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THE INTERFERON INDUCED SERINE/THREONINE PROTEIN KINASE, PKR, IS REGULATED BY THE INFLUENZA VIRUS ACTIVATED PROTEIN, P58^{IPK}, AND THE MOLECULAR CHAPERONES, HSP40 AND HSP70

by

Mark Wallace Melville

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by Michael G Katze
Chairperson of Supervisory Committee

Program Authorized
To Offer Degree Ph. D / Microbiology

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Doctoral Dissertation

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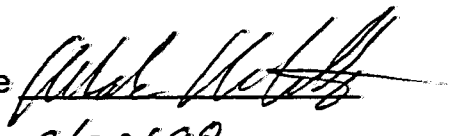
Signature 

Date 9/28/98

University of Washington

Abstract

THE INTERFERON INDUCED SERINE/THREONINE PROTEIN KINASE, PKR, IS REGULATED BY THE INFLUENZA VIRUS ACTIVATED PRORTEIN, P58^{IPK}, AND THE MOLECULAR CHAPERONES, HSP40 AND HSP70

By Mark Wallace Melville

Chairperson of Supervisory Committee: Professor Michael G. Katze
Department of Microbiology

P58^{IPK} is a TPR-containing protein with homology to the J-domain of DnaJ. P58^{IPK} was first recognized for its ability to inhibit the interferon-induced double-stranded RNA-activated serine/threonine protein kinase, PKR, in cells infected with influenza virus. PKR is activated in response to viral infection and downregulates translation initiation via phosphorylation of eukaryotic initiation factor 2 on the α-subunit. Many viruses have developed mechanisms to inhibit the kinase, but only influenza virus has taken advantage of the cellular inhibitor of PKR, P58^{IPK}. P58^{IPK} inhibits PKR through direct protein-protein interaction and prevents dimerization of the kinase. Influenza virus infection activates P58^{IPK} by dissociating it from its own inhibitor, originally termed I-P58^{IPK}.

We now show that I-P58^{IPK} is the molecular chaperone, heat shock protein 40 (Hsp40). P58^{IPK} and Hsp40 bind *in vitro* and *in vivo*, but dissociate upon infection with influenza virus, consistent with our hypothesis that P58^{IPK} is activated by dissociation from its inhibitor. Hsp40 is a mammalian homolog of

the DnaJ protein of *E. coli*, and assists its cognate partner, Hsp70, in folding and refolding nascent and denatured proteins. We now speculate that $P58^{IPK}$ recruits the molecular chaperone Hsp70 to refold (i.e. inactivate) the kinase. We show here that $P58^{IPK}$ binds specifically to the N-terminal half of Hsp70 (the ATPase domain). Furthermore, $P58^{IPK}$ regulates the ATPase activity and refolding activity of Hsp70 in *in vitro* assays. These findings support a model of $P58^{IPK}$ inhibition of PKR in which $P58^{IPK}$ stimulates Hsp70 to alter the conformation of PKR.

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ACKNOWLEDGMENTS

I want to express my extreme gratitude to the member of the Katze lab who, over the years, have helped me to achieve my dreams and grow in the process. A special thanks to Marlene and Cece who made everything possible, and a joy to do. I also want to thank my family, old and new, whose constant support and understanding has been a foundation for me throughout my education. Particularly to my wife, Catherine, who has the patience of a mountain. The friends I have made here in the Department and in Seattle have helped remind me that you need to look around once in a while, if only to remember where you are. Finally, thanks to my Advisor, Michael Katze, who had the courage to accept me into his lab and pursue my studies, the wisdom to discover when I was faltering and needed help, the generosity in spirit and scientific endeavors, and the trust that I would do what I set out to do.

DEDICATION

This dissertation is dedicated to the memory of my father, William Lyn Melville. His last wish was to see me graduate. He is with me now and forever.

CHAPTER 1: INTRODUCTION

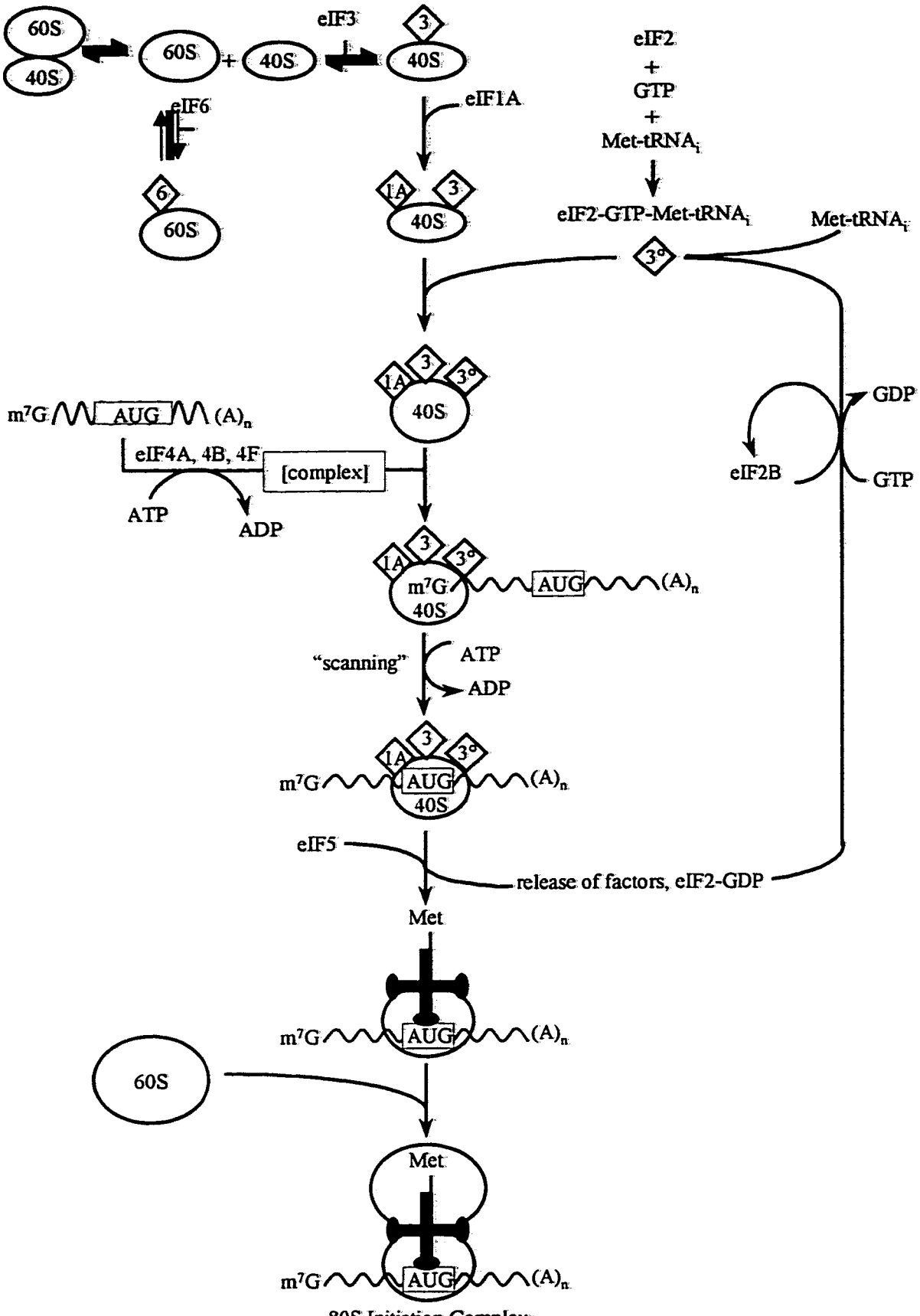
This doctoral dissertation details the characterization of the regulation of PKR, the serine/threonine protein kinase that is induced by interferon and activated by double-stranded RNA. PKR is most widely recognized for its ability to downregulate protein synthesis through phosphorylation of the α-subunit of eukaryotic initiation factor 2 in response to virus infection. The work presented here expands on the previous discovery that influenza virus infection activates a latent cellular inhibitor of PKR, called $P58^{IPK}$, to counteract the antiviral effects of the kinase. This work originated from studies of selective translation in influenza virus infected cells. Therefore, a brief overview of protein synthesis initiation and selective translation by influenza virus will help place this work in the proper biological context.

Overview of protein synthesis initiation

Messenger RNA translation consists of three steps: initiation, elongation, and termination. A model of eukaryotic translation initiation is shown in Fig. 1.1 (Merrick 1992). The initiation step is generally regarded as the rate limiting step in translation, and involves a large number of accessory proteins, or eukaryotic initiation factors (eIF's) (See reviews by (Merrick and Hershey 1996; Pain 1996)). Protein synthesis initiation begins when the complete ribosome is dissociated into its 40S and 60S subunits. This is stimulated by eIF-6, and eIF-

Figure 1.1. Schematic representation of eukaryotic translation initiation.

Adapted from Merrick 1992 Microbiol Rev.. See text for details.



80S Initiation Complex

3 binding to the 40S subunit prevents re-association of the subunits. The eIF-1A initiation factor also binds to the 40S subunit, and the resulting 43S complex is now ready to bind the eIF-2 ternary complex. Eukaryotic initiation factor 2 is a trimeric GTP-binding protein that forms a ternary complex with GTP and the initiator methionine – tRNA. The function of eIF-2 is to charge the 40S ribosomal subunit with the initiator methionine and thus form the 43S preinitiation complex. The preinitiation complex is joined by the mRNA and multi-subunit cap-binding complex. The cap-binding complex recognizes the 5' end of the mRNA by virtue of the m^7GTP cap structure, and subunits within the cap-binding complex melt secondary structures in the 5' UTR. This assists the 43S preinitiation complex in scanning to what is usually the first AUG codon. When this occurs, GTP brought in with eIF-2 is hydrolyzed by eIF-5, and the initiation factors are released. The release of initiation factors leaves the preinitiation complex free to bind the 60S ribosomal subunit, and translation ensues. The GDP moiety on eIF-2 is exchanged for GTP by the guanine nucleotide exchange factor (also called GEF), eIF-2B, thus recharging the factor for another round of protein synthesis. This is the critical step of protein synthesis initiation with respect to the translational control properties. As discussed next, regulation of protein synthesis occurs primarily at the initiation step, and usually involves one of two initiation factors, eIF-4E or eIF-2, or both. Because these factors are relevant for understanding translational control

mechanisms in influenza virus infected cells as well as conditions of cellular stress, they are discussed in the next section.

Mechanism of translational control: covalent modification of eIF-4E and eIF-2

The initiation factor eIF-4E is a component of the eIF-4F, cap-binding protein complex. The activity of this complex is modified by phosphorylation of the eIF-4E and eIF-4G subunits (Morley et al. 1991) as well as at the level of complex formation/dissociation (Duncan and Hershey 1989; Duncan and Hershey 1987; Lamphear and Panniers 1991; Lamphear and Panniers 1998). The three eIF-4E binding proteins (eIF-4E BP1, BP2, and BP3) inhibit protein synthesis initiation by blocking the assembly of eIF-4E into the eIF-4F cap-binding protein complex (Haghighat et al. 1995; Lin et al. 1994; Pause et al. 1994; Poulin et al. 1998). The affinity of the eIF-4E BP's for the initiation factor is controlled by the phosphorylation state of the binding protein, with the hyperphosphorylated forms (of BP1 and BP2) having a lower affinity for eIF-4E (Gingras and Sonenberg 1997). Thus, phosphorylated eIF-4E BP's indicate efficient translation. Phosphorylation of eIF-4E itself also enhances translation initiation, and has been observed during mitosis, following stimulation with growth factors, tumor necrosis factor-α (TNF-α), and hormones (see review by Sonenberg (Sonenberg 1996)).

In contrast to eIF-4E, phosphorylation of eIF-2α decreases translation initiation rates. Phosphorylated eIF-2α forms a stable complex with eIF-2B, and

prevents recycling of the initiation factor (Clemens 1994). This depletes the pool of functional eIF-2B because eIF-2 is present at a higher molar ratio (Clemens 1994). With no eIF-2 recycled back to the GTP bound form, protein synthesis initiation is blocked. Thus, even small changes in the phosphorylation state of eIF-2α can severely inhibit protein synthesis. Kinases that phosphorylate the α-subunit of eIF-2 have been identified in several systems (for reviews see (de Haro et al. 1996; Hinnebusch 1994)). The interferon-induced kinase, PKR, is one of the most well characterized eIF-2α kinases, and highlights the connections between translational control, viral infection, growth control, and cellular stress.

Influenza virus strategies of translational control

Our interest in translational control and PKR led us to study the molecular mechanisms of influenza virus infection. Influenza virus utilizes several mechanisms that ensure viral mRNA's are efficiently and preferentially translated (for reviews, see (Garfinkel and Katze 1993; Garfinkel and Katze 1994; Katze 1996)). In order to maintain translational competence in virally infected cells, influenza virus has evolved a mechanism to avoid the antiviral effects of the protein kinase, PKR. This was first demonstrated in co-infection experiments with the adenovirus mutant strain *dl*331. Adenovirus *dl*331 is deleted the PKR-inhibitory molecule, VAI RNA, and thus is sensitive to the antiviral effects of interferon (Thimmappaya et al. 1982). Experiments

confirmed that the defect in adenovirus *dl*331 was in eIF-2α hyperphosphorylation, as the mutant virus efficiently replicated in cells expressing a non-phosphorylatable form of eIF-2α (Davies et al. 1989), or upon addition of eIF-2 or eIF-2B (Reichel et al. 1985; Schneider 1984). However, replication of *dl*331 was rescued by co-infection with influenza virus (Katze et al. 1984; Katze et al. 1986b). Thus, influenza virus contains a mechanism to downregulate PKR activity in infected cells. More than maintaining efficient protein synthesis, however, influenza virus ensures that only viral mRNA is translated into protein.

Infection by influenza virus is characterized by a dramatic shutoff of host protein synthesis coupled with selective translation of influenza virus proteins (Katze and Krug 1984; Katze and Krug 1990). First, there is an approximate two-fold reduction in cellular RNA polymerase II transcription in cells infected with influenza virus (Katze and Krug 1984). The cellular mRNA's that are synthesized, however, are quickly degraded in the nucleus (Katze and Krug 1984). This is because the virus cleaves the 5' cap structure from nascent cellular mRNA (called "cap-snatching"), and uncapped messages rapidly degraded by nucleases (Krug et al. 1989). The "snatched" cap, and several nucleotides that are stolen in the process, acts as a primer for viral mRNA synthesis (Krug et al. 1989). In addition to preventing export of nascent cellular mRNA, influenza virus messages in the cytoplasm are preferentially translated over cellular mRNA (Garfinkel and Katze 1992; Katze et al. 1986a). Each

influenza virus mRNA contains a conserved sequence at the 5' end of the upstream leader sequence. The importance of the conserved sequence in the 5' UTR was demonstrated by a transfection/infection assay in which cells were transfected with a chimeric reporter gene and then infected with influenza virus. Those chimeras that contained the influenza virus 5' UTR were efficiently translated in the infected cells, while those lacking the viral 5' UTR were not (Garfinkel and Katze 1993). The 5' UTR functions in *cis* to promote translation, but also interacts with *trans*-acting factors that enhance translation (Garfinkel and Katze 1993; Park and Katze 1995). A *trans*-acting factor was recently identified in our laboratory as the RNA-binding protein G-rich sequence factor-1 (GRSF-1) (Park and Katze 1998). Finally, in cells infected with influenza virus the initiation factor eIF-4E is partially dephosphorylated, which lowers the rate of cap-dependent protein synthesis initiation (Feigenblum and Schneider 1993). This may enhance selective translation of influenza virus mRNA if these messages have a reduced requirement for initiation factors, as has been suggested (Katze 1996).

