in 30 μL of 50 mM Tris pH 8 by pipetting up and down. 300 μL of TENS [10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1 N NaOH, 0.5% SDS] were added and the contents mixed by inversion 10-12 times. 150 μL of 3 M NaOAc pH 5.2 were added and mixed again as before. The mixture was centrifuged for 5 – 10 minutes at maximum

speed and the supernatant was transferred to a fresh tube containing 900 µL 95% EtOH. Plasmid precipitation was allowed to occur over at least 30 minutes at –20°C and was then centrifuged for 10 minutes at maximum speed. 95% EtOH was removed, the pellet washed with 70 % EtOH and re-centrifuged. Supernatant was removed from the pellet; it was lyophilized and dissolved in 20 µL of TE [10 mM Tris-Cl pH 7.5, 1 mM EDTA]. This plasmid can be stored at –20°C.

TIK isolation

XL1-Blue strain of *E. coli* were transfected with His-TIK fusion construct in pQR47 (gift from Dr. Bell, Ottawa Regional Cancer Center). This construct encodes the first 98 amino acids of murine PKR (TIK) with some extra amino acids that are amino-terminal to the initiating methionine. The E. coli were grown to 1L volume overnight in LB broth with 50 mg/mL Ampicilin. Expression of the construct was induced by incubating with 0.48M IPTG for 3-3.5 hours. The bacteria were spun in sterile 1L Nalgene bottle for 15 minutes at 3500rpm, and the supernatant was removed. If required, the pellet was resuspended in 30 mL of sterile PBS, spun at 2800rpm for 15 minutes, flash frozen and stored at –70°C until needed. The rest of the protocol was acquired from Clontech along with the Talon Metal affinity resin. Batch isolation protocol was used with minor alteration s in the protocol to optimize the yield. Binding time was extended to 1 hr at 4°C. Acid pH washing

conditions were used with the optional wash being performed with 10 mL of pH 7.0 buffer. The construct was eluted with 100mM EDTA. The purified protein was flash frozen in liquid nitrogen and stored at –70°C.

Anti-PKR antibody production.

A 2-3 kg female New Zealand rabbit (Emilia) was pre-bled 3 times in the period of 6 weeks, then injected with 0.3 mg of purified TIK (from previous protocol) in 1mL of 50% PBS: 50% complete Frieund's Adjuvant inter-muscularly in two places. Similarly, Emilia was boosted two weeks later with 0.3 mg of purified TIK in 1mL of 50% PBS: 50% incomplete Freund's Adjuvant. Emilia was test-bled two weeks later and the serum was tested in a Western and a radio-immune precipitation. The antibody was finally collected by heart-puncture tested for specificity by western blot and IP (data not shown); the serum was isolated and stored in small aliquots at –70°C.

PKR activation with dsRNA

Appropriate amount of dsRNA (Poly I-C or S1 mRNA) was added to cold cellular lysates. The reaction was incubated for 2 minutes on ice and then subjected to *in vitro* kinase assay described above.

New assay

Lysates from NIH-3T3 monolayers were subjected to *in vitro* kinase assay, followed by immunoprecipitation. The pellet was washed as above, and then

in once in lysis buffer. The pellet was resuspended and incubated for appropriate amount of time in cellular lysates. The reaction was immediately boiled in PSB and analyzed on 10% SDS-PAGE.

Chapter 3. *Results*

Cells transfected with *Sos, v-erbB, H-ras* and *EJ-ras* support productive reovirus infection

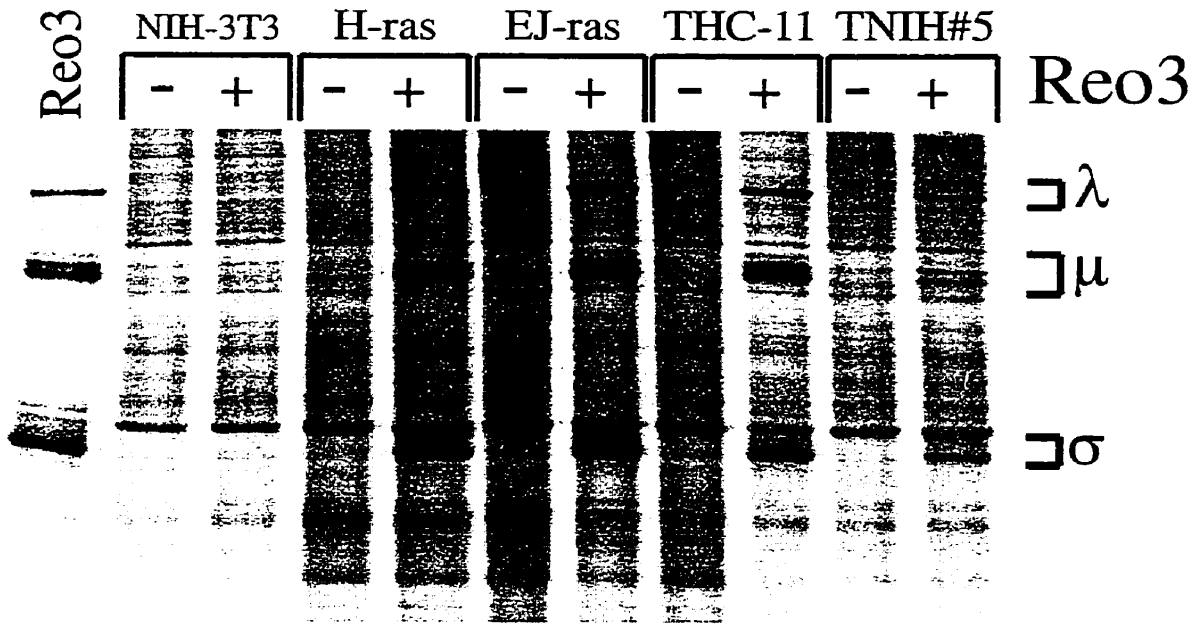
When I began my project in the lab, work was being done on the susceptibility of *v-erbB* transfected cells to reovirus infection (Strong and Lee, 1996) and the molecular characteristics of the requirements for this susceptibility were being elucidated. At this time as well as throughout this project, viral infectivity is measured by continuously labelling infected and uninfected cell monolayers with ^{35}S-methionine from 12 to 36 hours post-infection. The viral and host protein synthesis was then analyzed by 10% SDS-PAGE. It was observed that despite the fact that the level of host protein synthesis was similar in all of the cell lines used in the experiment, viral protein synthesis varied dramatically. With multiplicity of infection of 10 plaque forming units per cell, no detectable levels of reovirus proteins were produced in the parental NIH-3T3 cell line. NIH-3T3 cells overexpressing *Sos*, v-*erbB*, H-*ras* and EJ-*ras* oncogenes exhibited easily visible levels of viral protein synthesis as determined by comparing to the ^{35}S-methionine labelled reovirus serotype 3 that was prepared for use as a marker (Figure 7A). A similar figure appears in Strong *et al.*, 1998.

To conclusively show that reovirus proteins were being synthesized, the ^{35}S-methionine labelled lysates were immunoprecipitated with polyclonal antibody against the reovirus strain used in the lab. This antibody was prepared

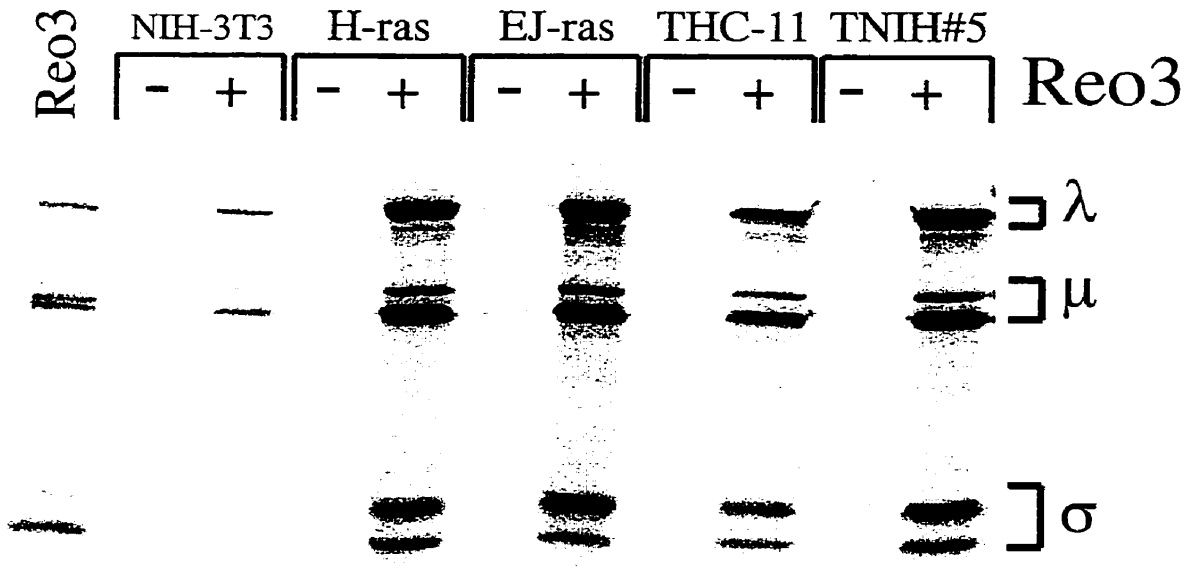
Figure 7:

Reovirus protein synthesis in infected and uninfected NIH-3T3, H-ras, EJ-ras, THC-11 and TNIH-#5 cells. Cells infected with reovirus (MOI ~ 10 PFU/cell) were labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. The lysates were (A) analyzed by 10% SDS-PAGE or (B) immunoprecipitated with polyclonal anti-reovirus antibody prior to 10% SDS-PAGE.

A


Reo3
NIH-3T3
H-ras
EJ-ras
THC-11
TNIH#5
- + - + - + - + - +
Reo3
λ
μ
σ

B


Reo3
NIH-3T3
H-ras
EJ-ras
THC-11
TNIH#5
- + - + - + - + - +
Reo3
λ
μ
σ

specifically for the purposes of this project as well as other ongoing studies in the lab. Though some viral proteins were detectable in the parental NIH-3T3 cells, the lysates from the transformed cell lines produced dramatically higher levels of reo3 proteins between 12 and 36 hours post-infection (Figure 7B). Low level of infection (~5%) in NIH-3T3 has been documented in earlier studies and is thought to result from spontaneous transformation of cell within the cell line (Strong *et al.*, 1998)

Reovirus susceptibility increases upon induction of Ras.

In order to eliminate the possibility that differential susceptibility to infection by reovirus results from long term transformation, and be able to focus on the Ras pathway, we used 2H1, a cell line with inducible H-*ras*. 2H1 cells are C3H 10T½ fibroblasts carrying c-H-*ras* gene under the control of the mouse metallothionein-I promoter which is inducible by 50 μM $ZnSO_4$ (Trimble *et al.*, 1986). Both the inducible and the parental (2H1 and C3H 10T½, respectively) cell monolayers were treated with 50 μM $ZnSO_4$ 24 hours prior to infection with 10 PFU/cell of reovirus. Starting at 12 hours post-infection, the monolayers were continuously labelled with ^{35}S-methionine for 24 hours. The lysates were harvested and immunoprecipitated with polyclonal anti-Reo3 antibody.

The 10% SDS-PAGE shows small amount of reo3 proteins being produced in infected parental cells with and without treatment with 50 μM $ZnSO_4$, as well as the untreated 2H1 cells. However, upon activation of H-*ras* by 50 μM $ZnSO_4$ 2H1

cell lysate produces an increased amount of viral proteins during the labelling period (Figure 8A). A similar figure appears in part in Strong *et al.*, 1998.

Phosphorylation of PKR is inversely proportional to productivity of infection

Phosphorylation pattern of proteins during infection of the parental cells as well as those with inducible *ras* were observed because PKR activation by phosphorylation has been implicated in the anti-viral response (Bischoff and Samuel, 1989). Monolayers were treated with 50 μM $ZnSO_4$ 24 hours prior to infection with 30 PFU/cell. Cell lysates were prepared at the time of infection as well as 24 and 48 hours post-infection and labelled with ^{32}P-phosphate in an *in vitro* kinase assay. The reactions were immunoprecipitated with polyclonal anti-PKR antibody that was prepared against the first 98 amino acids of murine PKR generously provided by Dr. J.C. Bell. The samples were analyzed by 10% SDS-PAGE.

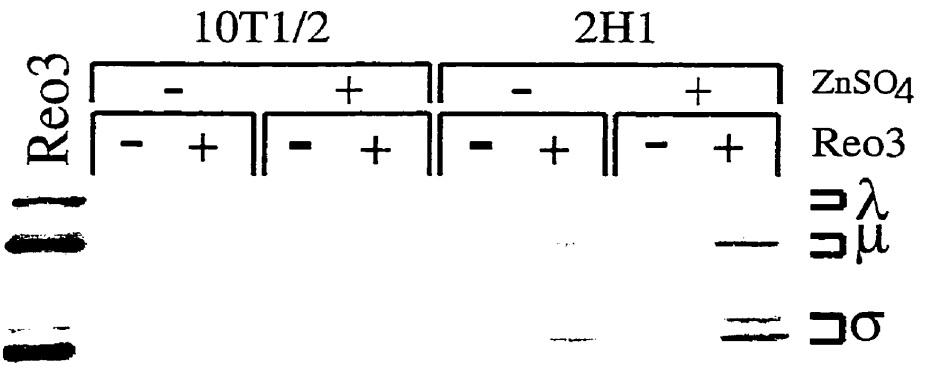
Though there is no significant difference in PKR phosphorylation between the 50 μM $ZnSO_4$ induced and uninduced parental 10T½ cells, PKR phosphorylation is drastically reduced at 48 hours post-infection in 2H1 cells that have been induced to express Ras. This correlates with increased productivity of infection in the *ras* expressing cells (Figure 8B).

Figure 8:

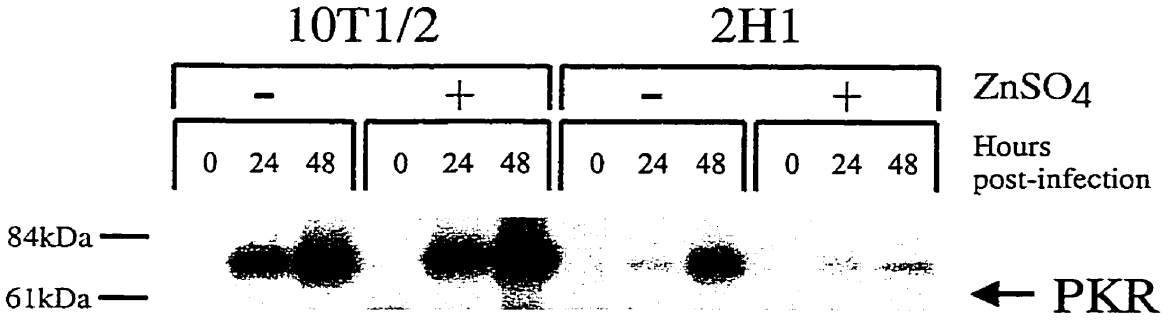
A. Reovirus protein synthesis in infected and uninfected C3H 10T½ and 2H1 cells upon treatment with $ZnSO_4$. Cells were treated with 50μM $ZnSO_4$ at 24 hours pre-infection with reovirus (MOI ~ 10 PFU/cell). Cells were labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. The lysates were immunoprecipitated with polyclonal anti-reovirus antibody prior to 10% SDS-PAGE.

B. PKR activation state in infected and uninfected C3H 10T½ and 2H1 cells upon treatment with $ZnSO_4$. Cells were treated with 50μM $ZnSO_4$ at 24 hours pre-infection with reovirus (MOI ~ 30 PFU/cell). At 48 hours post-infection, lysates were collected and *in vitro* kinase assay was preformed followed by immunoprecipitation with polyclonal anti-PKR antibody. The samples were analyzed by 10% SDS-PAGE.

A


10T1/2
2H1
ZnSO4
Reo3
- + - + - + - +
Reo3
λ
μ
σ

B


10T1/2
2H1
ZnSO4
- + - +
0 24 48 0 24 48 0 24 48 0 24 48
Hours post-infection
84kDa
61kDa
PKR

Determination of susceptibility of host cells occurs at the Ras not Raf-1 signal transduction level.

Having shown that elements in the pathway upstream of Ras confer infectibility on the 2H1 cells, we had to determine if elements downstream of Ras, such as Raf-1 had a similar effect. Monolayers of parental, H-ras and Raf-1 (22W1-4) transfected cells were infected with 2-20 PFU/cell and ^{35}S-methionine labelled continuously between 12 and 36 hours post-infection. The samples were analyzed on 10% SDS-PAGE.

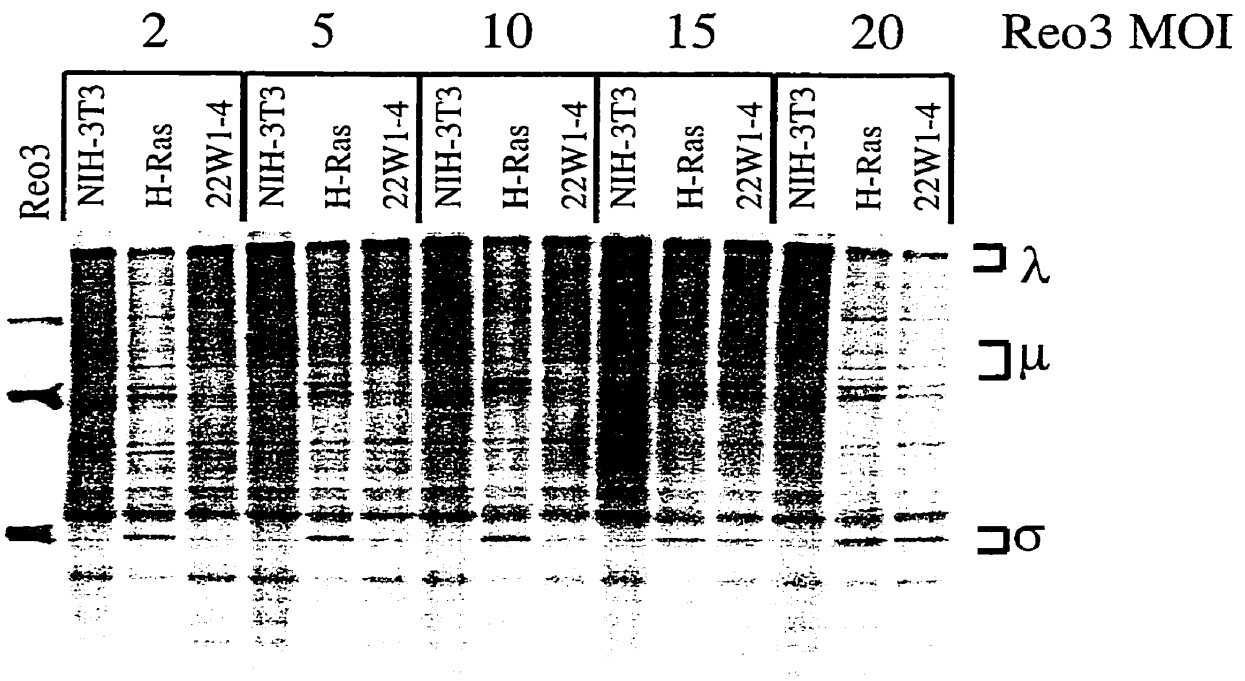
The parental NIH-3T3 cells, the negative control, showed none or very little viral protein synthesis in this time period. In H-ras, the positive control, large amount of viral proteins were produced independently of the multiplicity of infection. Raf-1 transfected cells (22W1-4), showed less viral synthesis than H-ras cells at moi of 15 PFU/cell or less. The decreased susceptibility of *raf-1* transformed cells suggests that H-ras is the most potent positive regulator of cellular susceptibility to reovirus infection. Viral protein synthesis in 22W1-4 cells at high moi may be explained by existence of feedback loop in the signaling pathway where Ras becomes indirectly activated (Figure 9).

Interferon-β decreases viral protein synthesis in susceptible cells

Since PKR is an interferon inducible kinase, we treated monolayers of NIH-3T3 and H-ras cells with 100 U/mL of IFN-β 18 hours prior to infection with 10 PFU/cell.

Figure 9:

Relative amount of reovirus protein synthesis during infection of NIH-3T3, H-ras and 22W1-4 cells. Cells infected with reovirus were labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. The lysates were analyzed by 10% SDS-PAGE.


2
5
10
15
20
Reo3 MOI
Reo3
NIH-3T3
H-Ras
22W1-4
λ
μ
σ

As previously, the cells were continuously ^{35}S-methionine labelled between 12 and 36 hours post-infection and then immediately analyzed on 10% SDS-PAGE (Figure 10A) or immunoprecipitated with polyclonal anti-reo3 antibody prior to SDS-PAGE (Figure 10B).

Treatment with interferon-β drastically decreased the amount of viral proteins produced in normally susceptible *ras* transformed cells. This is not surprising since interferon-β has been shown to increase expression of PKR in NIH-3T3 cells, thus mounting a stronger anti-viral response.

2-aminopurine increases the susceptibility of cells to reovirus infection.

The effect of 2-aminopurine, a specific PKR inhibitor (Hu and Conway, 1993), on cellular susceptibility to reovirus infection was determined. NIH-3T3 and H-ras cell monolayers were treated with 7.5 mM 2-aminopurine 24 hours prior to infection with 5 PFU/cell of reovirus. Continuous ^{35}S-methionine labelling between 12 and 36 hours post-infection showed a significant increase in reovirus protein synthesis both in normally non-susceptible NIH-3T3 and susceptible *ras* transformed cells (Figure 11, a similar figure appears in Strong *et al.*, 1998). This confirms that inhibition of PKR activity increases reovirus protein synthesis.

Figure 10:

Effect of interferon-β on reovirus protein synthesis during infection of NIH-3T3 and H-ras cells. Cells were treated with 100 U/mL of interferon-β 18 hours pre-infection, then infected with reovirus (MOI ~ 5 PFU/cell) and labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. The lysates were (A) analyzed by 10% SDS-PAGE or (B) immunoprecipitated with polyclonal anti-reovirus antibody prior to 10% SDS-PAGE.

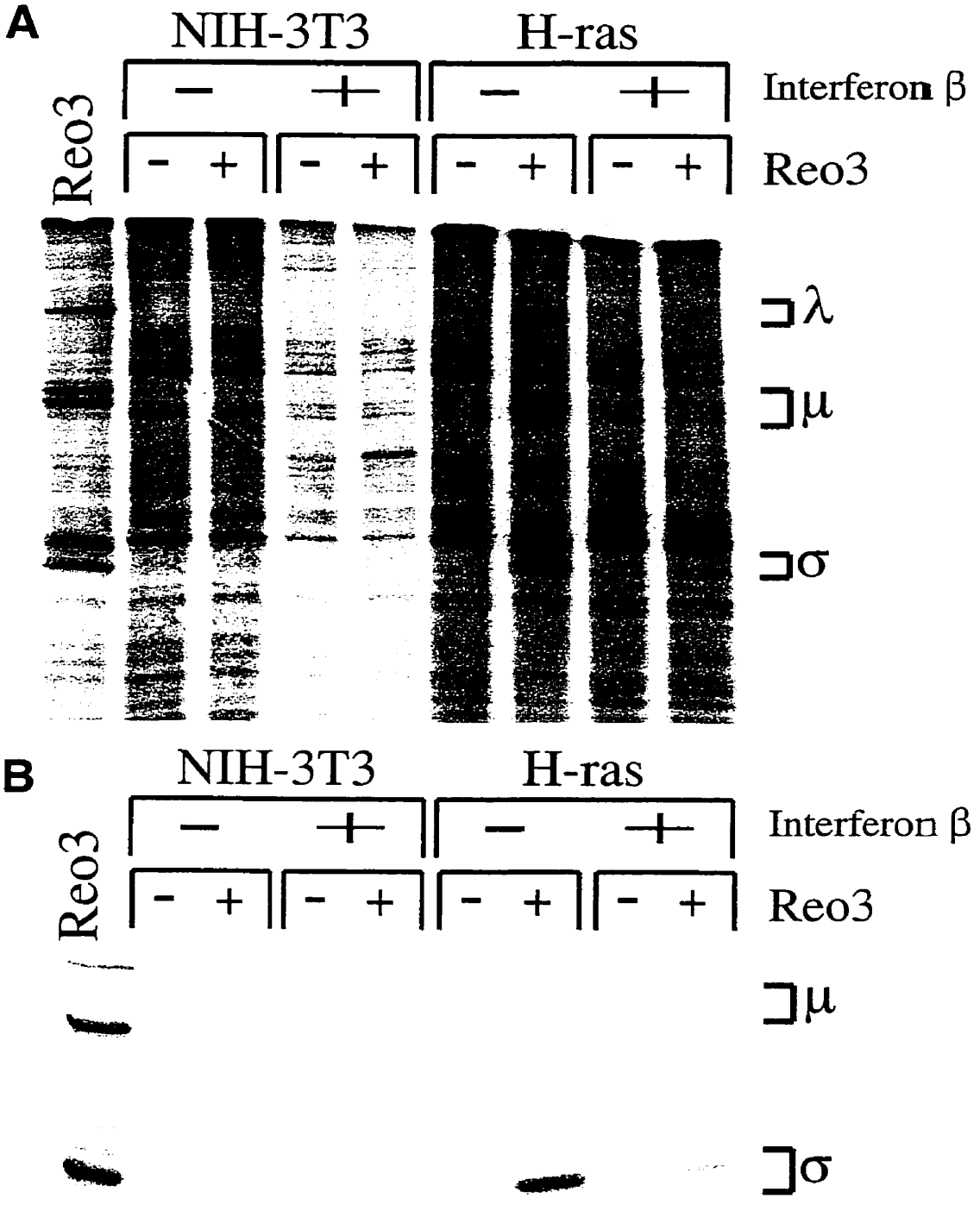
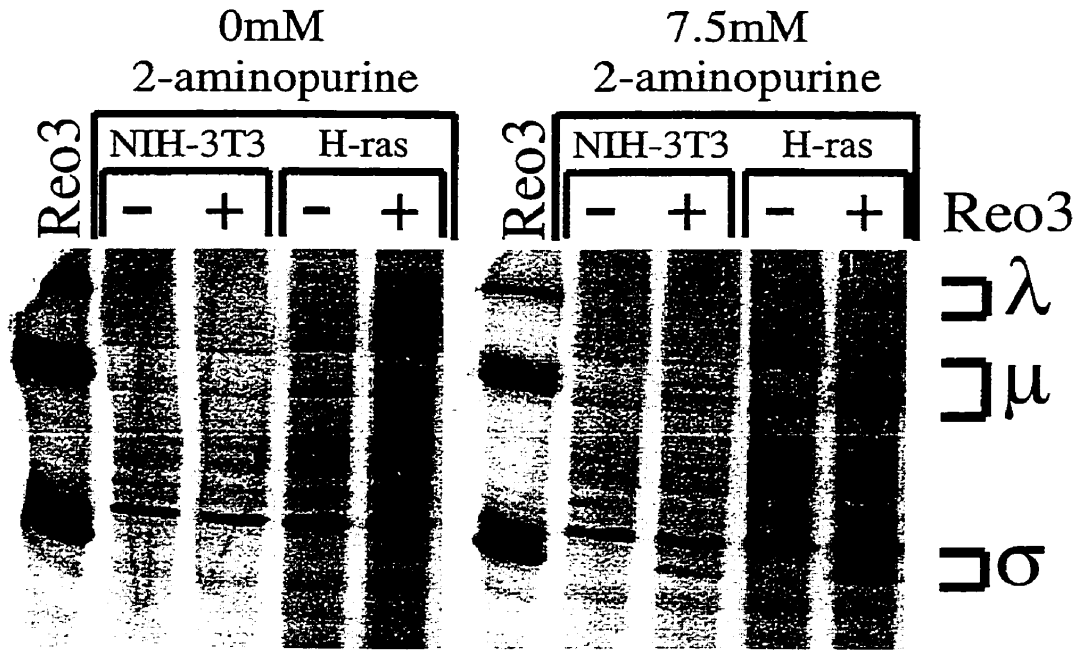