Overview of PKR and P58^{IPK}

The most well characterized role for PKR is downregulation of translation in response to viral infection (Reviewed in (Katze 1992; Katze 1993; Katze 1995; Mathews 1993)). Viral replication often produces double stranded or highly structured RNA molecules that activate the kinase (reviewed in (Mathews

1993)). For example, the secondary structure of HIV TAR RNA is sufficient to activate PKR (Edery et al. 1989; Maitra et al. 1994). Once activated, PKR phosphorylates eIF-2α to downregulate protein synthesis, and thus viral replication is impaired (O'Malley et al. 1986). Viruses that have no mechanism for down-regulating PKR are usually sensitive to the antiviral effects of interferon (For reviews see (Katze 1992; Katze 1993; Katze 1995)). As discussed below, virus strains or mutants that have lost their PKR-inhibitory gene(s) replicate less efficiently in cells treated with interferon. Other viruses simply do not encode a mechanism to downregulate PKR, and thus are sensitive to the antiviral effects of interferon. Two examples are encephalomyocarditis virus (Meurs et al. 1992) and Semliki Forest Virus (George et al. 1986; Stanton et al. 1989).

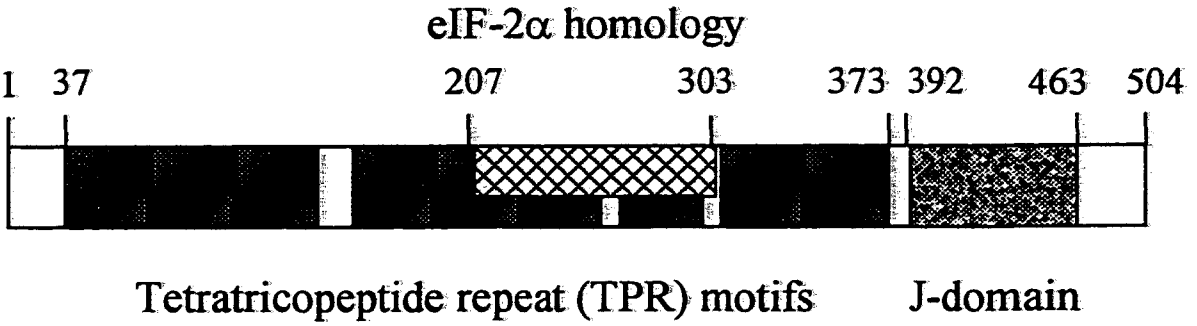
The wide array of strategies developed by viruses to downregulate the kinase highlights the importance of the antiviral properties of PKR. Of particular interest to our laboratory is the mechanism employed by influenza virus. The ability of influenza virus to downregulate PKR activity was first demonstrated in co-infection experiments with the adenovirus mutant strain *dl*331. Adenovirus *dl*331 is deleted the PKR-inhibitory molecule, VAI RNA, and thus is sensitive to the antiviral effects of interferon (Thimmappaya et al. 1982). Experiments confirmed that the defect in adenovirus *dl*331 was in eIF-2α hyperphosphorylation, as the mutant virus efficiently replicated in cells expressing a non-phosphorylatable form of eIF-2α (Davies et al. 1989), or upon

addition of eIF-2 or eIF-2B (Reichel et al. 1985; Schneider 1984). However, replication of *dl*331 was rescued by co-infection with influenza virus (Katze et al. 1984; Katze et al. 1986b). Thus, influenza virus contains a mechanism to downregulate PKR activity in infected cells.

P58^{IPK}, the influenza virus-activated cellular inhibitor of PKR

Lee and colleagues discovered that influenza virus activated a latent inhibitor of PKR, called P58^{IPK} (for inhibitor of protein kinase) (Lee et al. 1990; Lee et al. 1992). The cDNA for P58^{IPK} was cloned in 1994 by Lee et al, and a diagram of the structural components is shown in Figure 1.2 (Lee et al. 1994b). P58^{IPK} contains 9 tandemly arranged tetratricopeptide repeat (TPR) domains in the amino two-thirds of the molecule. These motifs consist of degenerate 34 amino acid repeat sequences that form two anti-parallel amphipathic α-helices that can interact with one another (Das et al. 1998). TPR repeats are predicted to mediate protein-protein interactions, and participate in a wide range of processes, from cell cycle regulation to steroid receptor signaling (for reviews, see Goebl 1991 TIBS, DF Smith 1998 Biol Chem, Lamb et al 1995 TIBS). Although in some cases TPR domains are interchangeable, there appears to be a degree of specificity. For example, Hsp70 binds the two TPR proteins, Hip and Hop (Frydman and Höhfeld 1997). While both proteins bind Hsp70 by virtue of their TPR motifs, they do not compete with one another (Frydman and Höhfeld 1997).

Figure 1.2. The structural domains of P58^{IPK}. The predominant structural domains of P58^{IPK} are indicated here. The protein contains 9 tandemly arranged tetratricopeptide repeat (TPR domains), indicated by the dark boxes. The central region of P58^{IPK} has homology to the α-subunit of eIF-2, the natural substrate of PKR (cross-hatched area). Finally, the C-terminus bears homology to the 70 amino acid J-domain of DnaJ.


eIF-2α homology
1
37
207
303
373
392
463
504
Tetratricopeptide repeat (TPR) motifs
J-domain

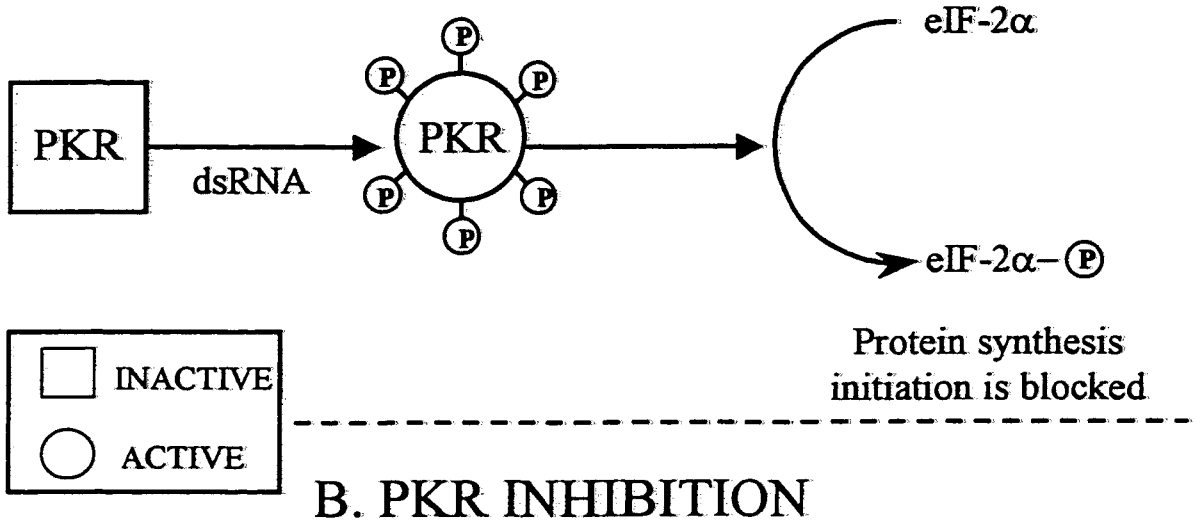
The central domain of $P58^{IPK}$, which includes TPR domains 5, 6, and 7, has weak homology to the natural substrate of eIF-2α. Notably, the homology region includes the analogous serine residue phosphorylated on eIF-2α by PKR. In addition to the tetratricopeptide repeat motifs, the C-terminal portion of $P58^{IPK}$ shares 41% identity with the J-domain of the DnaJ heat shock protein of *Escherichia coli*, placing $P58^{IPK}$ within the heat shock family of proteins (Cyr et al. 1994). DnaJ and its partner protein, DnaK, are prokaryotic homologs of the eukaryotic molecular chaperones, Hsp40 and Hsp70, described earlier. As with other J-domain proteins, $P58^{IPK}$ possesses the invariant HPD tripeptide critical for interaction with Hsp70.

Model of $P58^{IPK}$ – PKR pathway

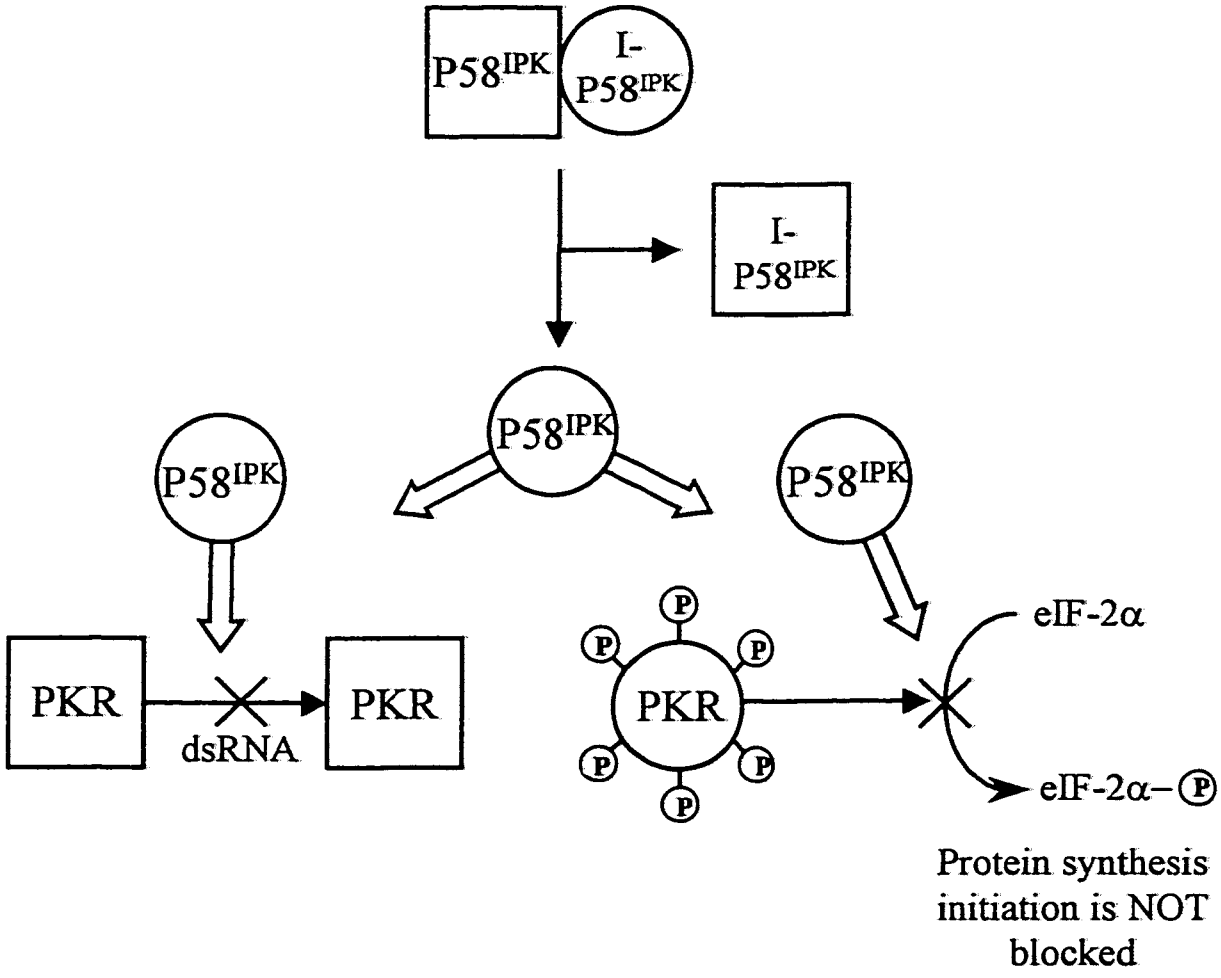
The model of the $P58^{IPK}$ – PKR pathway shown in Fig. 1.3 reflects what was known at the time this study began. PKR is normally latent in the cell, until it encounters an activator dsRNA molecule, such as that produced during viral replication. If the virus has no means of regulating PKR activity, the kinase is activated, phosphorylates the α-subunit of eIF-2, and protein synthesis is impaired. Previous work showed that influenza virus encoded a mechanism to downregulate PKR, as coinfection of influenza virus with adenovirus *dl*331 rescued the defective virus. Pursuing this observation, our laboratory purified a 58 kDa protein from influenza virus infected cells that could inhibit PKR in an *in vitro* assay (Lee et al. 1990). It was subsequently found that the protein, $P58^{IPK}$ was cellular in origin, and could be activated in extracts prepared from

Figure 1.3. Model of PKR – P58^{IPK} pathway. See text for details.

A. PKR ACTIVATION



B. PKR INHIBITION



uninfected cells by fractionating the extract by ammonium sulfate precipitation (Lee et al. 1992). Furthermore, $P58^{IPK}$ activity was "re-inhibited" by adding back one of the ammonium sulfate fractions. The conclusion from these experiments was that $P58^{IPK}$ was inactive in uninfected cells due to the presence of a reversible inhibitor, termed I-$P58^{IPK}$ (for inhibitor of **$P58^{IPK}$**). However, in cells infected with influenza virus or extracts fractionated by ammonium sulfate precipitation, the association between $P58^{IPK}$ and I-$P58^{IPK}$, and $P58^{IPK}$ was activated.

$P58^{IPK}$ inhibited the activation of PKR, as well as prevent phosphorylation of an exogenous substrate by kinase that has already been activated. Indeed, $P58^{IPK}$ is unique in the ability to inhibit both autophosphorylation and exogenous substrate phosphorylation. The mechanism of activation of $P58^{IPK}$ was unclear, but did not involve an increase in levels of the protein. This suggested that $P58^{IPK}$ was activated by some post-translational event, rather than by transcriptional activation. The mechanism of inhibition of PKR by $P58^{IPK}$ was equally unclear, as it did not involve sequestration of activator dsRNA, degradation of either dsRNA or PKR, or dephosphorylation of PKR (Lee et al. 1990; Lee et al. 1992).

We have since determined that PKR and $P58^{IPK}$ interact directly (Polyak et al. 1996). Deletion analysis of $P58^{IPK}$ revealed domains that are important for the inhibition of PKR, as well as interaction with a novel regulator of $P58^{IPK}$, as discussed below. Analysis of the eIF-2α homology region of $P58^{IPK}$ showed

that this region, specifically TPR domain 6, was required for the interaction with and inhibition of PKR (Lee et al. 1994b; Tang et al. 1996). A mutant lacking this region (ΔTPR6) was unable to interact with PKR *in vitro* or in a yeast two-hybrid analysis. It also failed to inhibit PKR *in vitro* or in *in vivo* assays using transfected cells or a yeast functional assay. Moreover, the ΔTPR6 mutant failed to disrupt dimerization of PKR in yeast two hybrid or lambda phage repressor assays (Tan et al. 1998). In contrast, deletion of TPR domain 7 enhanced $P58^{IPK}$ inhibition of PKR *in vivo* (Tang et al. 1996). TPR domain 7 is also the site of interaction of $P52^{rIPK}$ a negative regulator of $P58^{IPK}$. Deletion of the C-terminal ~80 amino acids found that the J-domain was dispensable for the inhibition of PKR *in vitro*, but was required *in vivo* (Tang et al. 1996). The nature of this apparent discrepancy was not entirely clear, but it was proposed that there might be another, positive regulatory factor that $P58^{IPK}$ required *in vivo*. The fact that the major role of the J-domain in other J-domain proteins is to stimulate the activity of Hsp70 suggested that the $P58^{IPK}$ pathway might involve molecular chaperones, such as Hsp70.