A
NIH-3T3
H-ras
Interferon β
Reo3
Reo3
λ
μ
σ
B
NIH-3T3
H-ras
Interferon β
Reo3
Reo3
μ
σ

Figure 11:

Effect of 2-aminopurine on reovirus protein synthesis during infection of NIH-3T3 and H-ras cells. Cells were treated with 7.5mM 2-aminopurine 24 hours prior to being infected with reovirus (MOI ~ 10 PFU/cell). Cells were labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. The lysates were analyzed by 10% SDS-PAGE.


0mM
2-aminopurine
7.5mM
2-aminopurine
Reo3
NIH-3T3
H-ras
- + - +
Reo3
Reo3
NIH-3T3
H-ras
- + - +
Reo3
λ
μ
σ

Total amount of PKR is comparable in susceptible (ras transformed) and non-susceptible cells and different multiplicity of infection.

In order to eliminate the possibility of degradation of PKR in *ras* transformed cells, we determined its total amount at 48 hours post-infection with 0, 20 and 30 PFU/cell. Monolayers of NIH-3T3 and H-ras cells were infected with appropriate PFU/cell and lysates were harvested at 48 hours post-infection. After performing a Western blot on these samples using the polyclonal anti-PKR antibody, it can be concluded that the levels of PKR are equivalent during infection of both *ras* transformed and parental cells (Figure 12).

PKR is phosphorylated in the presence of reovirus in non-susceptible cells but not in *ras* transformed cells.

Having established that the level of PKR remains constant, we then looked at its level of phosphorylation and the outcome of infection. Monolayers of NIH-3T3 and H-ras cells were infected with 30 PFU/cell and lysates were prepared 48 hours post-infection. The lysates were ^{32}P-phosphate labelled by the *in vitro* kinase assay and immunoprecipitated with the polyclonal anti-PKR antibody followed by 10% SDS-PAGE. Despite the attempts by the temperamental gel dryer to implode the gel, it can be seen that although PKR is phosphorylated in the parental, non-susceptible cells upon exposure to reovirus, no such event occurs in the *ras* transformed cells (Figure 13).

Figure 12:

Effect of multiplicity of infection on total amount of PKR during infection of NIH-3T3 and H-ras cells. Cells were infected with reovirus, and harvested at 48 hours post-infection. The lysates were separated on 10% SDS polyacrylamide gel, transferred to nitrocellulose membrane and blotted with polyclonal anti-PKR antibody. The results were visualized by probing with the secondary HRP bound antibody and the ECL detection kit

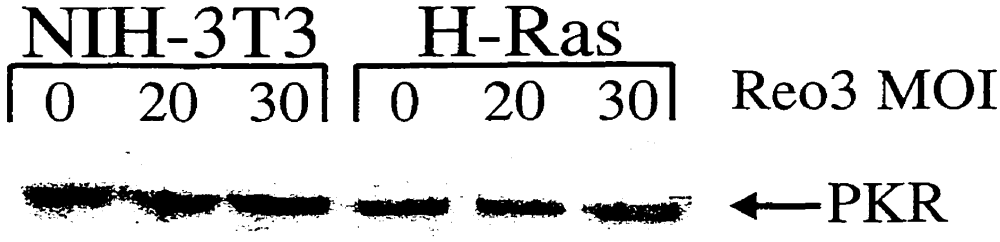
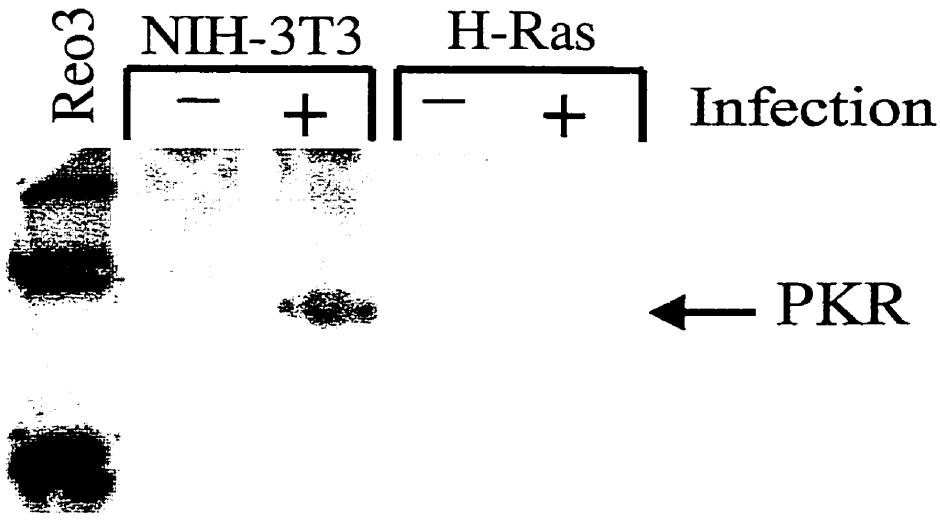

NIH-3T3
H-Ras
0 20 30
0 20 30
Reo3 MOI
PKR

Figure 13:

PKR activation state in infected and uninfected NIH-3T3 and H-ras cells. Cells were infected with reovirus (MOI ~ 30 PFU/cell). At 48 hours post-infection, lysates were collected and *in vitro* kinase assay was performed followed by immunoprecipitation with polyclonal anti-PKR antibody. The samples were analyzed by 10% SDS-PAGE.


Reo3
NIH-3T3
H-Ras
−
+
−
+
Infection
PKR

Reovirus protein synthesis in the first 24 hours of infection of parental and *ras* transformed cells.

To get a better understanding of the time involved in the infection process, infection of NIH-3T3 and H-ras monolayers with 10 PFU/cell was synchronized and the cells were pulse labelled with ^{35}S-methionine for 1 hour at 6 hour intervals post-infection. The samples were analyzed by 10% SDS-PAGE. Parental cells contained no ^{35}S-methionine labelled viral proteins, as expected from previous data (Figure 7). Reovirus proteins in *ras* transformed cells were visible to a small extent at 6 hours post-infection and gradually increased in intensity with time (Figure 14).

Total amount of PKR remains constant throughout the 48 hours of infection in parental and *ras* transformed cells.

In order to confirm that the total amount of PKR is also constant at different times post-infection, a Western blot with polyclonal anti-PKR antibody was performed on lysates of NIH-3T3 and H-ras cells at 0, 24 and 36 hours post-infection. Figure 15 shows that PKR protein levels remains constant in parental and *ras* transformed cells throughout infection.

Figure 14:

Reovirus protein synthesis at different times during infection of NIH-3T3 and H-ras cells. Cells infected with reovirus (MOI ~ 10 PFU/cell) were labelled with ^{35}S-methionine for 1 hour pulses at 0, 6, 12, 18, and 24 hours post-infection. The lysates were analyzed by 10% SDS-PAGE.

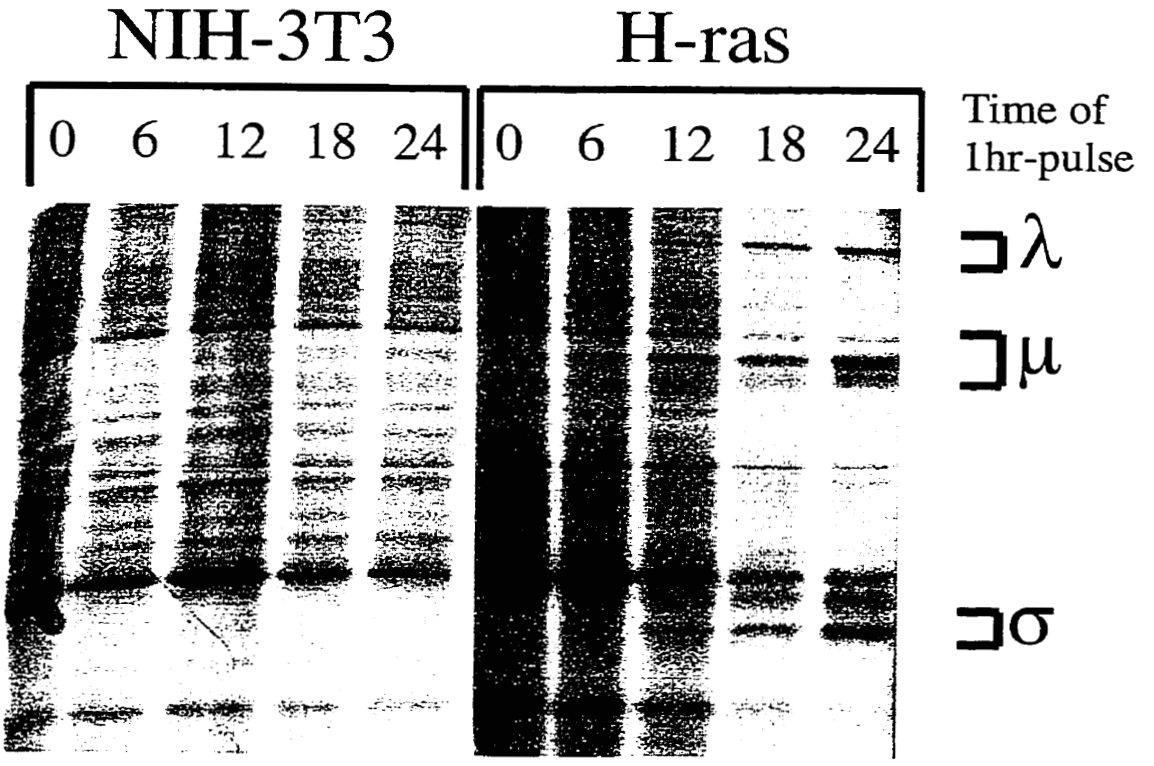
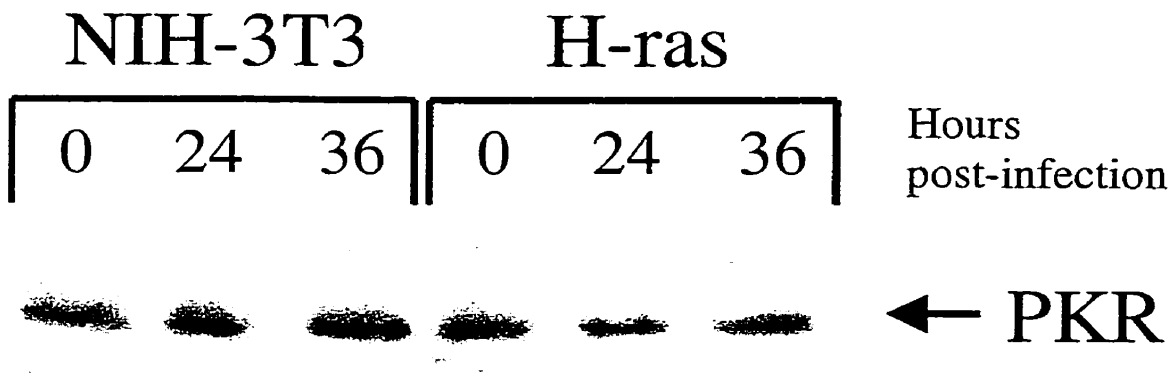

NIH-3T3
H-ras
0 6 12 18 24
0 6 12 18 24
Time of 1hr-pulse
λ
μ
σ

Figure 15:

Total amount of PKR at different times during reovirus infection of NIH-3T3 and H-ras cells. Cells were infected with reovirus (MOI ~30 PFU/cell), and harvested at 0, 24 and 36 hours post-infection. The lysates were separated on 10% SDS polyacrylamide gel, transferred to nitrocellulose membrane and blotted with polyclonal anti-PKR antibody. The results were visualized by probing with the secondary HRP bound antibody and the ECL detection kit.