PKR – an interferon-induced serine/threonine protein kinase

To place this work in its proper context, a more detailed description of PKR is necessary. PKR is a critical component of the interferon-stimulated host defense response against infection by viruses (Reviewed in (Gale, Jr. and Katze 1998b; Katze 1995)). PKR is expressed constitutively in cells at a low

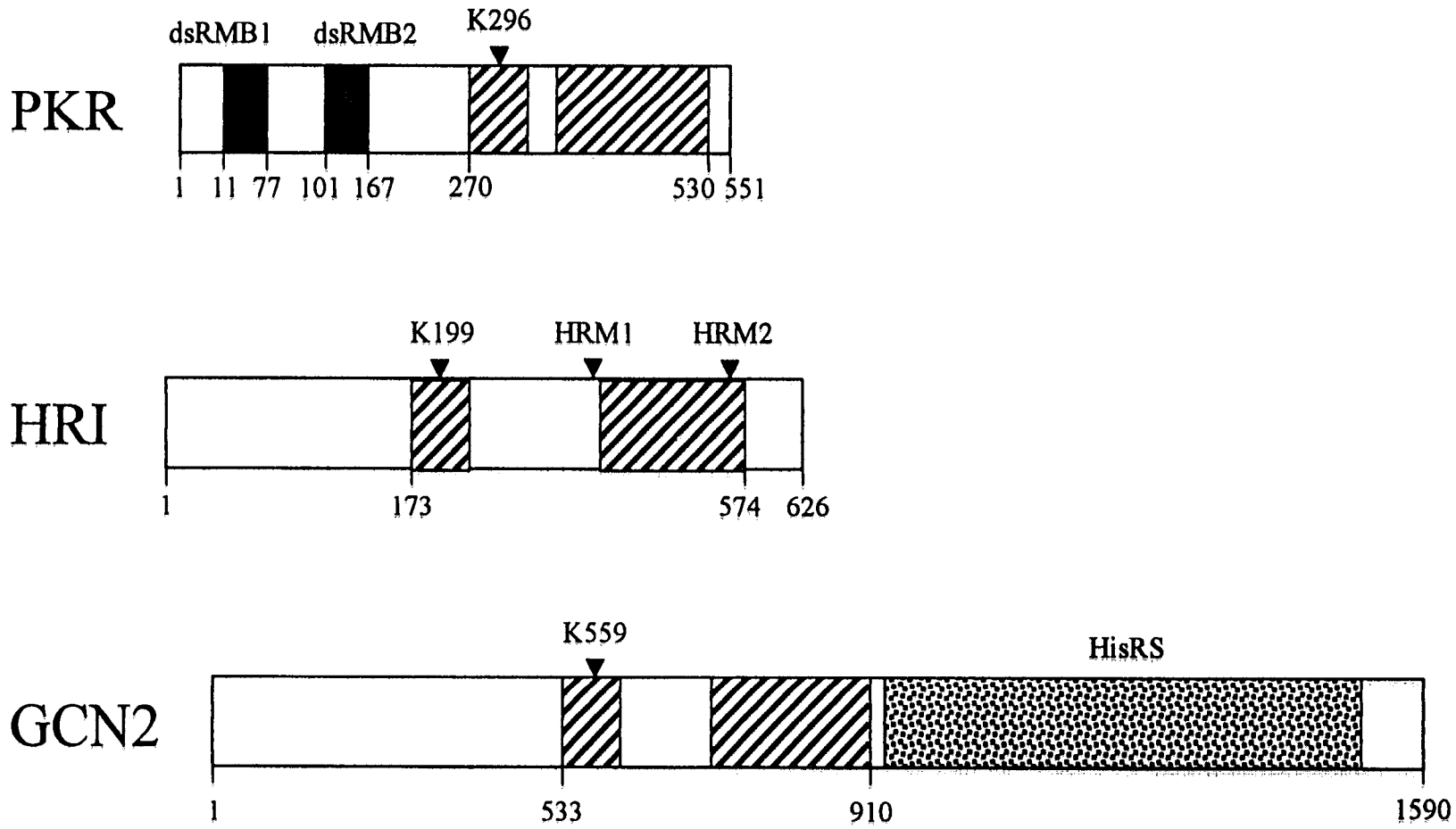
basal level, and the protein is primarily in a latent state (Dever et al. 1993; Petryshyn et al. 1983). In cells treated with interferon, however, the level of PKR is increased 5-10 fold, although this can vary between cell types (Roberts et al. 1976; Samuel 1979; Zilberstein et al. 1976). A detailed analysis of the promoter region of the PKR gene from mouse by Tanaka and Samuel showed that PKR may be regulated by many different pathways, including interferons, NF-IL6, NF-κB, and E2F (Tanaka and Samuel 1994). Although it is clear that interferons are certainly important for the induction of PKR, it is not known whether the other promoter regions are important for the regulation of PKR *in vivo*. There is evidence that PKR may also downregulate its own translation, thus forming an autoregulatory loop. Cells transfected with wild type PKR have lower levels of the kinase than cells transfected with an inactive PKR mutant (K296R), despite expressing similar levels of mRNA (Thomis and Samuel 1992). It should be noted that endogenous PKR is able to downregulate protein synthesis of a transfected gene (Giantini and Shatkin 1989; Kaufman 1985; LLoyd and Shatkin 1992; Seliger et al. 1992; Svensson and Akusjarvi 1985). Moreover, expression of an inhibitor of PKR, such as the transdominant mutant PKR, K296R, will actually enhance translation of a transfected gene (LLoyd and Shatkin 1992; Seliger et al. 1992). It is tempting to speculate that the proximity of PKR to its own mRNA immediately following translation provides a convenient mechanism to regulate the levels of PKR protein.

PKR is an eIF-2α kinase

The interferon-induced double-stranded RNA-activated serine/threonine protein kinase, PKR (also referred to as p68, dsRNA-PK, DAI, dsI, P1 kinase, and p1/eIF-2α kinase (Clemens et al. 1993)), is divided into 2 domains; a C-terminal catalytic domain and an N-terminal regulatory domain (Fig. 1.4). The catalytic domain of PKR contains the 11 conserved subdomains typical of serine/threonine protein kinases (Hanks et al. 1988; Meurs et al. 1990). This also includes the ATP-binding domain and invariant lysine residue, located at position 296. Indeed, mutation of this lysine to arginine (K296R) renders PKR catalytically inactive (Sharp et al. 1993). The regulatory domain contains two double stranded RNA (dsRNA) binding motifs, termed dsRBM1 and dsRBM2 (Green and Mathews 1992; Katze et al. 1991). The dsRBM's are each approximately 60 amino acids long and share homology with other dsRNA-binding proteins (see review by (Clemens and Elia 1997)). The N-terminal domain, dsRBM1, is a higher affinity binding site for dsRNA, and is necessary and sufficient for activation of PKR (Schmedt et al. 1995).

Once PKR is activated, the kinase phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2α) on serine residue 51. This essentially inactivates the factor, which results in a block in translation at the initiation step. In addition to PKR, there are other protein kinases that phosphorylate eIF-2α, and they share similar catalytic domains, including a conserved insertion

Figure 1.4. Catalytic and regulatory domains of three eIF-2α kinases. The structural domains of the three eIF-α kinases, PKR, HRI, and GCN2, are represented in this figure. The kinase domains are represented by the diagonally striped boxes. The division of the kinase domain into two sections represents the insertion sequence between subdomains V and VI. The conserved lysine residue that participates in the phosphotransfer reaction is also indicated in each kinase. The regulatory domains of each kinase are also represented. The two dsRNA-binding domains of PKR (dsRMB1 & 2) are shown in black (top). In the middle is HRI, with the heme recognition motifs indicated (HMR1 & 2). Finally, the histidyl-tRNA synthetase homology region (HisRS) of GCN2 is also shown (bottom).



Adapted from De Haro et al 1996 FASEB J

sequence located between subdomains V and VI (Hanks and Hunter 1995; Samuel 1993). Described next are two well-characterized eIF-2α kinases, the mammalian kinase, HRI (*h*eme *r*egulated *i*nhibitor of translation), and GCN2 of *Saccharomyces cerevisiae* (See (de Haro et al. 1996) for a review of eIF-2α kinases). These kinases highlight the connection between eIF-2α phosphorylation and the cell's response to environmental stresses.

HRI, also called HCR, is an eIF-2α kinase found primarily in reticulocytes, that is activated by heme deprivation (Clemens 1996; Crosby et al. 1994). The function of HRI is to shut down protein synthesis, most of which is dedicated to globin production, in the absence of heme (Chen et al. 1994; London et al. 1976). In addition to heme deprivation, HRI is activated in response to heat shock, as well as treatment with chemical stressors (Duncan 1996; Matts et al. 1991; Matts and Hurst 1992; Matts et al. 1993). Furthermore, both Hsp90 and the EC1 antigen (possibly in conjunction with Hsp70) bind to HRI, although the molecular details regarding these interactions and their roles in HRI regulation remain unclear (Gross et al. 1994; Matts and Hurst 1989). Thus, eIF-2α phosphorylation in erythrocytes is regulated in part by molecular chaperones and the stress response.

The yeast eIF-2α kinase, GCN2, also responds to stress, as it is activated in response to amino acid starvation. The kinase contains a region of homology to the histidyl-tRNA synthetase in its C-terminus (Wek et al. 1989).

When cells are starved for amino acids significant amounts of uncharged tRNA accumulate, which bind to GCN2 at the synthetase homology region (Wek et al. 1995). This activates the kinase, which phosphorylates eIF-2α, reducing the rate of translation initiation (Dever et al. 1992). The transcription factor, GCN4, contains four short upstream open reading frames (uORF's) in the 5' leader of the mRNA. Under normal growth conditions, the translating ribosome will initiate at the first uORF, terminate, and reinitiate at one of the downstream uORF's (Abastado et al. 1991). The GCN4 open reading frame is not translated in this case. When translation initiation is compromised, such as when eIF-2α phosphorylation levels are high, reinitiation is less efficient. That means many ribosomes bypass uORF's 2-4, and reinitiate at the *bona fide* GCN4 AUG. As mentioned above GCN4 is a transcriptional activator, and induces the transcription of amino acid biosynthetic genes. Thus, through eIF-2α phosphorylation, yeast are able to induce expression of genes for amino acid synthesis under conditions of amino acid starvation.

PKR is activated by binding double-stranded RNA (dsRNA)

Binding of dsRNA at the N-terminal dsRNA-binding domains stimulates autophosphorylation of PKR, which activates the kinase (Feng et al. 1992; Galabru et al. 1989; McCormack et al. 1992; Patel and Sen 1992). To efficiently activate the kinase, RNA molecules must have a double stranded region of ~50 base pairs or longer (Manche et al. 1992). However, it does not appear that the double stranded regions need be contiguous. Cech and

colleagues showed that activating RNA molecules may contain non-Watson–Crick A–G mismatches in the double stranded regions (Bevilacqua et al. 1998). PKR can be activated by synthetic duplex RNA, such as poly I:C, and this is often used to activate the kinase *in vitro*. The concentration of dsRNA is also critical, and follows a bell-shaped activation curve. That is, the kinase fails to activate when the concentration of dsRNA is too low or too high (Levin et al. 1980). The optimal range of dsRNA that activates PKR varies, depending upon the specific RNA in question. For example, synthetic duplex RNA (poly I:C) activates PKR between .01μg/ml and 10μg/ml (Baglioni et al. 1981). In contrast, a cellular RNA, termed R-RNA (for **R**egulatory **RNA**), activates PKR in the range of .1μg/ml to 100μg/ml (Petryshyn et al. 1997). In addition to dsRNA, PKR can be activated by other polyanions, such as heparin and dextran sulfate (George et al. 1996; Hovanessian and Galabru 1987; Patel et al. 1994). However, a physiological role for these molecules in the activation of PKR remains to be demonstrated.

PKR activation requires autophosphorylation

Once activator dsRNA binds, PKR autophosphorylates several serine and threonine residues (Galabru and Hovanessian 1987; Taylor et al. 1996). Not all phosphorylation sites are critical for kinase activity, however, and some sites seem to work cooperatively. For example, mutation of either serine-242 or threonine 255 to alanine had no significant effect on PKR activity, while changing threonine 258 to alanine somewhat reduced kinase activity. If either

S242A or T255A was combined with a T258A mutant, however, PKR activity was reduced even more than the T258A mutant alone. Moreover, a triple mutant displayed slightly less activity than either double mutant. These three sites are all found in the serine/threonine rich region between the N-terminal dsRNA binding region and the C-terminal catalytic domain. Additional sites that effect kinase activity are found in the so-called "activation loop", present in most protein kinases that are regulated by phosphorylation (reviewed in (Johnson et al. 1996)). The activation loop is located in the catalytic region, between kinase subdomains VII and VIII. There is usually a conserved Ala-Pro-Glu (APE) tripeptide located 5-10 amino acids C-terminal to the phosphorylation site(s) (Johnson et al. 1996). The PKR activation loop deviates slightly in that it contains an SPE tripeptide, rather than the APE sequence (Meurs et al. 1990). Within the N-terminal amino acids of the activation loop, however, are three potential phosphorylation sites: Thr-446, Ser-448, and Thr-451. Mutations at either Thr-446 or Thr-451 reduce kinase activity, demonstrating that autophosphorylation in the activation loop is important for PKR activity (Romano et al. 1998).

Dimerization is critical for PKR activity

In addition of autophosphorylation, biochemical and genetic evidence indicate that PKR requires dimerization for full activity. Studies of PKR activation showed second order kinetics, indicating that activation was accompanied by dimerization (Kostura and Mathews 1989). The first direct

evidence that PKR dimerizes was a gel filtration analysis in mouse L cells that showed that the kinase exists as a dimer in the cytosol, and a monomer in the ribosome associated fraction (Langland and Jacobs 1992). PKR dimerization has also been observed by two hybrid analysis (Ortega et al. 1996; Patel et al. 1995; Tan et al. 1998), *in vitro* binding assays (Patel et al. 1996), Far-Western analysis (Tan et al. 1998), and the λ repressor fusion system (Tan et al. 1998). Clearly, dimerization is important for PKR activity because proteins that disrupt dimerization also inhibit the kinase (Tan et al. 1998). However, it is not clear whether dimerization is required for activation of PKR (i.e. autophosphorylation), or the activity of the kinase (i.e. phosphorylation of exogenous substrate). The former hypothesis is supported by studies showing PKR autophosphorylation actually occurs through transphosphorylation of one PKR molecule by another (Ortega et al. 1996; Thomis and Samuel 1993; Thomis and Samuel 1995). However, dimerization also plays a role in the phosphorylation of exogenous substrates by PKR, as inactive PKR mutants are transdominant inhibitors of wild type PKR *in vivo*, (Barber et al. 1995; Koromilas et al. 1992; Meurs et al. 1993). PKR is not unique in regulating its activity through dimerization. Indeed, this phenomenon has been observed in other kinases, including Raf-1 kinase (Farrar et al. 1996), casein kinase II (Boldyreff et al. 1996), and cGMP- and cAMP-dependent kinases (Taylor et al. 1990).

Post-translational regulators of PKR

What follows is a discussion of the large number of cellular and viral mechanisms that regulate the activity of PKR. The number and variety of methods to regulate the kinase are an indication of how important it is to regulate PKR activity. A number of PKR regulators have been identified (Table 1.1), and they can be classified by their origins, either cellular or viral, and by their nature, either RNA or protein. The roles of PKR in growth regulation and signal transduction, i.e. in the absence of viral infection, suggest that there are endogenous activators of the kinase. For example, R-RNA, mentioned earlier was identified in 3T3-F442A cells, and may transiently activate PKR during differentiation (Li and Petryshyn 1991; Petryshyn et al. 1997).

Given the consequences of activating PKR, the ability to turn off the kinase is just as important as activating it. Several cellular inhibitors of PKR have been identified and characterized (see Table 1.1). Several of these factors are suspected or confirmed regulators or PKR in viral infection, cellular stress, or growth regulation, and they are described in more detail below. First, the human Alu repeat RNA, an RNA polymerase III transcript like adenovirus VAI, inhibits PKR in *in vitro* and *in vivo* assays (Chu et al. 1998). It is intriguing to note that Alu transcription is increased by cellular stress, as well as infection with several human viruses (Jang and Latchman 1992b; Jang et al. 1992a; Liu et al. 1995; Panning and Smiley 1993). One such protein is the *ras*-activated inhibitor found in *ras*-transformed cells (Mundschau and Faller 1992;

Table 1.1. Molecules that regulate PKR activity

Source	Gene Product	Effect	Target/Mechanism	Reference
Cellular RNA molecules				
	3' UTR of α-tropomyosin	activator	activates PKR	Davis and Watson 1996
	Alu repeat RNA	inhibitor	regulate PKR during stress?	see text
	R-RNA	activator	activates PKR during 3T3-F442A differentiation	see text
Cellular protein molecules				
	dRF	inhibitor	unknown; prevents differentiation in adipose cells	Judware and Petryshyn 1991; Judware and Petryshyn 1992
	La autoantigen	inhibitor	sequester activator dsRNA	Xiao et al. 1994
	$P58^{IPK}$	inhibitor	disrupt dimerization of PKR	see text
	PACT	activator	binds dsRBM of PKR	Patel and Sen 1998
	ras-activated inhibitor	inhibitor	inhibits PKR in *ras*-transformed cells	see text

Table 1.1. (continued)

Source	Gene Product	Effect	Target/Mechanism	Reference
	TRBP	inhibitor	sequester activator dsRNA; Bind directly to PKR?	see text
Viral RNA molecules:				
Adenovirus	symmetrical transcripts	activator	binds dsRBM of PKR	Maran and Mathews 1988
	VAI RNA	inhibitor	binds dsRBM; prevents	see text
Epstein-Barr virus	EBNA RNA	activator	binds dsRBM of PKR	Elia et al. 1996
	EBER RNA's	inhibitor	binds dsRBM; prevents interaction with activator RNA	see text
Hepatitis Delta agent	ssRNA genome (collapsed circle)	activator	binds dsRBM of PKR	Robertson et al. 1996; Circle et al. 1997
HIV-1	TAR RNA	activator/inhibitor*	binds dsRBM of PKR	see text
Reovirus	S1 mRNA 5' UTR	activator	binds dsRBM of PKR	Bischoff and Samuel 1989

Table 1.1. (continued)

Source	Gene Product	Effect	Target/Mechanism	Reference
Viral protein molecules				
Baculovirus	PK2	inhibitor	disrupts dimerization?	see text
HCV	NS5A	inhibitor	disrupts dimerization	see text
Influenza virus	NS1	inhibitor	sequester activator dsRNA	Schmechel et al. 1997
orf virus	OVIFNR	inhibitor	sequester activator dsRNA	Haig et al. 1998
Reovirus	σ3	inhibitor	sequester activator dsRNA	see text
Rotavirus	NSP3	inhibitor	sequester activator dsRNA	Langland et al. 1994
Vaccinia virus	E3L	inhibitor	sequester activator dsRNA	see text
	K3L	Inhibitor	pseudosubstrate for PKR	see text

Mundschau and Faller 1994). In contrast, the TAR-RNA binding protein (TRBP) may inhibit PKR by multiple mechanisms. TRBP is a dsRNA-binding protein, and may sequestor activator RNA molecules, much like reovirus σ3 and vaccinia virus E3L (Park et al. 1994). However, TRBP also interacts directly with PKR (Cosentino et al. 1995), and this interaction apparently does not require dsRNA (Benkirane et al. 1997). This suggests that TRBP may have another, dsRNA-independent mechanism for inhibition of PKR.

Virus encoded inhibitors of PKR

Viruses have developed a wide range of strategies to counteract the deleterious effects of PKR in infected cells. Most viruses studied thus far encode gene produces, either RNA or protein, which directly inhibit PKR (see Table 1.1). For example, adenovirus encodes a short dsRNA (VAI RNA) molecule that binds, but does not activate the kinase, thus blocking activation of PKR by other dsRNA molecules (Kostura and Mathews 1989; Mathews 1990; Mathews and Shenk 1991; Schneider 1996). EBV also encodes a set of small RNA molecules, called EBER RNA's, that may inhibit PKR activation during replication (Clarke et al. 1990). The EBER RNA's are similar to adenovirus VAI RNA in that they are RNA polymerase III transcripts (Kieff and Liebowitz 1990), they are of a similar size (Kieff and Liebowitz 1990), and they inhibit PKR by binding the DRBM of the kinase and preventing interaction with activator dsRNA (Elia et al. 1996). Despite the ample evidence that EBER RNA's can inhibit PKR activity, the physiological role of these molecules remains uncertain.

This is because deletion of EBER RNA's from the EBV genome does not make the virus more sensitive to the antiviral effects of interferon (Swaminathan et al. 1992).

The virally encoded protein inhibitors of PKR utilize mechanisms distinct from the RNA inhibitors. Reovirus encodes a dsRNA-binding protein, σ3, that inhibits PKR by binding and sequestering dsRNA that could activate the kinase (Giantini and Shatkin 1989; Imani and Jacobs 1988; Langland et al. 1994). The significance of reovirus σ3 in the regulation of PKR was confirmed when it was shown that transfection of the gene encoding σ3 rescued the replication of adenovirus *dl*331 (Thimmappaya et al. 1982). Vaccinia virus encodes a similar dsRNA-binding protein, termed E3L, that sequesters dsRNA in order to inhibit PKR activation (Akkaraju et al. 1989; Chang et al. 1992; Watson et al. 1991). Deletion of the gene encoding E3L renders vaccinia virus more sensitive to the antiviral effects of interferon (Beattie et al. 1991), while replacement of E3L with the gene encoding reovirus σ3 rescues the interferon-sensitive phenotype (Beattie et al. 1995).

A second mechanism to inhibiting PKR activity is for the virus to synthesize eIF-2α-like molecules, or pseudosubstrate molecules. This is the mechanism of PKR inhibition utilized by a second PKR-inhibitor encoded by vaccinia virus, the K3L gene product (Beattie et al. 1991; Carroll et al. 1993; Davies et al. 1992; Gale, Jr. et al. 1996; Kawagishi-Kobayashi et al. 1997;

Schneider 1996). As with vaccinia virus E3L, deletion of K3L also renders the virus more susceptible to the antiviral effects of interferon (Beattie et al. 1995). The ubiquity of PKR inhibitors also extends to insect viruses. The baculovirus *Autographa califonica* multiply-embedded nuclear polyhedrosis virus encodes a 25kDa protein, called PK2, with homology to the catalytic domain of eIF-2α kinases, but lacking subdomains I-VI (Ayres et al. 1994; Morris et al. 1994). The truncated kinase interacts directly with PKR, and inhibits both PKR and the yeast kinase, GCN2 (Dever et al. 1998). PK2 is included among the class of pseudosubstrate inhibitors of PKR because mechanism of inhibition appears to be to prevent PKR from interacting with substrates, both other PKR molecules and eIF-2α. It is not clear at this time, however, that this is truly the case.

Lastly, a new class of PKR inhibitors has emerged which are characterized by the ability to inhibit dimerization of the kinase. The hepatitis C virus (HCV) non-structural protein 5A (NS5A) appears to inhibit PKR through this mechanism (Gale, Jr. et al. 1997). There are several strains of HCV, most of which are resistant to interferon therapy (Hoofnagle 1994; Iino et al. 1994). Studies of a large number of different HCV isolates mapped the site of interferon resistance to a region of NS5A, called the ISDR (interferon sensitivity determining region) (Enomoto et al. 1995; Enomoto et al. 1996). We investigated the possibility that the mechanism of interferon resistance was through the PKR pathway. NS5A did indeed inhibit PKR in both *in vitro* and *in vivo* assays, as well as interact directly with the kinase (Gale, Jr. et al. 1998a;

Gale, Jr. et al. 1997). Moreover, if the ISDR was deleted, NS5A was unable to inhibit or interact with PKR (Gale, Jr. et al. 1997).

Viruses that use cellular mechanisms to downregulate PKR

Some viruses do not encode molecules that directly inactivate PKR, but utilize host mechanisms to downregulate the activity of the kinase. The most well characterized example of this is the mechanism employed by influenza virus. Research from our laboratory showed that influenza virus infection activates the latent cellular inhibitor of PKR, P58IPK (Lee et al. 1990; Lee et al. 1992). Our laboratory has also shown that the picornavirus, poliovirus, promotes the degradation of PKR by activating a cellular protease that specifically degrades PKR late during infection (Black et al. 1989; Black et al. 1993).

Roles for the PKR pathway in the absence of viral infection

PKR was originally discovered and characterized as an antiviral protein, but recently we have seen the roles for PKR expand into signal transduction, growth control, and cellular stress. Translational control through eIF-2α phosphorylation appears to be the dominant mechanism through which PKR mediates its effects. However, new potential substrates have been identified, and this opens new molecular pathways that may be subject to regulation by PKR.

PKR has roles in signal transduction

Several studies have implicated PKR as having signal transduction functions (reviewed in (Williams 1995)). The platelet-derived growth factor signal transduction pathway requires the activity of PKR for the induction of immediate early gene transcription (Hall et al. 1989; Mundschau and Faller 1995; Zullo et al. 1985). There are also indications that PKR is a regulator of the NF-κB signal transduction pathway (NF-κB reviewed in (Siebenlist et al. 1994)). This is supported by evidence that MEF's from PKR knockout mice (Yang et al. 1995), or ablation of PKR by antisense RNA (Maran et al. 1994). As further evidence for a role for PKR in NF-κB activation, PKR can phosphorylate the NF-κB inhibitory subunit, IκB, *in vitro* (Kumar et al. 1994). Phosphorylated IκB is quickly degraded *in vivo*, and this exposes a nuclear localization signal on NF-κB (Brown et al. 1995). Consequently, NF-κB translocates to the nucleus and promotes transcription of its target genes.

PKR regulates cell growth and death

Several studies indicate that PKR has growth suppressive properties. Expression of PKR in yeast leads to increased eIF-2α phosphorylation, a decrease in protein synthesis, which causes growth suppression (Chong et al. 1992). Similarly, PKR may help mediate the growth suppressive properties of interferon in mammalian cells (Chong et al. 1992; Jaramillo et al. 1995; Koromilas et al. 1992). The kinase may also function in cell growth and

development, as PKR has a role in adipocyte differentiation (Li and Petryshyn 1991). Apoptotic cell death is regulated in part by PKR, as well (Lee and Esteban 1994). It was demonstrated by Der and colleagues that MEF's derived from *PKR*$^{0/0}$ mice were resistant to dsRNA-, TNF-α-, and LPS-induce apoptosis (Der et al. 1997). Finally, a number of studies have shown that PKR is likely a tumor suppressor protein (Basu et al. 1997; Clemens 1992; Koromilas et al. 1992; Lengyel 1993). Indeed, overexpression of a non-functional PKR mutant leads to malignant transformation through transdominant inhibition of endogenous PKR (Barber et al. 1995; Meurs et al. 1993; Sharp et al. 1993). The mechanism of transformation is likely through eIF-2α phosphorylation, as other studies have determined that expression of a non-phosphorylatable form of eIF-2α is also transforming (Donzé et al. 1995). Although it remains to be proven, it is hypothesized that a general upregulation of protein synthesis, e.g. when eIF-2α is under phosphorylated, leads to enhanced translation of some weakly translated messages (Lodish 1974). It should be noted that many proto-oncogene messages are normally translationally repressed because they contain long 5' leader sequences (Sonenberg 1993).

Overexpression of PKR in mammalian cells has always proven difficult, and it now appears this is because the kinase stimulates apoptosis. Using an inducible expression system, Lee and Esteban demonstrated that overexpression of wild type PKR, but not the K296R mutant, leads to apoptotic

cell death (Lee and Esteban 1994). Specifically, PKR may play a role in dsRNA- and TNF-α-induced apoptosis (Der et al. 1997; Kibler et al. 1997; Yeung et al. 1996).

P58^{IPK} induces malignant transformation

The nature of P58^{IPK} as an inhibitor of PKR, and the observation that PKR may be a tumor suppressor protein, prompted our laboratory to study the oncogenic potential of P58^{IPK}. As expected, overexpression of P58^{IPK} in NIH-3T3 fibroblasts did indeed lead to malignant transformation of these cells (Barber et al. 1994). These cells also displayed lower levels of eIF-2□ phosphorylation than control cells, confirming that P58^{IPK} was functional and inhibiting PKR. A follow up study by our laboratory, however, revealed that there may be a second mechanism for P58^{IPK}-induced transformation. Overexpression of the 'inactive' ΔTPR6 P58^{IPK} mutant also led to malignant transformation (Tang and Katze 1998). Interestingly, the kinetics of transformation differed between wtP58^{IPK} and ΔTPR6, with wtP58^{IPK} inducing oncogenesis more rapidly and severely. The difference in kinetics, combined with the observation that the eIF-2α phosphorylation state is unchanged in ΔTPR6-transformed cells, suggests that P58^{IPK} has a second pathway for transforming cells. Moreover, preliminary indications are that this second transforming pathway requires the J-domain, suggesting a role for molecular chaperones in the transforming activity of P58^{IPK}.

PKR, protein synthesis, and cellular stress

In response to environmental stress, cells undergo a general reduction in protein synthesis that occurs at the translation initiation step. The shutoff of protein synthesis occurs through altering the phosphorylation state of the initiation factors, eIF-4E and eIF-2α. In cells subjected to heat shock, eIF-4E is dephosphorylated, thus reducing its activity, and the eIF-4F complex may also dissociate (Duncan 1996). Protein synthesis initiation is further impaired by concurrent phosphorylation of eIF-2α. As discussed above, phosphorylation of eIF-2α prevents formation of the 43S preinitiation complex, and thus blocks translation. Each of the three identified eIF-2α kinases has demonstrated the ability to downregulate translation in response to cellular stress. The yeast kinase, GCN2, is activated by amino acid starvation, as discussed above. The heme regulated kinase, HRI, is inactive in heme-supplemented lysates, and is often found associated with Hsp90 (Matts and Hurst 1989; Matts et al. 1992). In heat shocked lysates, or lysates treated with chemical stressors, HRI is liberated from Hsp90, and thus activated (Matts et al. 1991; Matts et al. 1992; Matts et al. 1993). There may be role for other heat shock protein in the regulation of HRI, as the kinase is known to bind to Hsp70-family proteins, p56, and the EC1 antigen (Matts et al. 1992; Matts and Hurst 1992).

PKR also downregulates translation in response to cellular stress. Early on, it was thought that PKR was responsible for the increased eIF-2α

phosphorylation in heat shocked cells (Duncan and Hershey 1984; Duncan and Hershey 1989; Saito 1990). Recent studies confirmed that PKR is indeed activated by cellular stress. Specifically, PKR inhibits translation in response to chemicals that elicit stress in the endoplasmic reticulum (Brostrom et al. 1996; Prostko et al. 1995; Srivastava et al. 1995). Although the phenomenon of PKR activation is well established, it is not known how the kinase is activated, or how it is downregulated during recovery from stress. Indeed, downregulating translation is merely the first step for a cell to survive a cellular stress.

Once protein synthesis is compromised, the translation of heat shock messages is enhanced. Perhaps due to this seeming contradiction, the selective translation of heat shock messages has been extensively studied (reviewed in (Duncan 1996)). The precise mechanisms for the selective translation are not well understood, but appear to be a combination of length, conserved sequences, and a lack of secondary structure. Once heat shock proteins are induced, and the stress has passed, the cell enters the recovery stage. During recovery, translation of non-heat shock messages returns to normal, typically 4-6 hours for a moderate to severe heat shock. The temporal control of recovery is significant for our work because we are attempting to correlate protein complex formation and dissociation with activity.