NIH-3T3
H-ras
0 24 36
0 24 36
Hours post-infection
PKR

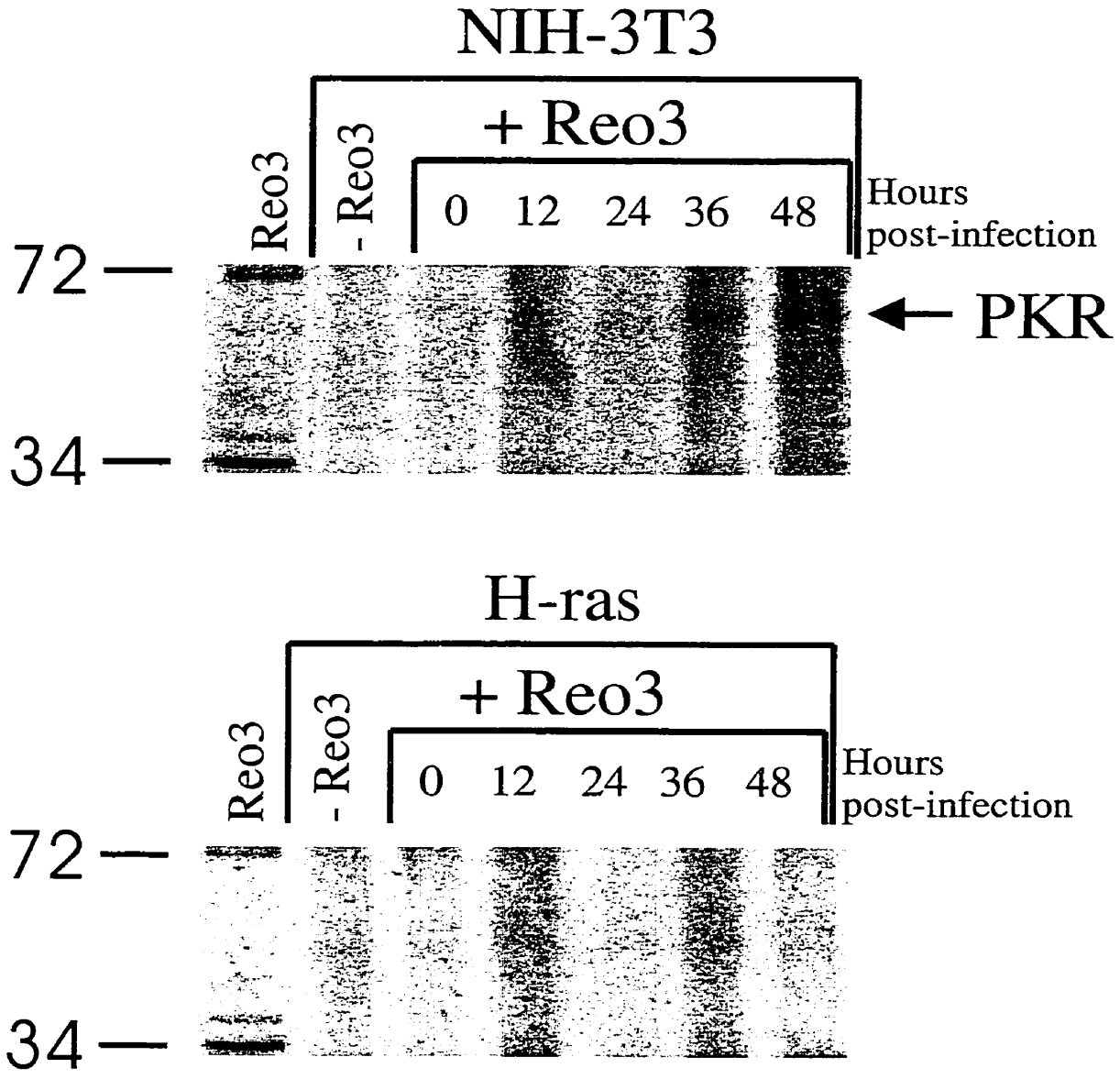
PKR becomes visibly phosphorylated at 36 to 48 hours post-infection in non-susceptible parental but not ras transformed cells

Having confirmed that PKR levels remain constant during reovirus infection, we again turned to its phosphorylation level. Monolayers of NIH-3T3 and H-ras cells were infected with 30 PFU/cell and harvested every 12 hours post-infection. To determine the phosphorylation state of PKR, the lysates were labelled with ^{32}P-phosphate and immunoprecipitated with polyclonal anti-PKR antibody. PKR was not phosphorylated in the *ras* transformed cells, however phosphorylated PKR was present in the non-susceptible cells after 36 hours post-infection (Figure 16).

Looking at this figure, the following question arises. Considering the infectious process in NIH-3T3 cells is theoretically halted immediately after primary transcription about 2 hours post-infection (Strong *et al.*, 1998), why is PKR only phosphorylated at 36 hours post-infection? The answer to this lies in the compartmentalization of PKR activation at low multiplicity of infection and the low sensitivity of the *in vitro* kinase assay. When much larger amounts of lysate are used, a faint phosphorylated PKR band is visible at 6-8 hours post-infection (data nor shown). However, at normal conditions, once reovirus progeny from the 5% of the infectable NIH-3T3 cells infect the surrounding cells (secondary infection), PKR activation become much more wide spread and is then picked up by the assay.

Figure 16:

PKR activation state at different times during reovirus infection of NIH-3T3 and H-ras cells. Cells were infected with reovirus (MOI ~ 30 PFU/cell). At 0, 12, 24, 36, and 48 hours post-infection, lysates were collected and *in vitro* kinase assay was preformed followed by immunoprecipitation with polyclonal anti-PKR antibody. The samples were analyzed by 10% SDS-PAGE.


NIH-3T3
+ Reo3
Reo3
- Reo3
0 12 24 36 48
Hours post-infection
72
34
PKR
H-ras
+ Reo3
Reo3
- Reo3
0 12 24 36 48
Hours post-infection
72
34

Reovirus infection-dependent phosphorylation of high molecular weight proteins in *ras* transformed cells.

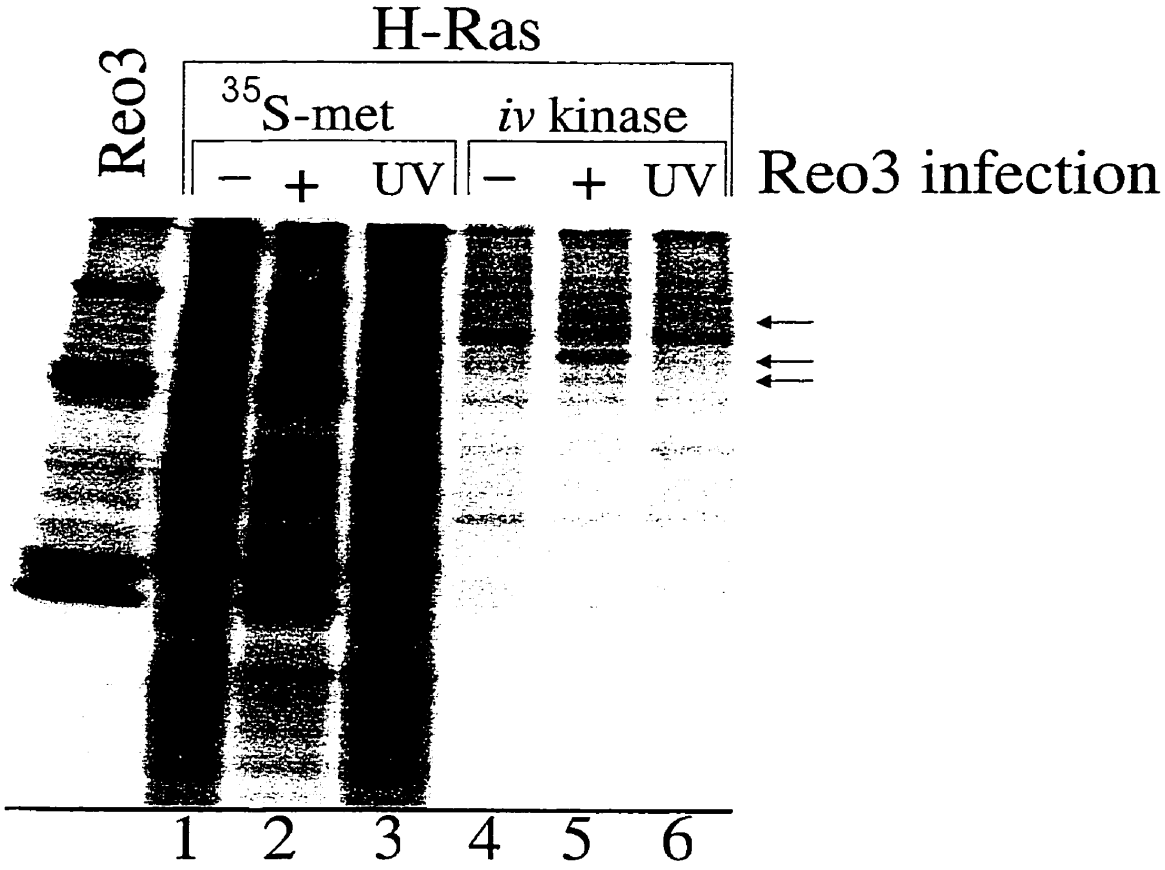
In Strong *et al.*, 1998 we had observed that a high molecular protein (~100 kDa) is phosphorylated during infection of susceptible cells. Since the *in vitro* kinase assay using ^{32}P-phosphate-γATP produces fuzzy bands that further decrease the assay's sensitivity, a more expensive ^{33}P-γATP was used for the next two *in vitro* kinase experiments. Monolayers of *ras* transformed cells were infected with live and UV inactivated reovirus.

The cells infected with 10 PFU/cell were continuously ^{35}S-methionine labelled between 12 and 36 hours post-infection and analyzed on 10% SDS-PAGE (Figure 17, lanes 1-3). As expected from previous data, reovirus proteins were only produced in cells infected with live reovirus (lane 2).

The H-ras cells infected with 30 PFU/cell were harvested 48 hours post-infection. The lysates were labelled with ^{33}P-phosphate -γATP in the *in vitro* kinase assay and analyzed on 10% SDS-PAGE (Figure 17, lanes 4-6). Three high molecular weight phosphorylated bands were detectable only in the *ras*-transformed, susceptible cells infected with live virus (lane 5). So just as in non-susceptible cells, the presence of live reovirus in *ras*-transformed cells induces infection-dependent phosphorylation events.

Figure 17:

Reovirus protein synthesis and phosphorylation pattern in infected and uninfected H-ras cells. Cells were infected with reovirus (MOI ~ 10 PFU/cell lanes 1-3, and MOI ~30 PFU/cell lanes 4-6). In lanes 1-3 cells were labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. In lanes 4-6, lysates were collected at 48 hours post-infection, labeled with ^{33}P-phosphate as per *in vitro* kinase assay. The samples were analyzed by 10% SDS-PAGE. The arrows indicate the high molecular weight phosphorylation events in H-Ras cells during infection.