Overview of the molecular chaperones Hsp40 and Hsp70

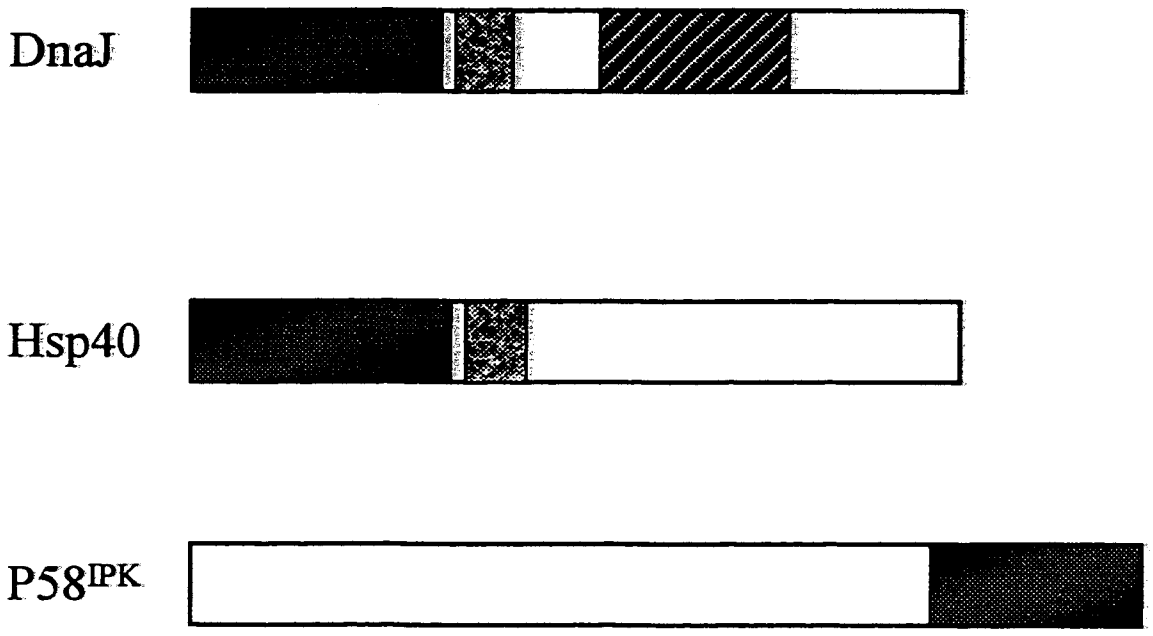
The molecular chaperones, originally identified as proteins induced by heat (so-called 'heat shock proteins'), are a highly conserved super-family of proteins whose main function is to prevent denaturation and aggregation of proteins and to promote the proper folding of proteins (reviewed in (Morimoto et al. 1994)). The major protein families that comprise the molecular chaperone super-family are the Hsp40 (J-domain proteins), Hsp70, and Hsp90 proteins. Hsp40 and Hsp70 are cognate partner proteins that function together in protein transport, folding of nascent peptide chains, and protection and recovery of cells from environmental stress. The Hsp90 family protects denatured proteins from aggregation during stress, and maintains them in a state competent for refolding once conditions in the cell return to normal.

We have discovered that two molecular chaperones, heat shock protein 40 (Hsp40) and heat shock protein 70 (Hsp70), are components of the PKR pathway. Therefore, a brief description of the molecules is required.

Hsp40 and Hsp70: structure and function

Hsp40 is a member of the J-domain family of proteins, as characterized by the presence of its N-terminal J-domain. This large protein family is minimally defined by the presence of the J-domain, but many members share other conserved regions, as well (for a review of J-domain proteins, see (Cheetham and Caplan 1998)). The prototype J-domain protein, DnaJ of *E. coli*, contains three notable structural domains (Fig. 1.5). These include the N-

Figure 1.5. J-domain proteins may contain different structural determinants. Shown here are three J-domain proteins, representing each class of J-domain proteins (see Table 1.2). DnaJ (top) contains the conserved J-domain, G/F domain, and C-terminal cysteine-rich region. Hsp40 (middle) contains a J-domain and G/F region. The C-terminus, shares homology with the DnaJ C-terminus, but lacks the cys-rich region. $P58^{IPK}$ (bottom) only shares homology with DnaJ in the J-domain. Note that the J-domain is C-terminal in $P58^{IPK}$. This is not uncommon among other proteins that only contain a J-domain.


DnaJ
Hsp40
P58IPK
J-domain
G/F domain
Cys-rich region

terminal J-domain, a glycine/phenylalanine rich region (G/F domain) immediately C-terminal to the J-domain, and a cysteine-rich region in the C-terminal half of the molecule. Not all J-domain proteins contain all three regions; many share only two, and others possess only the J-domain itself (Table 1.2). The function of the G/F region is not entirely understood, but it appears to stimulate the activity of the J-domain itself (Cheetham and Caplan 1998). The cys-rich region of DnaJ recognizes denatured proteins and binds to them (Banecki et al. 1996; Szabo et al. 1996). The J-domain is a conserved region of approximately 70 amino acids that forms three α-helices separated by a loop region, which contains an invariant HPD tripeptide (Cheetham and Caplan 1998; Kelley 1998; Pellecchia et al. 1996; Qian et al. 1996). The conserved tripeptide interacts directly with Hsp70 to stimulate ATPase activity and refolding of denatured substrate (Tsai and Douglas 1996). The mammalian DnaJ homologue, Hsp40, does not contain the cys-rich region.

Hsp70 and Hsp40 function together to promote nascent chain folding of translating proteins, protein transport, and secretion (Becker and Craig 1994; Beissinger and Buchner 1998; Hartl 1996; Johnson and Craig 1997; Martin and Hartl 1997; Mayer and Bukau 1998; Welch 1991). They are also a critical part of the stress response system, and help to prevent the denaturation of proteins during cellular stress, and refold proteins that have been denatured as a result of cellular stress. Proteins that are improperly or partially folded or denatured often have exposed hydrophobic regions. The C-terminal refolding domain of

Table 1.2. Members of the J-domain protein family

	Type I	Type II	Type III
Eubacteria	DnaJ	CbpA NolC	DjlA
Archaea	DnaJ		
Virus			T antigen
Yeast	YDJ1 MDJ1 SCJ1 XDJ1	SIS1 zuotin CAJ1 HLJ1 YIR004w YJR097w	Sec63 JEM1 YJL162c YNL227c YFR041c
Plant	ANJ1* ATJ1	D3	
Animals#	HDJ2 Tid56	HSJ1a&b Hsp40(Hdj1)	$P58^{IPK}$ MTJ1 auxilin csp MIDA1

* Plant homologue of YDJ1 – identified in many plant species

Not including Expressed Sequence Tags (ESTs)

Adapted from Cheetham and Caplan 1998

Hsp70 recognizes and binds short (8-11 amino acid) hydrophobic stretches. The normally low ATPase activity of the N-terminal domain of Hsp70 is stimulated approximately 2-fold when a substrate binds. The hydrolysis of ATP to ADP promotes a conformational change in Hsp70 that stabilizes the interaction between the chaperone and its substrate. The mechanics of the refolding event are not entirely clear, but it is thought that the association with Hsp70 provides an environment in which the protein can properly fold. The role of Hsp40 in this reaction is to enhance the refolding efficiency of Hsp70 by stimulating the ATPase activity even further, up to 10-fold.

Many J-domain proteins have functions in addition to regulating Hsp70 activity (reviewed in (Kelley 1998)). These are generally of the Type III class, such as $P58^{IPK}$. The importance of the J-domain in $P58^{IPK}$ was demonstrated *in vivo*, but the molecular details remain to be worked out. For two of the Type III J-domain proteins, however, the function of the J-domain has been well characterized.

First is the clathrin-uncoating factor, auxilin, which contains a C-terminal J-domain homology region. The J-domain is required for the clathrin uncoating activity of auxilin, as well as interaction with Hsc70 (Jiang et al. 1998; King et al. 1997). The current hypothesis is that auxilin targets Hsc70 to specific sites on the clathrin basket, and stimulates the chaperone to refold the clathrin molecule, and thus uncoat the vesicle (Ungewickell et al. 1997). Indeed, deletion of the J-domain of auxilin abrogates clathrin uncoating (Ungewickell et

al. 1995). Furthermore, the clathrin-uncoating activity can be blocked by other J-domain proteins, such as yeast Ydj-1 and human Hsj-1 (King et al. 1997; Cheetham et al. 1996). Presumably, this is because these general chaperones do not contain a clathrin-binding domain, and thus compete with auxilin for Hsc70.

The second co-chaperone is the tumor antigen (T antigen) of the polyomavirus, simian virus 40 (SV40), which has a J-domain at its N-terminus. Work by Kelley and Georgopoulos showed that the T antigen J-domain could functionally substitute for the J-domain in DnaJ of *E. coli* (Kelley and Georgopoulos 1997). More recently, a reciprocal experiment found that the J-domain from Hsj-1 could functionally substitute for the T antigen J-domain (Zalvide et al. 1998). The T antigen also contains a binding site for the retinoblastoma tumor suppressor protein (pRb), which the T antigen may inactivate through direct protein-protein interaction. However, recent reports demonstrate that the J-domain of the T antigen is critical for the inactivation of pRb and malignant transformation (Harris et al. 1998). Srinivasan *et al.* proposed that the T-antigen targets Hsc70 to pRb, and stimulates the chaperone to alter the conformation of pRB (Srinivasan et al. 1997). This tenet is supported by a report showing that a functional J-domain in the T antigen is critical for the accumulation of free E2F and activation of E2F promoter sequences (Harris et al. 1998). Thus, the T antigen and auxilin systems

highlight the potential of J-domain co-chaperone proteins to target specific proteins for refolding by Hsp70.

Specific Aims

When we began this study, the cellular inhibitor of $P58^{IPK}$, I-$P58^{IPK}$, was partially purified (Lee et al. 1992). The goal of this project was to complete the purification and identification of I-$P58^{IPK}$. If I-$P58^{IPK}$ were a novel protein, it would be cloned, sequenced, and characterized. However, if I-$P58^{IPK}$ were a known protein, we would proceed immediately to the characterization step. Furthermore, it was our hope that the characterization of I-$P58^{IPK}$ would also lead to some idea as to the role of $P58^{IPK}$ in the absence of influenza virus infection.

Specific Aim 1: Purify and identify I-$P58^{IPK}$, the cellular inhibitor of $P58^{IPK}$.

The cellular inhibitor of $P58^{IPK}$ was biochemically purified from uninfected Madin-Darby Bovine Kidney cells by cell fractionation and column chromatography. The purified product contained two major proteins; mitochondrial aspartate aminotransferase (mAAT) and the molecular chaperone, Hsp40. Purified, recombinant Hsp40, but not mAAT, displayed I-$P58^{IPK}$ activity.

Specific Aim 2: Characterize the P58^{IPK} - Hsp40 interaction.

P58^{IPK} and Hsp40 directly interacted, as determined by in vitro binding experiments and co-immunoprecipitation in vivo. Confirming the hypothesis that influenza virus infection activates P58^{IPK}, the interaction between P58^{IPK} and Hsp40 was disrupted by influenza virus infection.

Specific Aim 3: Characterize the role of P58^{IPK} as a co-chaperone protein.

P58^{IPK} interacted with Hsp70, as determined by GST-pulldown and two-hybrid+ analyses. The interaction required Hsp40 and ATP. P58^{IPK} regulated the ATPase activity and refolding activity of the molecular chaperone, thus defining P58^{IPK} as a co-chaperone protein.

CHAPTER 2: IDENTIFICATION OF THE MOLECULAR CHAPERONE, HSP40, AS THE P58^{IPK}-INHIBITOR FACTOR, I-P58^{IPK}

Introduction

The interferon (IFN)-induced, serine-threonine protein kinase, PKR, is a critical component of the host defense response against virus infection (Katze 1996; Meurs et al. 1990). PKR becomes autophosphorylated and activated in response to double-stranded RNA (dsRNA), and functions to block protein synthesis by phosphorylating the α-subunit of eukaryotic initiation factor 2 (eIF-2α) (Galabru and Hovanessian 1987). Translational regulation by PKR may play a role in growth regulation, as expression of PKR is growth suppressive in yeast (Dever et al. 1993). Furthermore, expression of a dominant negative PKR mutant has been shown to transform NIH-3T3 cells, suggesting PKR may function as a tumor suppressor protein (Barber et al. 1995; Koromilas et al. 1992; Meurs et al. 1993). The kinase has also been hypothesized to play a role in inducing apoptosis (Lee and Esteban 1994; Lee et al. 1997) and regulating adipocyte differentiation (Li and Petryshyn 1991). The platelet-derived growth factor signal transduction pathway has been found to require the activity of PKR in the induction of immediate early gene transcription (Mundschau and Faller 1995) Finally, PKR has been suggested to be a regulator of the NF-kB signal transduction pathway (Maran et al. 1994).

Several cellular regulators of PKR have been previously described (Lee and Katze 1994a), but the best characterized is $P58^{IPK}$ (IPK, inhibitor of protein kinase) (Lee et al. 1994b). $P58^{IPK}$ was first identified as a PKR inhibitor that is activated during influenza virus infection. $P58^{IPK}$ has since been purified, cloned, and extensively characterized (Korth et al. 1996; Lee et al. 1994b). $P58^{IPK}$ is a member of the tetratricopeptide repeat family of proteins, containing 9 tandemly-arranged 34 amino acid repeats. Tetratricopeptide repeat domains are predicted to form amphipathic helices that may play a role in protein-protein interaction (Lamb et al. 1995). Several tetratricopeptide repeat proteins have been identified thus far, but few have been assigned functional roles (for a review, see (Goebl and Yanagida 1991)). In addition to the tetratricopeptide repeat motifs, the C-terminal portion of $P58^{IPK}$ shares homology with the J-domain of the DnaJ heat shock protein of *Escherichia coli*, placing $P58^{IPK}$ within the heat shock family of proteins (Cyr et al. 1994). Finally, we found $P58^{IPK}$ to display oncogenic properties, possibly through its ability to down-regulate PKR (Barber et al. 1994).

The $P58^{IPK}$-PKR regulatory pathway is highly complex. Evidence that $P58^{IPK}$ is regulated by its own inhibitor, earlier defined as I-$P58^{IPK}$ includes the following: (*i*) $P58^{IPK}$ activity was undetectable in uninfected cells; (*ii*) $P58^{IPK}$ activity, but not physical protein levels, increased upon influenza virus infection; (*iii*) $P58^{IPK}$ activity was unmasked in uninfected cell extracts following biochemical fractionation with ammonium sulfate ($P58^{IPK}$ activity was found in

the 40-60% ammonium sulfate fraction); and (*iv*) $P58^{IPK}$ activity was repressed by adding back the 60-80% ammonium sulfate fraction (which contains I-$P58^{IPK}$ activity) (Lee et al. 1992). Given the above observations, we hypothesized that infection by influenza virus released $P58^{IPK}$ from I-$P58^{IPK}$, thus allowing $P58^{IPK}$ to directly act on PKR and inhibit the protein kinase. The goal of the present study was to identify I-$P58^{IPK}$ using biochemical purification and *in vitro* functional assays for I-$P58^{IPK}$ function. We can now report that the molecular chaperone, Hsp40, also known as Hdj-1, both inhibits and interacts with $P58^{IPK}$, strongly suggesting that the cellular stress pathway participates in PKR regulation during influenza virus infection.