H-Ras
Reo3
35S-met
iv kinase
– + UV
– + UV
Reo3 infection
1 2 3 4 5 6

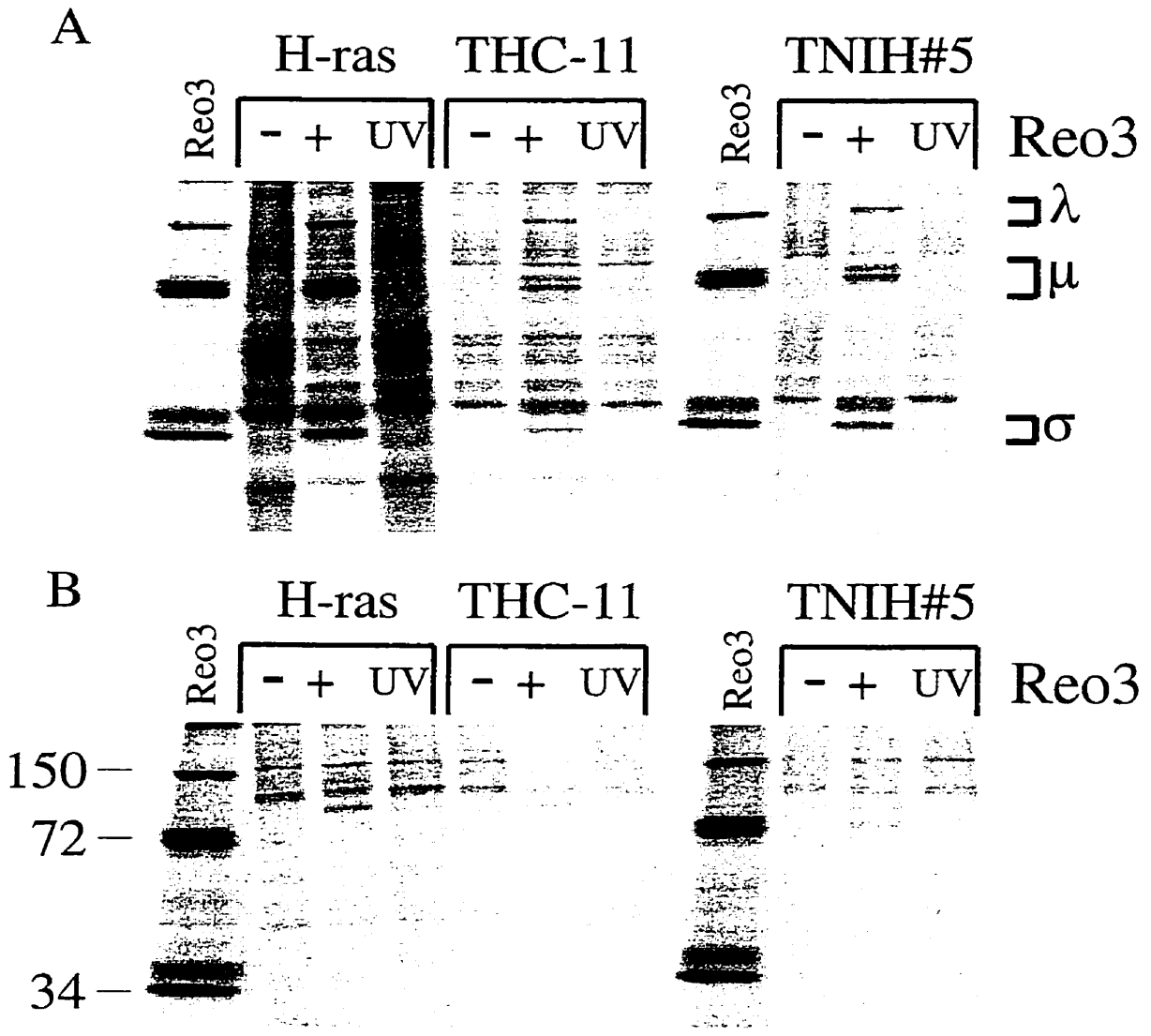
Reovirus infection-dependent phosphorylation of high molecular weight proteins in *Sos, v-erbB* and *ras* transformed cells.

In order to determine whether the intensity of the phosphorylation of these high molecular weight proteins correlates with the efficiency of infection, the same experiment as for the previous figure was performed on *Sos-, v-erbB-* and *ras*-transformed cells side by side. Indeed, their phosphorylation correlated perfectly with the amount of viral protein synthesis in the cells (Figure 18).

We then turned our attention to the nature of inhibition of PKR phosphorylation in *ras* transformed cells. At what level of PKR regulation does this inhibition take place? We have established that it is a post-translational event, since the amount of PKR protein is equal regardless of the cells' susceptibility to reovirus infection (Figures 12 and 15). Is PKR phosphorylation prevented or reversed in these cells? To answer this question we incubated equal amounts of the lysates together after establishing the phosphorylation state of PKR in each lysate.

Figure 18:

Reovirus protein synthesis and phosphorylation pattern in infected and uninfected H-ras, THC-11 and TNIH-#5 cells. Cells were infected with reovirus (MOI ~ 10 PFU/cell (A), and MOI ~30 PFU/cell (B)). In A, cells were labelled with ^{35}S-methionine between the hours of 12 and 36 hours post-infection. In B, lysates were collected at 48 hours post-infection, labeled with ^{33}P-phosphate as per *in vitro* kinase assay. The samples were analyzed by 10% SDS-PAGE.


A
H-ras
THC-11
TNIH#5
Reo3
- + UV
- + UV
Reo3
- + UV
Reo3
λ
μ
σ
B
H-ras
THC-11
TNIH#5
Reo3
- + UV
- + UV
Reo3
- + UV
Reo3
150
72
34

PKR is not degraded when lysates of the *ras* transformed cells are incubated with the lysates of parental cells.

First however, we had to confirm that the total amount of PKR was not altered during this type of incubation. Monolayers of NIH-3T3 and H-ras cells were infected with 30 PFU/cell and harvested 48 hours post-infection. Infected and uninfected NIH-3T3 and H-ras lysates were mixed and incubated for 0 - 35 minutes at 37ºC. The reactions were analyzed by Western blot with polyclonal anti-PKR antibody and visualized with the ECL detection kit. The level of PKR remains relatively constant during incubation of all combinations of lysates (Figure 19).

No conclusive results can be obtained from the literal 'mixing' of ^{32}P-phosphate labelled lysates.

An attempt was made at a very simple 'quick and dirty', though as it turned out, not at all informative mixing experiment. An *in vitro* kinase assay was performed on both infected and uninfected NIH-3T3 and H-ras cells after which the lysates were mixed and incubated for 35 minutes at 37°C. As expected from previous data, PKR was phosphorylated in infected NIH-3T3 and not in the *ras* transformed cells. Upon incubation of NIH-3T3 lysates with infected and uninfected H-ras lysates, however, there is no significant change in PKR phosphorylation (Figure 20).

Figure 19:

Total amount of PKR at different times during incubation of infected and uninfected NIH-3T3 and H-ras lysates. Cells were infected with reovirus (MOI ~30 PFU/cell), and lysates were prepared at 48 hours post-infection and incubated in different combinations for 0 - 35 minutes at 37ºC. The samples were separated on 10% SDS polyacrylamide gel, transferred to nitrocellulose membrane and blotted with polyclonal anti-PKR antibody. The results were visualized by probing with the secondary HRP-bound antibody and the ECL detection kit.

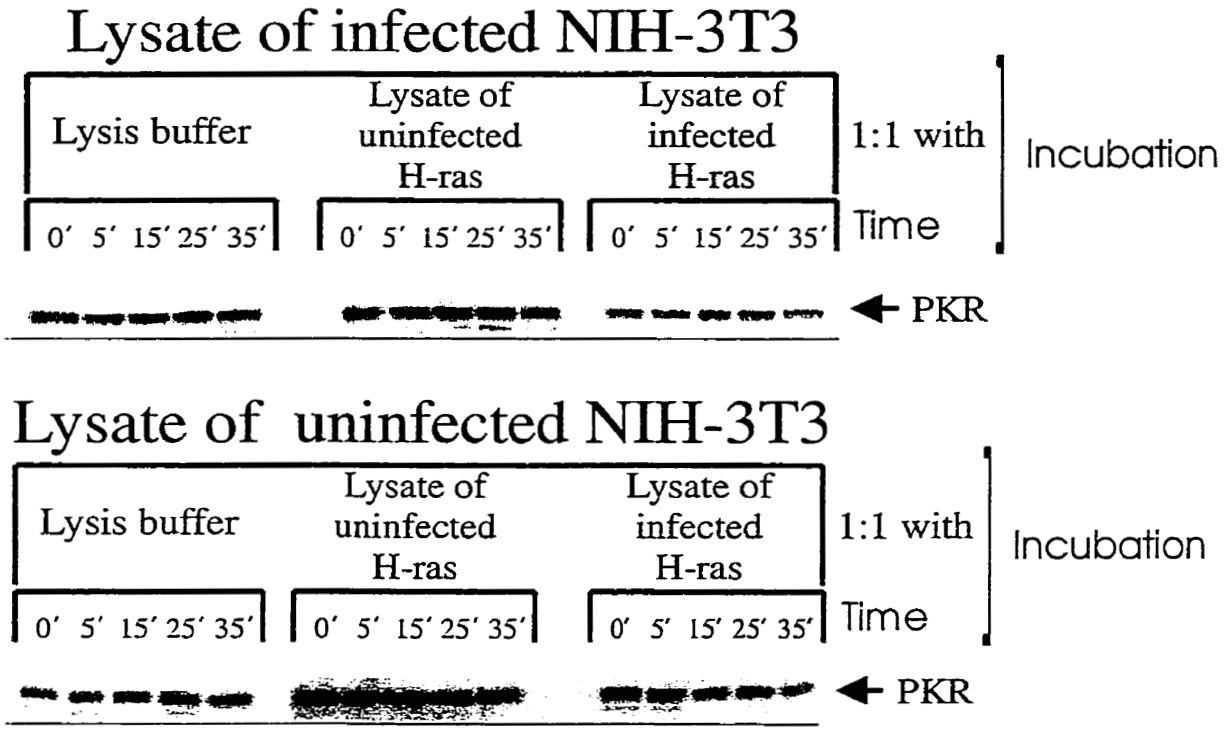
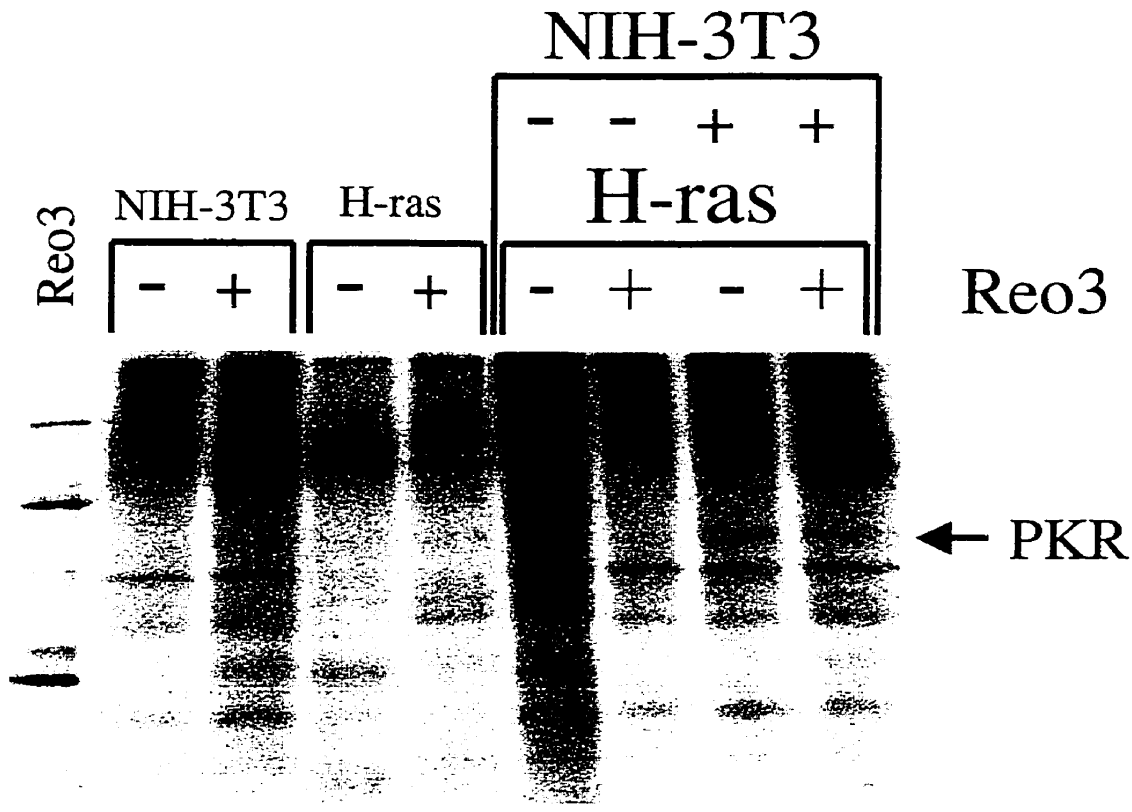

Lysate of infected NIH-3T3
Lysis buffer
Lysate of uninfected H-ras
Lysate of infected H-ras
1:1 with
Incubation
0' 5' 15' 25' 35'
0' 5' 15' 25' 35'
0' 5' 15' 25' 35'
Time
PKR
Lysate of uninfected NIH-3T3
Lysis buffer
Lysate of uninfected H-ras
Lysate of infected H-ras
1:1 with
Incubation
0' 5' 15' 25' 35'
0' 5' 15' 25' 35'
0' 5' 15' 25' 35'
Time
PKR

Figure 20:

Phosphorylation pattern during incubation of infected and uninfected NIH-3T3 and H-ras lysates. Cells were infected with reovirus (MOI ~30 PFU/cell), and lysates were prepared at 48 hours post-infection, labelled with ^{32}P-phosphate by the *in vitro* kinase assay and incubated in different combinations for 35 minutes at 37ºC. The samples were analyzed by 10% SDS PAGE.


NIH-3T3
- - + +
H-ras
NIH-3T3
H-ras
Reo3
- + - + - + - +
Reo3
PKR

This can be taken as a negative result and one can conclude that *ras* transformed cells have no ability to dephosphorylate activated PKR. However, it is of value to analyze the efficiency and sensitivity of this type of experiment. The shortcomings of the *in vitro* kinase assay have already been discussed. These shortcomings are compounded by the tremendous amount of choice of conditions in the mixing step. The ratio of the lysates in the mixture, the time of harvesting, the mixing conditions, the conditions and duration of incubation, even the optimal multiplicity of infection would all have to be optimized in order to have a hope of gathering some conclusive results.

Even then, we must consider the fact that the cell lysate preparation does not disrupt molecular interactions that exist at the time of harvesting. So, lets for a moment, assume that the active inhibitor in H-ras lysates is co-localized with intrinsic PKR. Introduced external PKR may not have the ability to compete the intrinsic PKR to show any significant or realistic effect. It is also possible that phosphorylated PKR in NIH-3T3 cell lysate is bound to its regulators which will also diminish the effect.

Overall, results from any experiment with this many potential interferences and unknowns must be taken with much skepticism and distrust. We thus chose to focus not on the optimization of conditions for the mixing experiment but on the development of a new assay with fewer problems.

PKR is phosphorylated by in vitro addition of Poly(I)-poly(C) and S1 mRNA to NIH-3T3 and H-ras lysates

An uninfected monolayer of NIH-3T3 cells was harvested and lysates were prepared as previously described. Different concentrations of dsRNA were added at 4ºC. After 2 minutes the *in vitro* kinase assay was performed on the samples followed by immunoprecipitation with polyclonal anti-PKR antibody and SDS-PAGE. PKR phosphorylation increased gradually with increasing final concentrations of dsRNA. The activation efficiency of PKR as a result of its natural activator S1 mRNA was slightly higher than the artificial poly(I)-poly(C) at the same concentrations (Figure 21). This is not surprising since S1 mRNA is a pure species of RNA with constant length of dsRNA stretches that have been shown to efficiently activate PKR, whereas Poly(I)-poly(C) is a mixture of dsRNA of different length some of which may be inhibitory to PKR dimerization.

Phosphorylation of PKR in H-ras cells under identical conditions was faintly detectable (Figure 21B).

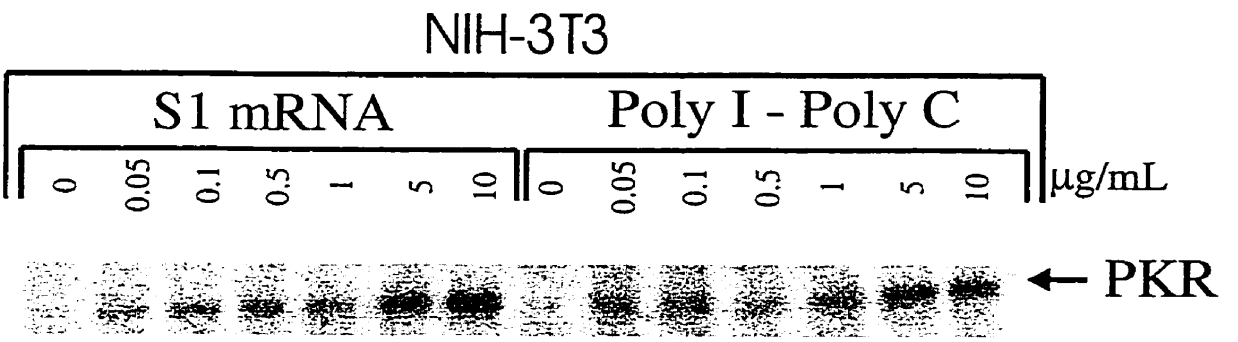
PKR is phosphorylated by in vitro addition of 10 µg/mL S1 mRNA to parental, ras, v-erbB and Raf-1 transformed cell lysates

Uninfected monolayers of NIH-3T3, H-ras, THC-11 and 22W1-4 cells were harvested and lysates were prepared as previously described. S1 mRNA was added to the final concentration of 10µg/mL. After a 2 minute incubation at 4ºC the *in vitro* kinase assay was performed on the samples followed by immunoprecipitation with polyclonal anti-PKR antibody and SDS-PAGE.

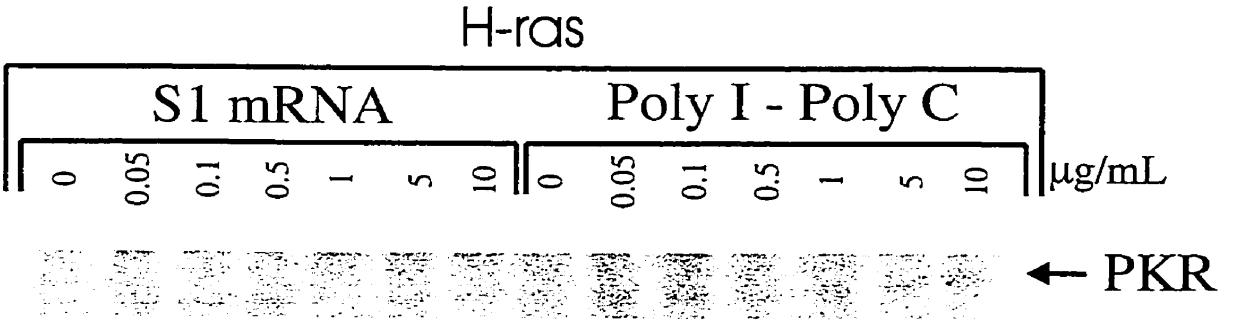
Figure 21:

Phosphorylation of PKR by dsRNA. NIH-3T3 (A) and H-ras (B) cell lysates were prepared, incubated with different concentrations of dsRNA (S1 mRNA or Poly(I)-Poly(C)) for 2 minutes on ice. *In vitro* kinase assay was performed on the reactions followed by immunoprecipitation with anti-PKR antibody and 10% SDS PAGE.

A


NIH-3T3
S1 mRNA
Poly I - Poly C
0
0.05
0.1
0.5
1
5
10
0
0.05
0.1
0.5
1
5
10
µg/mL
← PKR

B


H-ras
S1 mRNA
Poly I - Poly C
0
0.05
0.1
0.5
1
5
10
0
0.05
0.1
0.5
1
5
10
µg/mL
← PKR

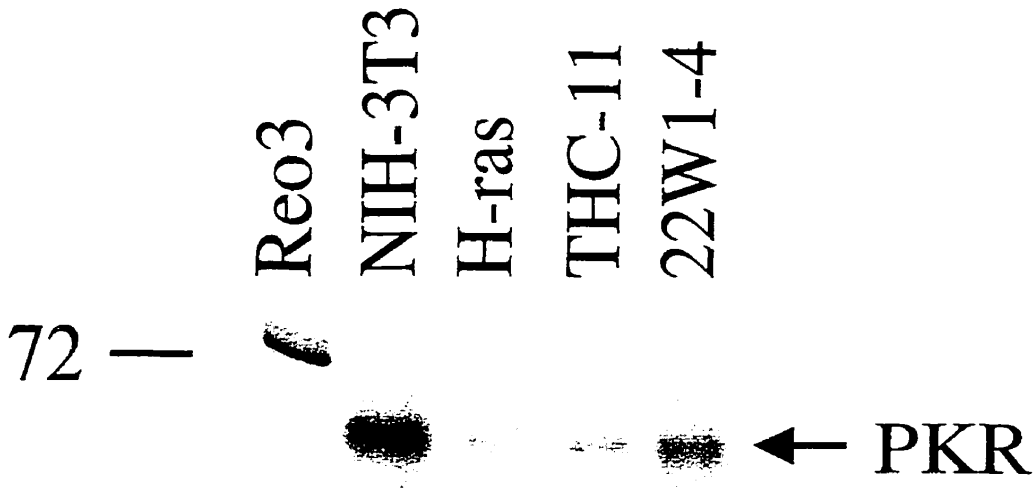
The ability of S1 mRNA to induce PKR phosphorylation in lysates of uninfected parental and transformed cells correlated inversely with the susceptibility of these cells to reovirus infection. NIH-3T3 parental cells have been previously shown to be least susceptible show a superior phosphorylation state of PKR upon addition of 10μg/mL S1 mRNA (Figure 22).

Two possibilities exist that may explain this result. It is statistically possible that *Pkr* genes in H-ras, THC-11, and 22W1-4 cells are mutated and so that PKR is not capable of being phosphorylated. Given the fact that these cell lines were each derived from NIH-3T3 (with wild-type PKR) by different labs and procedures and that there are varying degrees of phosphorylation of PKR populations in these cell lysates, as opposed to its absence or presence, this is very unlikely. A much more plausible scenario is the existence of the inducible cellular inhibitor that decreases the phosphorylation state of PKR whether during infection or upon addition of dsRNA as in this experiment.

It should be noted that regardless of which of these two explanations is in fact descriptive of the nature of the events, this is the first time that difference in PKR regulation has been shown in cells that have not been exposed to live virus.

Figure 22:

Phosphorylation of PKR by dsRNA in NIH-3T3, H-ras, THC-11 and 22W1-4. Cell lysates were prepared, incubated with 10μg/mL of S1 mRNA for 2 minutes on ice. *In vitro* kinase assay was performed on the reactions followed by immunoprecipitation with anti-PKR antibody and 10% SDS PAGE.


72
Reo3
NIH-3T3
H-ras
THC-11
22W1-4
PKR

Experimental design of the new assay

The experimental design of the new assay came out of the necessity to eliminate some of the uncontrollable freedoms in the 'mixing experiments' as well as the need to establish whether this inhibition occurs prior to or post-phosphorylation of PKR. The general concept was to introduce ^{32}P-phosphate labelled PKR into lysates of cells with different susceptibilities to reovirus infection and determine if there was a de-phosphorylation event.

It has been shown that PKR in uninfected NIH-3T3 lysates can be easily phosphorylated *in vitro* by addition of 10μg/mL S1 mRNA (Figure 21). The importance of the volume of lysate used (let us call it X μl) will become evident later in this chapter. This reaction was performed in the presence of ^{32}P-phosphate-γATP as for the *in vitro* kinase assay used previously. The ^{32}P-phosphorylated PKR was partially purified by immunoprecipitation with polyclonal anti-PKR antibody as described above. After 3 washes, the pellet was washed one more in the lysis buffer. This washed pellet was then resuspended and incubated in desired lysates under different conditions.

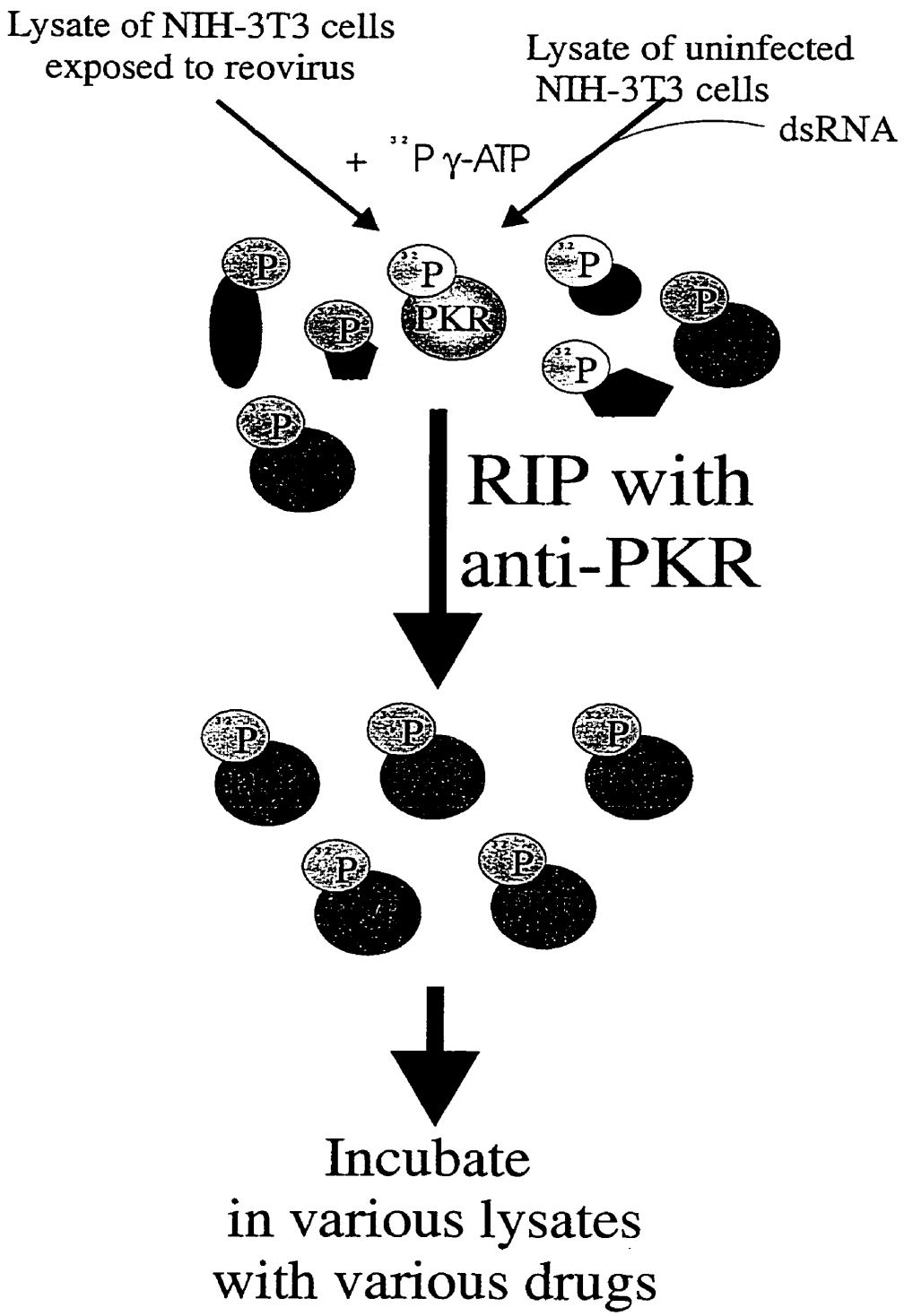


Figure 23: Schematic of the experimental design of the new assay.