Materials and Methods

Cells and Bacterial Strains. Madin-Darby bovine kidney (MDBK) cells were grown in monolayer cultures in 850-cm^2 roller bottles at 37°C in Dulbecco's Modified Eagle (DME) medium supplemented with 10% calf serum, 2mM L-glutamine and 10mM HEPES buffer. Glutathione-S-transferase (GST)-tagged fusion protein (GST-$P58^{IPK}$) was prepared as described (Lee et al 1994 MCB). For the binding experiments, full length human Hsp40 was expressed from the T3 promoter of pBluescript (Stratagene) harbored in *E. coli* strain XL-1.

Purification of I-$P58^{IPK}$. Monolayer MDBK cells (2×10^{10}) were harvested at ~90% confluency, as described (Lee et al. 1990). Cytoplasmic extracts were centrifuged at 100,000 x *g* for 1 hour in a Beckman Ti 70.1 rotor.

The supernatant (S-100) was fractionated as described (Lee et al. 1990) and assayed for I-P58^{IPK} activity. The active fraction was applied to a MonoQ HR 10/10 fast protein liquid chromatography (FPLC) anion-exchange column (Pharmacia). Proteins were eluted batch-wise with 300mM and 500mM KCl. Fractions were dialyzed into buffer B and assayed for I-P58^{IPK} activity. The active fraction was resuspended into buffer A (75mM Tris-CH_3COOH pH 9.3) and dialyzed against the identical buffer. The dialysate was applied to a MonoP HR 5/20 FPLC chromatofocusing column (Pharmacia). Proteins were eluted on a linear pH gradient of 9.3 - 6.0 with 10% Polybuffer-96 (Pharmacia) adjusted to pH 6.0. Fractions were collected and pooled protein peaks were dialyzed into buffer B [20mM Tris-HCl/100 mM KCl/0.1 mM EDTA/1 mM DTT/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/5% glycerol] and assayed for the ability to inhibit P58^{IPK} activity.

***In vitro* Assays for I-P58^{IPK} Activity.** Two assays were developed to detect I-P58^{IPK} activity, one using crude and partially purified components, and a second using highly purified components. The assay using crude components was used to screen for I-P58^{IPK} activity in the ammonium sulfate and MonoQ fractions and is described in ref. (Lee et al. 1992).

The assay using purified components was used to test for I-P58^{IPK} activity in the MonoP fractions and in the purified mitochondrial aspartate aminotransferase (mAAT) and Hsp40 preparations. Fractions were mixed with GST-P58^{IPK} purified from *E. coli* for 10 minutes at 30°C. PKR, purified from

IFN-treated Daudi cells, was added, and the reaction was incubated at 30°C for 10 minutes. The total reaction volume was 30µl in buffer with final concentrations as follows: 2mM HEPES pH 7.5 / 16.7mM Tris-HCl / 56.7mM KCl / 40mM NaCl / 2mM $MgCl_2$ / 2mM $MnCl_2$ / 1mg/ml BSA / 1.4mM DTT / 6µg/ml Aprotinin / 0.02mM PMSF / 0.05mM EDTA / 6.6% glycerol / 2mM ATP. Poly I:C dsRNA was added at a final concentration of .1µg/ml, in addition to 5µCi (1 Ci = 37GBq) [γ-^{32}P]ATP, and the reaction was incubated at 30°C for 10 min. Calf thymus histone protein IIA (10µg) was added in the presence of 10µCi [γ-^{32} P]ATP, and the reaction was incubated at 30°C for 20 minutes. We earlier found a perfect correlation between the ability of PKR to phosphorylate histones and its natural substrate eIF-2α (Katze et al. 1988). The reaction was stopped with the addition of 30µl stop buffer [2 x protein disruption buffer (150mM Tris-HCl, pH 6.8 / 2.6M 2-mercaptoethanol / 3.7% SDS / 18.5% glycerol / 17mM EDTA / 20µg/ml RNase A) at 60°C], boiled for 5 minutes, and analyzed by SDS-PAGE (14% gel). The degree of substrate phosphorylation was visualized by autoradiography and quantified by PhosphorImager analysis.

mAAT and Hsp40. Recombinant purified Hsp40 was prepared as described (Zylicz et al. 1985). Polyclonal antiserum to Hsp40 was prepared using recombinant protein as immunogen (unpublished data). Recombinant mitochondrial aspartate aminotransferase (mAAT) protein was kindly provided by Marino Martinez-Carrion (University of Missouri, Kansas City, KS), and

purified as described (Altieri et al. 1989). The mAAT antibody was provided by Dr. P.D. Berk, prepared as described (Zhou et al. 1992).

Western Analysis of P58^{IPK}. *In vitro* kinase reactions were performed as described above (without [γ-^{32}P]ATP). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter, according to the procedure of Towbin and coworkers (Towbin et al. 1979). Filters were blocked in phosphobuffered saline (PBS) containing 5% nonfat dry milk, and subsequently probed with monoclonal antibodies specific for P58^{IPK} (2F8 and 9F10) at 1:25 each (Polyak et al. 1996). Blots were washed 4 times with .1% Tween-20 in PBS and hybridized with secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (GIBCO/BRL). Blots were washed repeatedly with .3% and .1% Tween 20 in PBS and the signal was visualized by enhanced chemilumenescence (Amersham).

Two-Dimensional Non-Equilibrium pH Gel Electrophoresis (NEpHGE). Analyses were carried out essentially as described by O'Farrell and coworkers (O'Farrell et al. 1977). Protein samples were prepared as follows, Lammeli sample buffer (6X) was added to a final concentration of .5X and samples were boiled for 5 min. RNase A (1μg) was added and samples were incubated at room temperature for 15 min, then boiled for 2 min. RNase A (10μg) and 10μg BSA were then added as pH markers, and 3μl 3M 2-mercaptoethanol was added. Samples were lyophilized in a Speed Vac

(Savant). Lyophilized samples were resolubilized with 30µl NEpHGE sample buffer (9.5M urea, 2.0% NP-40, 5% β-mercaptoethanol, 2% Servalyte Ampholines, pH 2-11 (Serva)), and incubated for 10 minutes at 30°C. Samples were loaded onto 1.5mm x 15cm tube gels (5% acrylamide, .2% bis-acrylamide, 2% Servalytes, pH 2-11, 2% NP-40, 8M urea), overlaid with .5x NEpHGE sample buffer, and electrophoresed in a Hoefer DE-102 Tube Gel Electrophoresis Apparatus for 2400 volt-hours. After the first dimension run, the tube gels were extruded and layered onto a 1.5mm x 14cm slab gel, and overlaid with .5% SeaKem agarose (FMC) containing .2x Lammeli sample buffer, and analyzed by SDS-PAGE (12.5% gel).

***In Vitro* Transcription and Translation.** Hsp40 plasmid DNA was prepared using a Wizard Mini-Prep kit (Promega) as per the manufacturer's protocol. Full length capped Hsp40 transcript was prepared in a mMessage mMachine Large Scale *In vitro* Transcription Kit (Ambion, Austin, TX) using the T3 promoter of pBluescript. A linear transcript was generated by cleavage at a *Hin*dIII site immediately downstream of the Hsp40 coding sequence. Transcript length and purity was examined by PAGE on a 5% acrylamide 7.5M urea denaturing gel. Message-dependent rabbit reticulocyte lysate was prepared as previously described (Katze et al. 1991). The *in vitro* transcribed product (140ng) was used to program the recitulocyte lysate in the presence of [^{35}S] methionine, as previously described (Katze et al. 1991). The amount of translated product was determined by scintillation counting of trichloroacetic

acid-precipitable protein. The translated product was also visualized by SDS/PAGE and autoradiography.

Results

Purification of the Cellular Inhibitor of P58^{IPK}.

We purified the inhibitor of P58^{IPK} using standard biochemical purification and two independent *in vitro* functional assays. The procedure for the purification is described outlined in Figure 2.1. Briefly, cytoplasmic extracts, prepared from approximately $2x10^{10}$ MDBK cells, were subjected to centrifugation at 100,000 X *g*. The supernatant (S-100) was further fractionated by ammonium sulfate precipitation. The 60-80% ammonium sulfate fraction was found to inhibit P58^{IPK} (data not shown; Table 2.1), and was further purified using a MonoQ FPLC anion-exchange column. The MonoQ fractions were then tested for their ability to inhibit P58^{IPK}. Fractions were mixed with partially purified P58^{IPK} (present in the 40-60% ammonium sulfate fraction). Following a brief incubation, these components were mixed with extracts prepared from IFN-treated 293 cells (as a PKR source). PKR was immunoprecipitated from the mix and assayed for activity. An inhibitor of P58^{IPK} would be expected to reverse the P58^{IPK} mediated inhibition of PKR, resulting in a stimulation of PKR activity and enhanced histone phosphorylation. The 100mM KCl MonoQ flow through fraction contained the highest I-P58^{IPK} activity, restoring nearly 60% of PKR function (Fig 2.2*B*).

Figure 2.1. Outline of I-P58^{IPK} purification scheme. See text for details

I-P58^{IPK} purification scheme

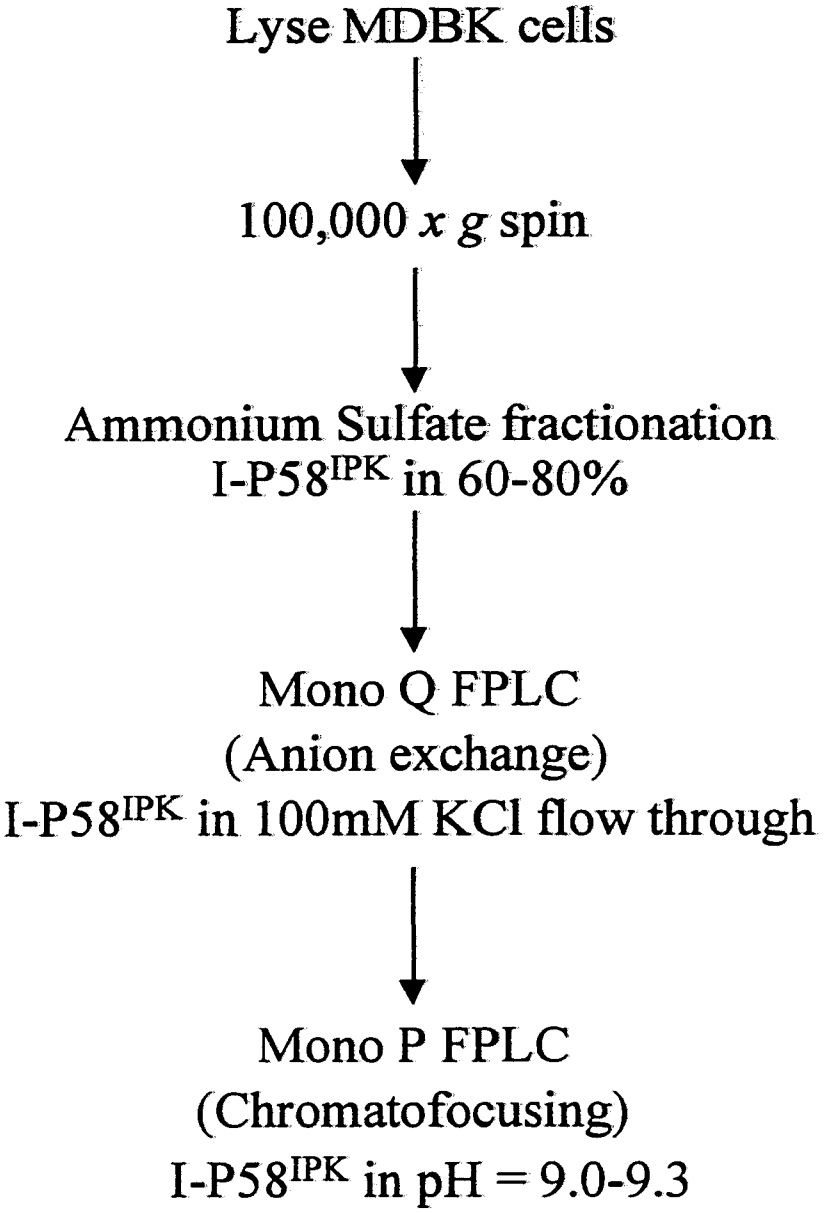


Table 2.1. Summary of the purification of the inhibitor of P58^{IPK}

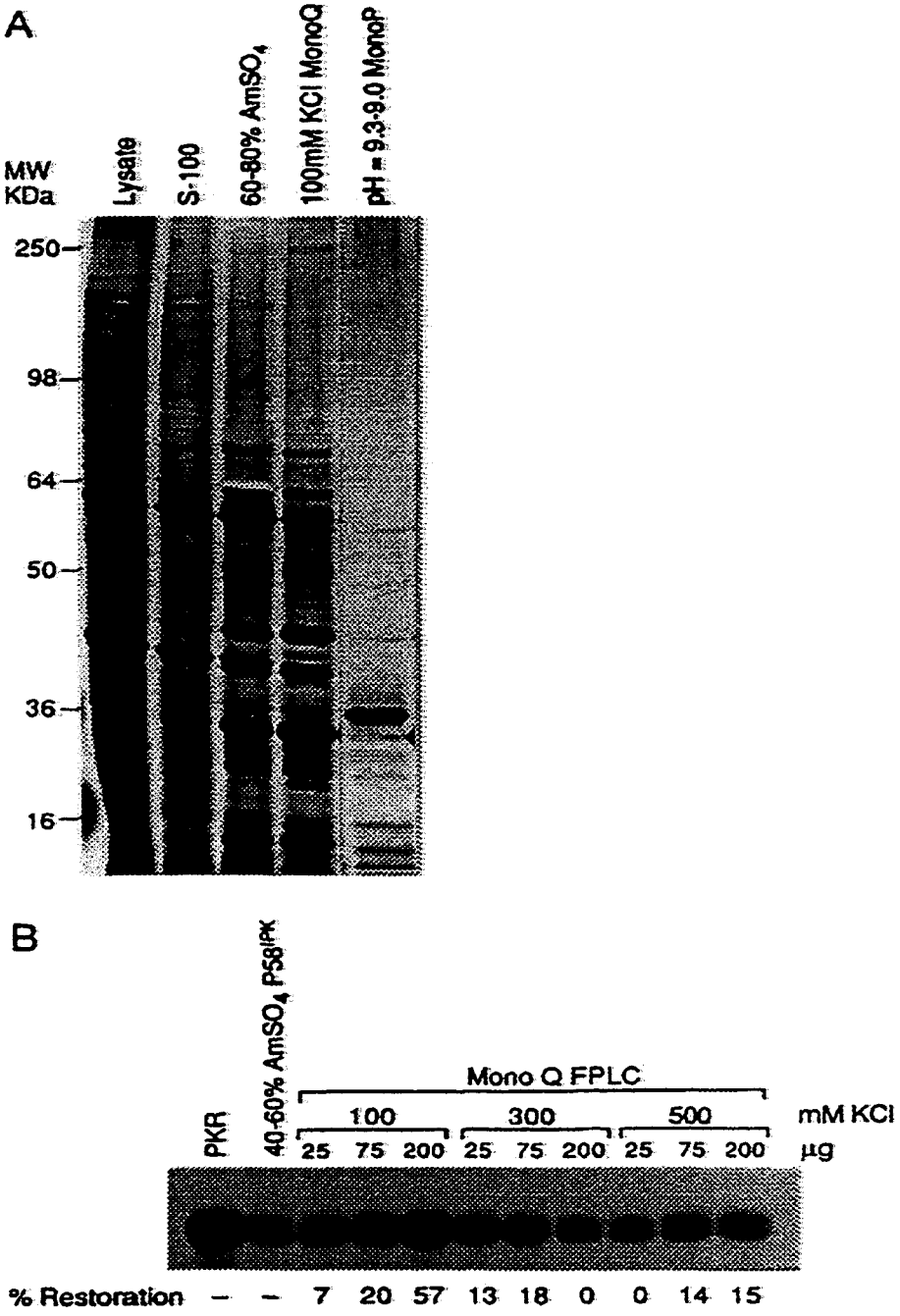
Purification step	Volume, ml	Protein,* mg	Total Activity,† units	Specific Activity,‡ units/mg	Purification, -fold	Yield, %
Cytoplasmic extract	265.00	1439.00	—	—	—	—
100,000 x *g*	255.00	1201.00	—	—	—	—
Ammonium Sulfate	16.50	110.00	4939	44.9	1.0	100
MonoQ FPLC	28.00	55.00	3212	58.4	1.3	65
MonoP FPLC	9.46	.036	445	12364	275	9

*Protein was measured by the Micro BCA protein assay (Pierce) except for the MonoP fractions in which protein was estimated from silver-stained gels.