Phospho-PKR dephosphorylation in cellular lysate directly correlates with susceptibility of the cells to reovirus infection

The pellet of phospho-PKR (isolated from 30 µl of NIH-3T3 lysate) was incubated in 30 µl of non-susceptible parental cell lysate as well as *ras*, *v-erbB*, *Sos*, and *Raf-1* transformed relatively susceptible cell lysates for 35 minutes at 37°C. The reactions were stopped by boiling in PSB and analyzed by 10% SDS-PAGE. Note: the amount of PKR that was introduced to the lysates is comparable to the amount during interferon treatment.

Two observations can be made from Figure 24: the decrease of apparent molecular weight of PKR after incubation in all of the lysates, as well as the difference in its phosphorylation state between the different lysates. The increase in migration rate of PKR upon incubation with any cellular lysate may be a result of a general dephosphorylation event (Laurent *et al.*, 1985) that is not related to cell's susceptibility to reovirus infection. This is supported by the fact that the change in PKR migration after incubation in non-susceptible (NIH-3T3) and highly susceptible (H-ras) lysates was equivalent. Any number of general cellular phosphatases may dephosphorylate PKR on sites that are non-essential to its anti-viral effect. Based on this argument, this migration change will no longer be addressed in this thesis.

The second observation from this experiment, however is much more interesting to this investigation. Having originally introduced equal amount of

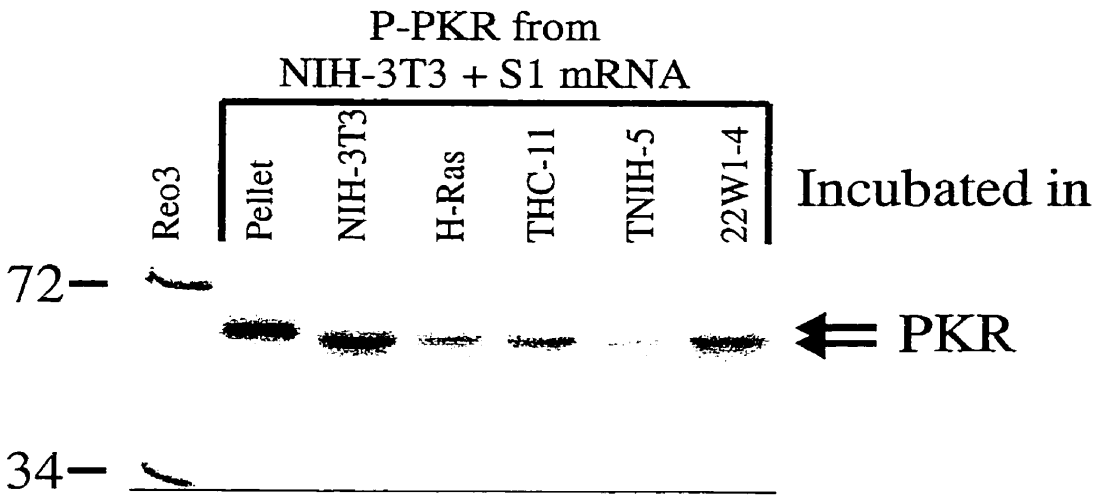
phosphorylated PKR into each of the different lysates (represented in the 'pellet' lane), the dephosphorylation of PKR can be attributed directly to the nature of the lysate. It should be noted that the extent of PKR dephosphorylation is directly proportional to the cellular susceptibility to reovirus infection.

Prior to this investigation, it was unclear whether *ras* transformed NIH-3T3 cells inhibited PKR activity by interfering with its dimerization and autophosphorylation or by dephosphorylating it and preventing its further inhibitory activity. This is the first time that inhibition of PKR activity in reovirus susceptible NIH-3T3-derived cells has been shown to occur after activation of PKR by viral RNA. This supports the possibility of existence of a soluble ras-induced factor which promotes dephosphorylation of PKR.

Extremely faint bands were observed in the higher molecular weight range in the lanes where the PKR dephosphorylation was the most severe. In order to be able to better visualize these phosphoproteins we doubled the amount of ^{32}P-phosphorylated PKR relative to the volume of cell lysates.

Figure 24:

Degree of dephosphorylation of PKR in NIH-3T3, H-ras, THC-11 and 22W1-4 lysates. PKR from NIH-3T3 cell lysate was ^{32}P-phosphorylated by addition of 5 μg/mL S1 mRNA and then immunoprecipitated with anti-PKR antibody. The pellet was incubated in equivalent amount of NIH-3T3, H-ras, THC-11 and 22W1-4 lysates for 35 minutes at 37ºC. The reactions were analyzed by 10% SDS PAGE.


P-PKR from
NIH-3T3 + S1 mRNA
Reo3
Pellet
NIH-3T3
H-Ras
THC-11
TNIH-5
22W1-4
Incubated in
72—
PKR
34—

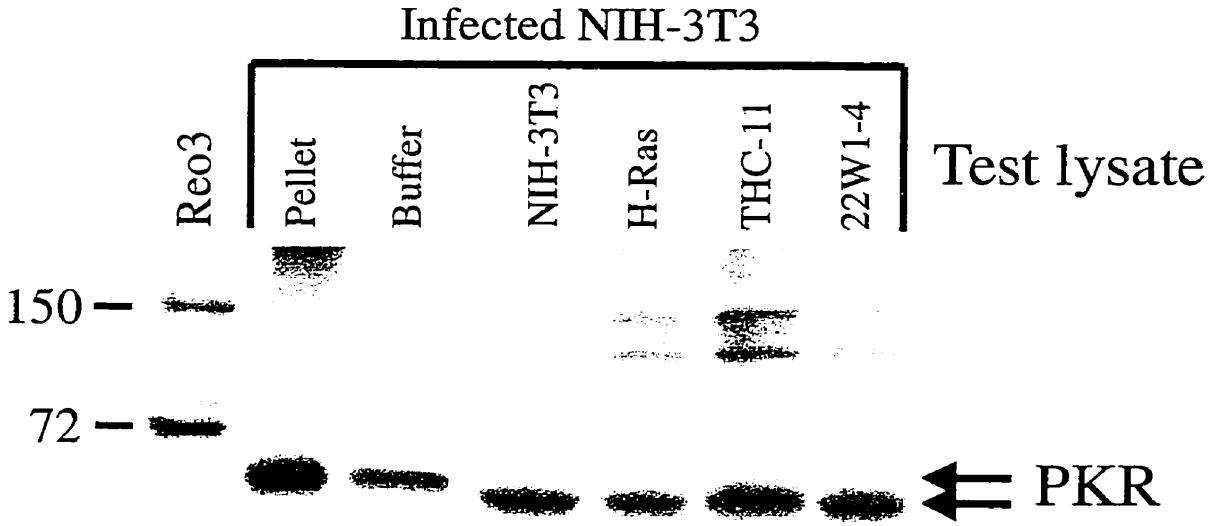
Phosphorylation of a high molecular weight protein is inversely proportional to the phosphorylation state of PKR.

An identical experiment as for the previous experiment (Figure 24) was performed with the exception of the relative volumes of the lysates. The amount of PKR introduced into 30 μL of cell lysate was doubled. As before the reactions were incubated, boiled in PSB and analyzed by 10% SDS-PAGE. Note: the amount of PKR is still within the levels that have been observed in the cells, in fact, it is theoretically lower than 16 hours after induction by interferon-β.

The phosphorylation of two proteins of high molecular weight was visible when phospho-PKR was incubated in lysates of cell that are susceptible to reovirus infection. Intriguingly, these bands were phosphorylated to a lesser extent in Raf-1 transformed cell lysate than in the more susceptible *ras* and *v-erbB* transformed cell lysates. These phospho-proteins were not introduced with the pellet, and no phosphorylation of these bands was detectable in the buffer alone or the NIH-3T3 lysate (Figure 25). Thus, at the very least, this is an event specific to the *ras*, *v-erbB* and (to a lesser extent) *raf-1* transformed cells.

Figure 25:

Phosphorylation of a high molecular weight protein is inversely proportional to dephosphorylation of PKR in NIH-3T3, H-ras, THC-11 and 22W1-4 lysates. PKR from NIH-3T3 cell lysate was ^{32}P-phosphorylated by addition of 5 μg/mL S1 mRNA and then immunoprecipitated with anti-PKR antibody. The pellet was incubated in one half the original amount of lysis buffer, NIH-3T3, H-ras, THC-11 and 22W1-4 lysates for 35 minutes at 37ºC. The reactions were analyzed by 10% SDS PAGE.


Infected NIH-3T3
Reo3
Pellet
Buffer
NIH-3T3
H-Ras
THC-11
22W1-4
Test lysate
150
72
PKR

Phosphorylation level of one of the high molecular weight proteins increases with time during incubation with ^{32}P-PKR.

Some insight into the kinetics of this reaction was obtained by observing it at different times of incubation. The experimental details were the same as for the previous experiment, with the exception of the duration of incubation with the lysate which was varied between 1 and 30 minutes. Phosphorylation of the top of the two high molecular weight bands was visible after 10, 20 and 30 minutes of incubation. Phosphorylation of the bottom of the two bands was faintly distinguishable after 20 minutes of incubation (Figure 26).

The possibility that the phosphorylation of the two bands was dependent on a phosphatase activity was too tempting to let go, and a 'quick and dirty' experiment where phosphatase inhibitors were added to the incubation was performed.

Sodium orthovanadate decreases the phosphorylation level of the top of the two high molecular weight bands.

Incubation of the phospho-PKR in lysates with the addition of phosphatase inhibitors resulted in slight decrease in phosphorylation level of the upper high molecular weight band. Though this result partially satisfies the above mentioned curiosity, much more work must be done in order to be able to conclude that the band in fact represents a tyrosine phosphatase.

Figure 26:

Time course of dephosphorylation of PKR and phosphorylation of the high molecular weight protein in NIH-3T3 and H-ras lysates. PKR from NIH-3T3 cell lysate was ^{32}P-phosphorylated by addition of 5 μg/mL S1 mRNA and then immunoprecipitated with anti-PKR antibody. The pellet was incubated in one half of the original amount of NIH-3T3 and H-ras lysates for 0 - 30 minutes at 37ºC. The reactions were analyzed by 10% SDS PAGE.