†One unit of activity is defined as the amount of protein required to cause 1% restoration of PKR activity in the presence of P58^{IPK}.

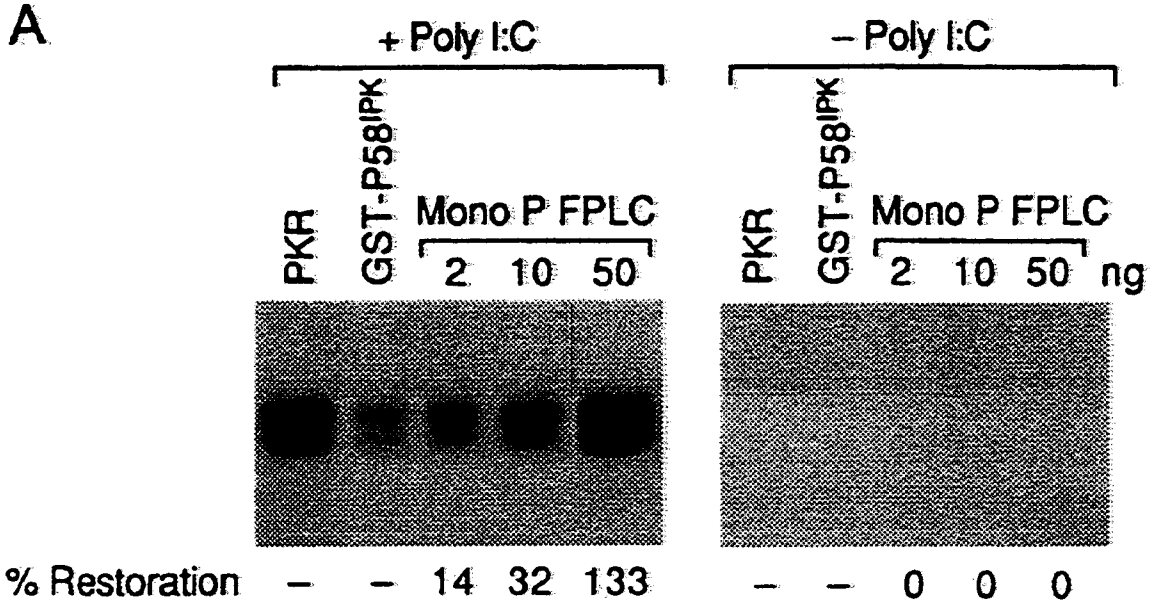
‡Specific activity for the MonoP fraction was corrected for the activity of GST-P58^{IPK} relative to the 40-60% $AmSO_4$ P58^{IPK} used in previous fractions.

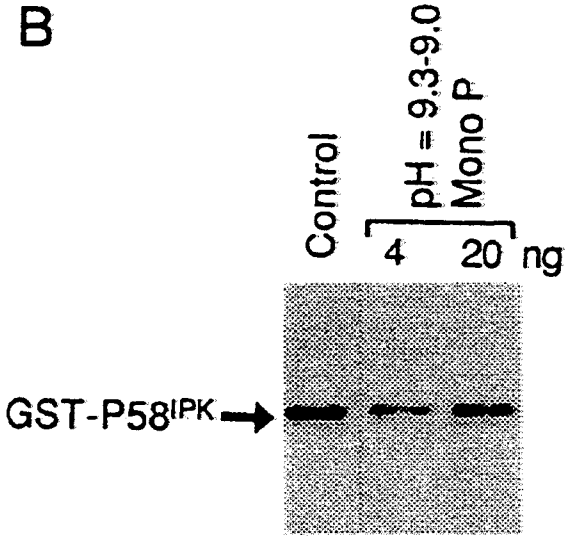
Figure 2.2. I-P58^{IPK} is a 38,000 dalton protein that inhibits P58^{IPK} activity in an in vitro assay. SDS-PAGE analysis of the fractions containing the inhibitor of P58 and an *in vitro* assay showing the inhibition of P58^{IPK} by the MonoQ FPLC fraction: (*A*) 12% SDS-PAGE analysis of, from left to right, cytoplasmic cell extract, 100,000 x *g* supernatant, 100mM KCl MonoQ FPLC flow through, and pH = 9.3-9.0 MonoP FPLC eluate. Proteins were visualized by silver staining (Bio Rad Silver Stain Kit). (*B*) *In vitro* assay examining I-P58^{IPK} activity in the MonoQ FPLC fractions. All reactions contain 100µl 293-IFN extract. 100% PKR activity is defined by incubation with buffer alone (Buffer lane), and 0% activity defined by incubation with P58^{IPK} alone (40-60% $AmSO_4$ P58). The remaining reactions contain 293-IFN, P58^{IPK}, in addition to the indicated amounts of the 100, 300, and 500mM KCl fractions of a MonoQ FPLC purification. The per cent restoration of PKR activity, as determined by PhosphorImager analysis, is shown at the bottom.


A
MW
KDa
Lysate
S-100
60-80% AmSO4
100mM KCl MonoQ
pH = 9.3-9.0 MonoP
250
98
64
50
36
16
B
PKR
40-60% AmSO4 P58IPK
Mono Q FPLC
100
300
500
mM KCl
25 75 200 25 75 200 25 75 200
µg
% Restoration – – 7 20 57 13 18 0 0 14 15

The active fraction was then applied to a MonoP FPLC chromatofocusing column. The eluted fractions were then tested for I-P58^{IPK} inhibitory activity using the alternative *in vitro* assay, composed of purified proteins. The second assay was developed because the crude component assay was unsuitable for screening the highly purified MonoP fractions. Conversely, the assay using purified components could not be used with the ammonium sulfate or MonoQ fractions due to contaminating kinases and phosphoproteins. Again we were testing for a reversal of P58^{IPK} mediated inhibition of PKR and thus a restoration of PKR activity. The dose-dependent activity was found to reside exclusively in the pH = 9.3-9.0 fraction (Fig 2.3*A* and data not shown). Only 50ng of the MonoP fraction was required to completely inhibit P58^{IPK} and restore essentially all the PKR activity. Restoration of PKR activity by the MonoP fraction required preincubation with recombinant P58^{IPK}, suggesting the protein was not acting on PKR itself. Control experiments, performed in the absence of dsRNA, showed this MonoP fraction did not activate PKR (Fig 2.3*A*). Furthermore, Western blot analysis, using P58^{IPK} monoclonal antibodies, showed that the reduction of P58^{IPK} activity was not due to proteolysis (Fig 2.3*B*). The active fractions throughout the purification were analyzed both for their specific activity (Table 2.1) and their polypeptide content (Fig 2.2*A*). The specific activity of the inhibitor of P58^{IPK} increased from approximately 45 units per mg of protein in the 60-80% $AmSO_4$ fraction to 12,364 units per mg in the MonoP fraction, reflecting a 275-fold increase during the purification procedure. Significantly,

Figure 2.3. Analysis of the MonoP purified I-P58^{IPK} activity. (*A*) Left panel shows the restoration of PKR activity in an *in vitro* assay using purified PKR and GST-P58^{IPK} by the pH 9.3-9.0 MonoP fraction by the indicated amounts of protein. 100% activity is defined as the activity of 1μl PKR alone (PKR), and 0% activity defined as PKR mixed with .5μl GST-P58^{IPK} (GST-P58^{IPK}). Right panel is the identical experiment minus dsRNA activator, showing the MonoP fraction does not activate PKR directly. The per cent PKR recovery is shown at the bottom. (*B*) Control assay showing pH 9.3-9.0 MonoP fraction does not degrade GST-P58^{IPK} under reaction conditions identical to panel A. The amounts of MonoP purified protein are indicated, and GST-P58^{IPK} was visualized by Western analysis.


A
+ Poly I:C
– Poly I:C
PKR
GST-P58IPK
Mono P FPLC
2 10 50 ng
% Restoration
– – 14 32 133
– – 0 0 0

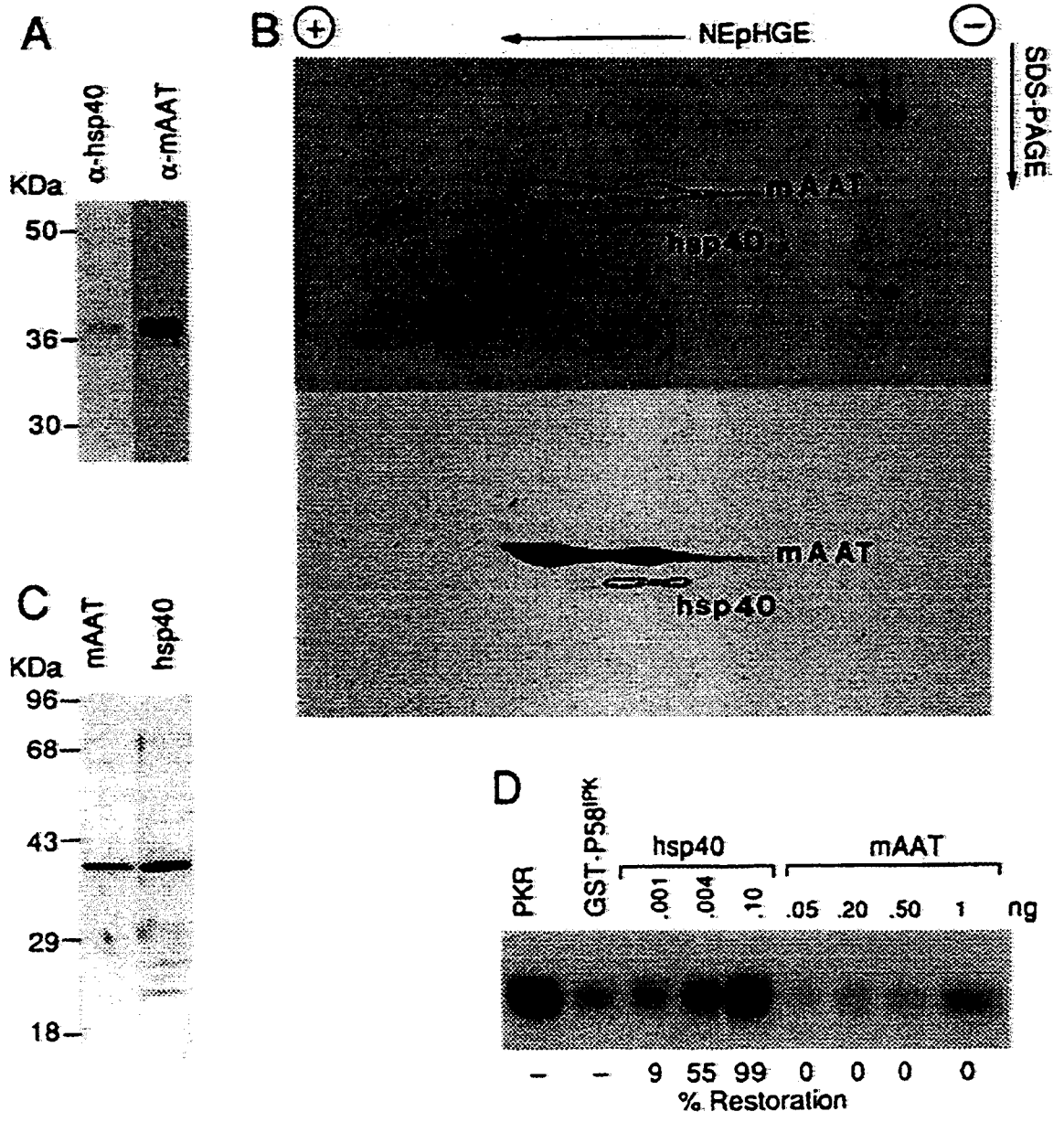

B
Control
pH = 9.3-9.0
Mono P
4 20 ng
GST-P58IPK

one major protein of M_r = 38,000 present in the highly active MonoP fraction was detected by silver staining.

Identification of I-P58^{IPK}.

To determine its identity, the MonoP purified protein was digested with the protease LysC and the peptide fragments subjected to microsequence analysis. Three of three peptide fragments were 100% identical to the bovine mitochondrial aspartate aminotransferase (mAAT; data not shown). In accordance with our MonoP analysis, mAAT is known to have an isoelectric point ≅ 9.0 and M_r ≅ 40,000 (Sonderegger et al. 1982). However, when we tested purified mAAT in our *in vitro* assay, we were unable to inhibit P58^{IPK} and restore PKR activity (Fig. 2.4*D*). Given these results, and the well documented enzymatic functions and subcellular localization of mAAT (Isola et al. 1995), we explored the possibility that we had copurified mAAT with the bona fide inhibitor of P58^{IPK}. Several observations led us to consider a member of the heat shock family of proteins, specifically the eukaryotic DnaJ homolog, Hsp40. First, the homology of P58^{IPK} to the DnaJ protein family suggests that P58^{IPK}, and thus any coregulator, may be involved in the stress response. Second, heat shock proteins interact primarily through direct binding, which is consistent with our evidence of how P58^{IPK} acts and how it is presumably regulated (Lindquist 1986). Finally and most importantly, the Hsp40 protein has a M_r ≅ 38,000 and pI ≅ 9.0, essentially identical to the protein identified in our MonoP purified fraction (Ohtsuka 1993).

Figure 2.4. Analysis of pH = 9.3-9.0 MonoP fraction and Hsp40 and mAAT. (*A*) Western analysis of MonoP fraction. The α-Hsp40 lane was probed with antibody to Hsp40, then the same blot stripped and reprobed with antibody to mitochondrial aspartate aminotransferase (α-mAAT). (*B*) Analysis of pH = 9.3-9.0 MonoP fraction by NEpHGE. .5μg of protein was analyzed by NEpHGE, as described in *Materials and Methods*. The second-dimension gel was transferred to nitrocellulose membrane and probed with antisera to hsp40 for Western analysis (top panel). The blot was stripped and reprobed with antisera to mAAT (lower panel). The mAAT positive band runs slightly more basic and has a higher apparent molecular weight than the Hsp40 positive band by this analysis, clearly indicating the single band seen by 1-D SDS-PAGE is composed of two proteins. (*C*) *In vitro* assay of purified hsp40 and purified mAAT. PKR activity is defined as in fig. 7A. Amounts of Hsp40 and mAAT used are shown, and reflect the relative levels seen in the MonoP purified fraction. The per cent PKR recovery is shown at the bottom.