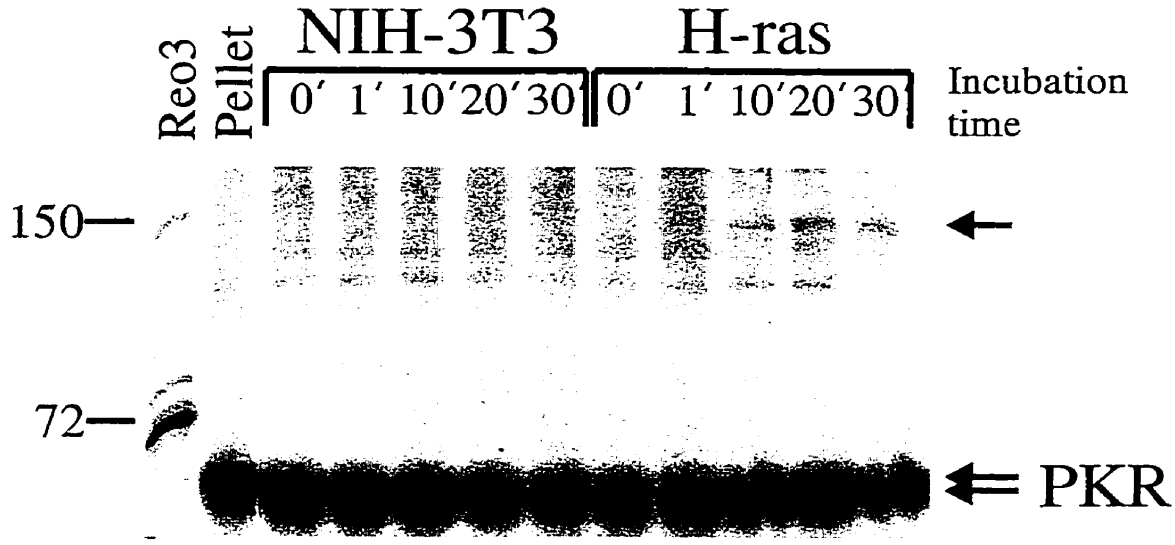
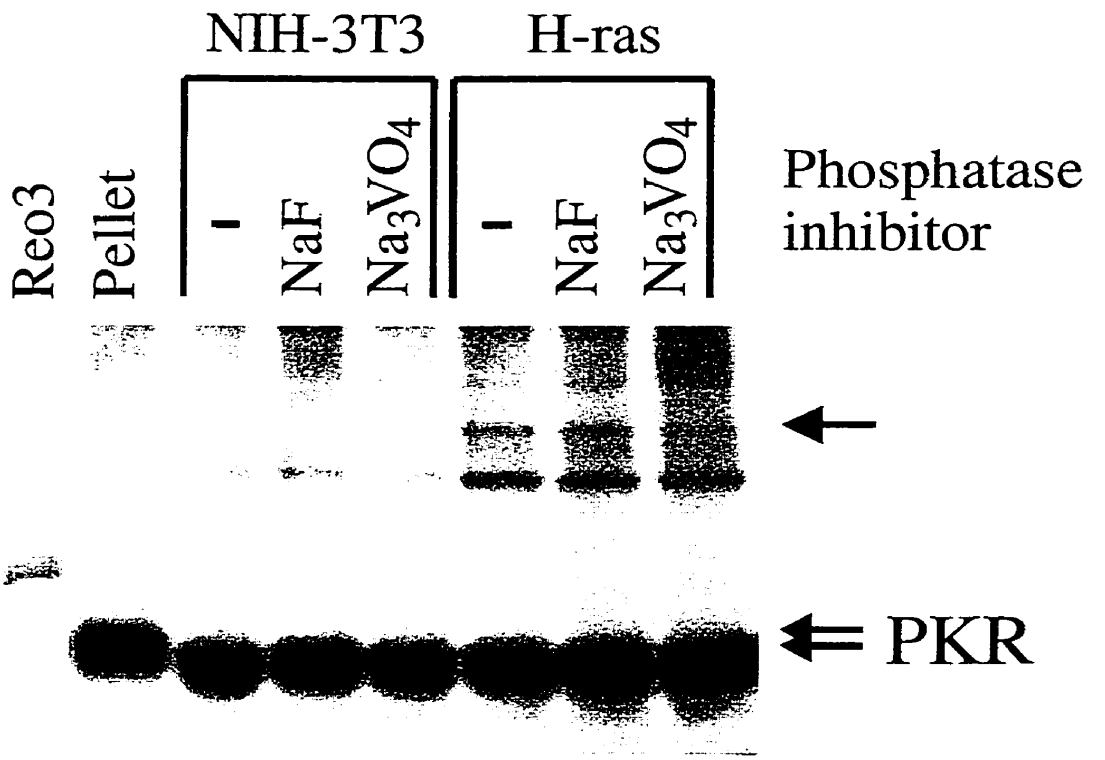

Reo3
Pellet
NIH-3T3
H-ras
0′ 1′ 10′20′30′
0′ 1′ 10′20′30′
Incubation time
150
72
PKR

Figure 27:

Effect of phosphatase inhibitors on phosphorylation of a high molecular weight protein in NIH-3T3 and H-ras lysates. PKR from NIH-3T3 cell lysate was ^{32}P-phosphorylated by addition of 5 μg/mL S1 mRNA and then immunoprecipitated with anti-PKR antibody. The pellet was incubated in one half the original amount of NIH-3T3, H-ras lysates for 35 minutes at 37ºC, in the presence or in the absence of 5 mg/mL NaF and Na_3VO_4. The reactions were analyzed by 10% SDS PAGE.


NIH-3T3
H-ras
Reo3
Pellet
-
NaF
Na_3VO_4
-
NaF
Na_3VO_4
Phosphatase inhibitor
PKR

Chapter 4. Final Discussion

This investigation began with confirmation of a number of well known general facts for specific conditions chosen for this project. It was confirmed that NIH-3T3 cells were relatively non-susceptible to reovirus serotype 3 and that *H-ras*, *EJ-ras*, *Sos* and *v-erbB* transformed NIH-3T3 cells supported productive viral infection. It was further demonstrated that even transient induction of Ras expression was enough to allow productive infection of reovirus. Overexpression of Raf-1, downstream effector of Ras, however was not sufficient to make the cells susceptible to reovirus infection to the same degree. Thus we have focused our attention on Ras itself.

Under all conditions, the amount of viral protein synthesis inversely correlated to the phosphorylation state of PKR upon introduction of live reovirus to the cells, while the total amount of PKR remained constant. This was observed in chemically untreated NIH-3T3 cells where PKR was clearly phosphorylated after infection. No PKR phosphorylation was detected in any of the susceptible cell lines. Dramatic reduction of PKR phosphorylation accompanied increased susceptibility of cells upon induction of Ras expression.

The inverse relationship between reovirus infection and PKR activation was also demonstrated. In other words, induction of PKR by interferon-β leads to decreased amount of reovirus protein synthesis, while inhibition of PKR by treatment with 2-aminopurine increased reovirus protein synthesis.

While PKR was consistently phosphorylated (as determined by its incorporation of ^{32}P-phosphate) upon the addition of live reovirus to non-susceptible cells, a slightly more sensitive assay was used to observe phosphorylation events occurring in all susceptible cells. ^{33}P-phosphate was shown to be incorporated into a number of high molecular weight bands upon infection of *H-ras*, *Sos*, and *v-erbB* transformed cells with live reovirus. This suggested that inhibition of PKR phosphorylation by activated Ras may possibly involve other intermediate phosphorylation events. An example of this type of ras-dependent regulation was previously reported in BALB-3T3 cells, however outside of the context of viral infection (Mundschau and Faller, 1992; 1994). Characterization of the phosphorylated bands was not pursued further, though it may be of interest in further studies.

The focus of the investigation was shifted to the *level* at which Ras exerts its PKR inhibitory effect. Up to this point, this has not been addressed in our lab or by other investigators. Considering the tremendously complex multi-level network of cellular and viral PKR regulation, it is important to determine at which point regulation of PKR by Ras feeds into this system. First, the mechanisms were segregated into those inhibiting PKR phosphorylation, and those inhibiting PKR activity *after* it has been phosphorylated and activated. The data collected in the field to this point in the investigation shed no light on this problem and a totally new approach had to be taken to be able to elucidate the situation any further.

We began by attempting to mix two lysates at a time in 1:1 ratios, with one of which containing already phosphorylated PKR. This 'index' lysate was chosen to be that of NIH-3T3 cells after introduction of live reovirus since it has been shown to exhibit high levels of PKR phosphorylation. Incubation of this lysate with lysates prepared from susceptible and non-susceptible cells produced no conclusive results. In fact the results proved inconsistent and rather confusing to interpret due to the possible side effects of the conditions on the outcome. Upon more detailed examination of its shortcomings (discussed in the Results Chapter), the whole experiment was abandoned in favour of the development of a new assay.

The idea of introducing phosphorylated PKR into a number of different lysates was retained from the failed experiment, however a number of parameters were greatly restricted. Most importantly, instead of introducing the crude lysate *containing* phosphorylated PKR, in the new assay ^{32}P-PKR was partially purified by immunoprecipitation prior to its incubation with cold lysates.

It is important to note that the only ^{32}P-phosphates that are brought into the reaction are those incorporated into the ^{32}P-PKR. Even if some unincorporated ^{32}P-phosphate-γATP non-specifically precipitated with the pellet, the phospho-PKR pellet was washed three times with 1 mL sarcosyl wash buffer and once with same volume of lysis buffer and its final dilution factor after all the washes would be more than 10^{-5} (generously assuming pellet volume to be 50 μL). Thus,

the only significant source of the radiolabelled phosphate in the reaction is the introduced ^{32}P-PKR.

With the help of this new assay, we were, for the first time, able to show that lysates of cells with overexpressed Ras (or activators upstream of Ras) are capable of *de*phosphorylating PKR after it has been phosphorylated. Furthermore, the extent of this ras-induced dephosphorylation directly correlates with the cell's susceptibility to reovirus infection. This supports the notion that Ras quantitatively reduces the amount of PKR phosphorylation and thus controls the susceptibility of the cell to reovirus infection.

Unexpectedly, another correlation was also observed throughout these experiments. Dephosphorylation of PKR in susceptible cells was accompanied by phosphorylation of two high molecular weight bands. The intensity of these bands increased with incubation time and the higher of the two was sensitive to inhibition by sodium orthovanadate, a tyrosine phosphatase inhibitor.

At this point, attempts to explain the exact mechanism by which the ^{32}P-phosphate becomes incorporated specifically into the high molecular weight proteins turn to pure speculation. Possibilities vary enormously. One feasible mechanism is the direct transfer of the ^{32}P-phosphate from PKR to the two proteins. Our search for a precedence for this type of reaction proved to be rather unsuccessful. Another possibility is that upon PKR dephosphorylation, the ^{32}P-phosphate is released into the lysate and is free to be incorporated into other proteins. A question to consider in this case is the remarkable specificity of the

final targets of these free ^{32}P-phosphates, or perhaps the pellet did bring in some amount of free ATP that was 'tangled' in it.

Despite tremendous room for further exploration, this investigation produced a number of positive outcomes. Existence of a number of reovirus infection-specific phosphorylation events (in addition to the ~100kDa phosphorylation band described in Strong *et al.*, 1998) was demonstrated in reovirus susceptible cells.

Some of the possible future avenues of research are the biochemical characterization of the HMW proteins and their isolation. The suggestion that Ras-dependent PKR regulation is in fact not dependent on reovirus infection is potentially a very important finding. This would connect the growth control mechanisms with that of regulation of apoptosis and tumour suppression. This would directly lead into the study of the Ras-PKR connection during normal cell processes.

On a more infection specific level, other regulators of PKR that may possibly be activated by various viruses (including reovirus) during infection. The role of ribosomes and cellular short RNAs may prove interesting and eluminating upon closer examination. There is a large amount of circumstantial evidence concerning this level of PKR regulation, but a closer look needs to be taken.

It became increasingly clear throughout this investigation that reovirus serotype 3 was opportunistic in nature and was not capable of overcoming the normal anti-viral response mounted by cells against it. This argues against prior suggestions that σ3 is the viral protein capable of sequestering dsRNA and

preventing PKR activation thus allowing progression of reovirus infection. The data presented in this investigation does not eliminate the possibility that σ3 does bind dsRNA *in vivo* during infection, and plays a minor role in PKR inhibition. However, it does abolish σ3 effect as the main 'protector' of reovirus replication who's action is sufficient to inhibit the potent PKR response. In fact, if σ3 was the only anti-PKR mechanism utilized by reovirus, it would be in a class of its own. None of the viruses rely solely on dsRNA sequestration as the main or only method of inhibition of PKR. As discussed in the introduction, vaccinia virus E3L dsRNA binding protein is accompanied in its 'battle' by K3L which binds the PKR active site preventing the binding of other substrates. Influenza NS1 dsRNA binding protein is also aided by the virus' ability to activate a cellular PKR inhibitor, $P58^{IPK}$.

Up to now, no second anti-PKR mechanism intrinsic to reovirus has been identified. Sequestration of dsRNA coming short of protecting the virus from anti-viral response mounted by un-transformed cells, reovirus is then only infects those cells which have lowered or absent PKR activity due to extracellular environment or specific transformation. This molecular evidence not only clarifies the described benign nature of the virus but also supports its use as an anti-cancer drug (as is being developed in our lab). Its absolute requirement for activated ras directs it exclusively to quickly proliferating cancer cells, and away from the healthy cells. This naturally occurring benign virus has long ago

achieved the goal of the early synthetic chemotherapeutic drugs which exhibited horrific side effects and extremely low effectiveness in eradicating cancer from the individual. Yet again, molecular biology, virology and biochemistry have helped to shed light on the mechanism of possible new treatments of some Cancers.

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