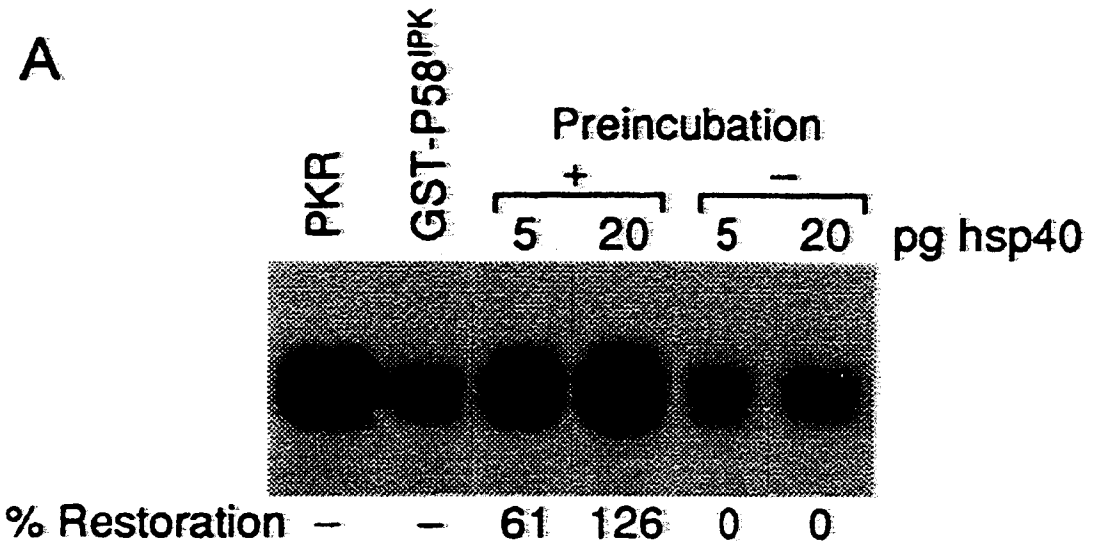

A
α-hsp40
α-mAAT
KDa
50
36
30
B
NEpHGE
SDS-PAGE
mAAT
hsp40
C
mAAT
hsp40
KDa
96
68
43
29
18
D
PKR
GST-P58IPK
hsp40
mAAT
.001
.004
.10
.05
.20
.50
1
ng
– – 9 55 99 0 0 0 0
% Restoration

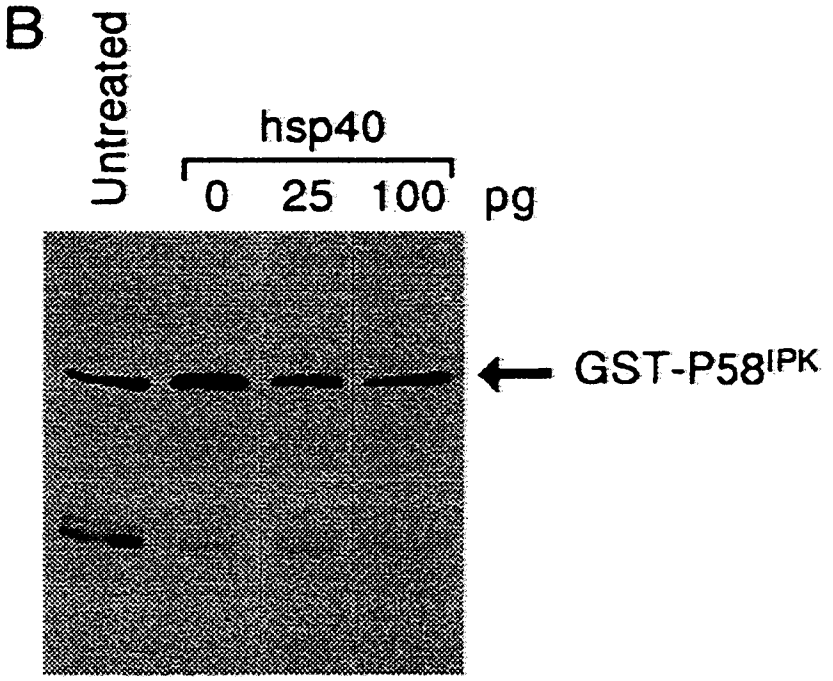
Initially, we probed the MonoP purified fraction with antisera to Hsp40 by Western analysis. We found the 38kDa protein band reacted with the Hsp40 antisera. As expected, antisera to mAAT also reacted with the polypeptide, and quantitative Western analysis showed mAAT present in a 50-fold higher amount (Fig. 2.4*A* and data not shown). This is consistent with the observation that peptide sequence information was obtained exclusively from mAAT, as the copurified Hsp40 represents a significantly smaller proportion of the purified fraction. Since the proteins were indistinguishable by one-dimensional electrophoresis, we attempted to separate the two proteins by 2-dimensional non-equilibrium gel electrophoresis (2-D NEpHGE), an established technique for Hsp40 analysis (Fig 2.4*B*) (Hattori and Kaneda 1993). Using Western blot analysis, we were able to distinguish the more abundant and basic mAAT from Hsp40, unequivocally demonstrating the presence of the two proteins in our purified preparation. The 2-D NEpHGE positions of the proteins were confirmed using purified Hsp40 and mAAT (data not shown).

Definitive proof that Hsp40, not mAAT, was I-P58^{IPK} required comparing their functional activity in our *in vitro* assay. Therefore, we obtained highly purified recombinant Hsp40 and mAAT. The purity of the two protein preparations was confirmed by silver staining of the SDS/PAGE (Fig 2.4C). Using our *in vitro* assay, which included native PKR and recombinant GST-P58^{IPK}, we found that highly purified, recombinant Hsp40 reversed P58^{IPK} inhibition of PKR in a dose dependent manner (Fig 2.4*D*). Importantly, purified

mAAT (tested at up to 10-fold higher amounts than Hsp40) failed to inhibit $P58^{IPK}$ activity. The lack of mAAT function also suggests that the I-$P58^{IPK}$ specific activity in Table 2.1 is severely underestimated due to the relatively low concentrations of Hsp40 in our purified preparation. However, this apparent low yield is not surprising considering published reports that suggest Hsp40 is a relatively non-abundant protein (Sugito et al. 1995; Yamane et al. 1995). Similar to our native purified preparation, recombinant Hsp40 failed to activate PKR in the absence of dsRNA (data not shown), and was unable to block $P58^{IPK}$ activity unless preincubated with $P58^{IPK}$ prior to addition of PKR (Fig. 2.5*A*). Finally, it was critical to determine whether decreased $P58^{IPK}$ function was due to Hsp40 induced $P58^{IPK}$ proteolysis. Hsp40 did not induce the degradation of recombinant $P58^{IPK}$ as determined by Western blot analysis (Fig 2.5*B*). The stoichiometry of the Hsp40 mediated inhibition of $P58^{IPK}$ deserves comment. While virtually equimolar amounts of $P58^{IPK}$ are required for PKR inhibition, we found that picogram quantities of Hsp40 were sufficient to inhibit nanogram amounts of $P58^{IPK}$. We feel this may indicate that Hsp40 is able to disrupt secondary or tertiary structures within $P58^{IPK}$, consistent with the role of Hsp40 as a molecular chaperone. Furthermore, the recent observation that $P58^{IPK}$ is able to homooligermize would suggest that even disruptions of quaternary structure could have a drastic effect on $P58^{IPK}$ activity (Gale, Jr. et al. 1996).

Figure 2.5. Control assays for Hsp40 (*A*) PKR activity is defined as in fig. 7A. Amounts of purified Hsp40 used are shown. *In vitro* assays were performed either identical to those previously described (+ preincubation), or PKR and GST-P58^{IPK} were mixed prior to addition of hsp40 (- preincubation). This shows the restoration of PKR activity (shown at the bottom) is dependent upon preincubation of Hsp40 with P58^{IPK}. (*B*) Control assay showing Hsp40 does not degrade GST-P58^{IPK}. GST-P58^{IPK} untreated, or mixed under *in vitro* assay conditions with the indicated amounts of Hsp40, was examined by Western analysis to confirm the down regulation of P58^{IPK} was not due to proteolysis.


A
PKR
GST-P58IPK
Preincubation
+
−
5
20
5
20
pg hsp40
% Restoration
−
−
61
126
0
0


B
Untreated
hsp40
0
25
100
pg
GST-P58IPK

Although Hsp40 antibody recognized our purified MonoP fraction and recombinant highly purified Hsp40 inhibited $P58^{IPK}$ activity *in vitro*, we cannot rule out that I-$P58^{IPK}$ is not Hsp40 itself, but a highly related protein, which is therefore reactive with the antibody and possesses inhibitory activity due to conserved Hsp40 functional domains. Unequivocal demonstration that I-$P58^{IPK}$ is Hsp40 requires microsequencing the homogeneously purified protein, a goal currently not attainable due to the mAAT contamination and the relatively low Hsp40 yields.

Discussion

Translational regulation in influenza virus infected cells may be analogous to translational control during conditions of heat shock or stress. In influenza virus infected cells, there is a dramatic shutoff of cellular protein synthesis leading to the selective translation of viral mRNA's (Katze 1996). In heat shocked or stressed cells, there similarly is often a complete cessation of "normal" cellular protein synthesis and a subsequent redirection of the translation of exclusively heat shock mRNA's (Duncan 1996). One might speculate that, in both virus infected and stressed cells, protein synthesis is initially inhibited through the transient activation of PKR and phosphorylation of eIF-2α. This report suggests that in the case of influenza virus infection, $P58^{IPK}$, normally bound to Hsp40, dissociates from Hsp40, and interacts with PKR to block eIF-2α phosphorylation and restimulate protein synthesis. Whether a

specific influenza viral gene product causes the dissociation of Hsp40 and $P58^{IPK}$ remains to be determined. The restart of mRNA translation in virus-infected cells during "recovery" is restricted to viral mRNA's, likely due to the *cis*-acting sequences present in the 5' untranslated region (UTR) which recruit cellular and/or viral *trans*-acting factors (Garfinkel and Katze 1993; Park and Katze 1995). We currently do not know whether the PKR/$P58^{IPK}$/Hsp40 pathway plays a role in the regulation of the translation of heat shock mRNA's, which also have unique elements within the 5' UTR thought to play a role in their selective translation (Duncan 1996). It is clear, however, that heat shock induces a transient increase in eIF-2α phosphorylation, at least in certain cell lines (Duncan and Hershey 1984). The regulatory pathways involving PKR and $P58^{IPK}$ are likely more intertwined with stress control given the recent discovery of a novel Hsp90-related protein which was identified as an additional $P58^{IPK}$ interactive and inhibitory protein by a yeast two-hybrid library screen (Gale, Jr. et al. 1998).

In addition to PKR, there are two other protein kinases which phosphorylate eIF-2α on serine 51, HRI (*h*eme *r*egulated *i*nhibitor of translation), present predominantly in reticulocytes, and GCN2 of *Saccharomyces cerevisiae*. Like PKR, there is evidence that both these kinases are regulated by stress (Hinnebusch 1994). The yeast GCN2 kinase is stimulated by deprivation of amino acids or purines. In addition to inhibiting general protein synthesis, GCN2 plays a role in selective translation and

specifically stimulates translation of GCN4, a transcriptional activator of amino acid biosynthetic genes. HRI is an eIF-2α kinase that is activated in rabbit reticulocytes by heme deprivation and stress conditions that elicit a heat shock response. Furthermore, both Hsp90 and the EC1 antigen (possibly in conjunction with Hsp70) bind to HRI, although the molecular details regarding these interactions and their roles in HRI regulation remain unclear (Matts and Hurst 1989).

This report is not the first example of viruses regulating or utilizing components of the stress pathway to aid in their replication. Perhaps the most elegant example has been documented in the bacteriophage system. Infection leads to an increased synthesis of stress proteins, such as GroEL and DnaK, in spite of a general decrease in total protein synthesis (Jindal and Malkovsky 1994). These stress proteins interact with viral components and aid in the correct assembly and replication of a variety of bacteriophages such as lambda, T4, and T5 (Jindal and Malkovsky 1994). In regard to eukaryotic viruses, there is substantial evidence that many viruses induce a stress response in host cells as detected by increased levels of particular stress proteins or their cognate mRNA's. In most cases, the induced stress proteins are members of the Hsp60, Hsp70, or Hsp90 families (Lindquist 1986). In some cases, a direct association between stress proteins and viral components has been demonstrated in virus-infected cells. Jianming and Seeger observed that hepatitis B virus requires Hsp90 as an essential host factor for virus replication

(Jianming and Seeger 1996). Other work has shown that an Hsp60 related protein, but not other heat shock proteins, was associated with purified HIV and SIV virions (Bartz et al. 1994). It is also notable that the HIV gag protein binds to cyclophilins, the expression of which is induced by heat shock and which appears to play a role in the stress response (Luban et al. 1993). Finally, our data suggest that influenza virus recruits Hsp40 as a mechanism for down-regulating the potential lethal effects of PKR on viral replication.

In closing, we found that Hsp40 functions as a $P58^{IPK}$ inhibitor, and in this way modulates the activity of PKR and mRNA translation. Hsp40 is a eukaryotic homologue of the bacterial DnaJ heat shock protein. Morimoto and colleagues have directly shown that Hsp40 can serve as a molecular chaperone, participating in the proper folding and assembly of selected polypeptides (Freeman and Morimoto 1996). Evidence also has been presented which shows that Hsp40 interacts with the translational machinery and may play an important role in the biogenesis of proteins, inducing the proper folding of polypeptides as they emerge from ribosomes (Frydman et al. 1994). Work on a yeast Hsp40 homologue, Ydj-1, now suggests another novel role for Hsp40; Hsp40 may influence the signal transduction and steroid signaling properties of Hsp90 (Kimura et al. 1995). Early related work by Edwards *et al.* also implicated heat shock as a mechanism for modulating steroid receptor activity (Edwards et al. 1992). Indeed, it has been revealed that steroid receptor may require Hsp40, in addition to Hsp90 and Hsp70, for

assembly and maintenance of the aporeceptor in the absence of ligand and for proper folding of the activated receptor after ligand binding (Caplan et al. 1995; Pratt and Welsh 1994). It is intriguing that Hsp40, like PKR, has therefore been implicated in two areas of gene regulation, translational control and signal transduction. The common player in these pathways is $P58^{IPK}$, which has been found to interact with both proteins. The evidence presented in the current report therefore points to potential roles for $P58^{IPK}$ not only in signal transduction, but also in the regulation of protein synthesis under conditions of cellular stress.

CHAPTER 3: THE MOLECULAR CHAPERONES HSP40 AND HSP70 ARE COMPONENTS OF THE PKR PATHWAY VIA INTERACTION WITH P58^{IPK}

Introduction

Viral infection and the stress response have been linked since DnaJ and DnaK were first characterized as accessory factors for λ phage replication in *E. coli* (Georgopoulos et al. 1990). In many cases, viral infection elicits a cellular stress response, similar to heat shock or chemical treatment. This has been demonstrated for vaccinia virus (Jindal and Young 1992) and Sindbis virus (Trgovcich et al. 1997). Many viruses also utilize specific molecular chaperones to aid in viral replication and assembly. For example, hepatitis B virus recruits Hsp90 for efficient replication (Hu and Seeger 1996; Hu et al. 1997). In addition, vaccinia virus (Jindal and Young 1992), adenovirus (Macejak and Luftig 1991), and rabies virus (Sagara and Kawai 1992) all enlist the aid of the molecular chaperone, Hsp70, during viral assembly.

The group of proteins known as molecular chaperones work together to promote nascent chain folding of proteins during translation, transport, and secretion (reviewed in (Morimoto et al. 1994)). Originally characterized as heat shock proteins, they constitute a critical part of the general cellular response to stress (Becker and Craig 1994; Welch 1991). The bacterial DnaJ and DnaK protein families and their eukaryotic counterparts, Hsp40 and Hsp70, protect cells by refolding proteins that have been denatured as a result of stress (Hartl

1996). However, recent studies have extended the role of molecular chaperones beyond nascent peptide chain folding and protection from cellular stress. For example, chaperones are required for assembly of steroid receptor complexes (Pratt and Welsh 1994; Pratt and Toft 1997), and are involved in apoptosis (Takayama et al. 1997) and kinase maturation (Kimura et al. 1997). In many cases, molecular chaperones are assisted by a number of proteins, referred to as co-chaperones, which interact in a specific manner with the chaperones to modify their activities (Mayer and Bukau 1998).

Our studies suggest that the cellular protein $P58^{IPK}$ may also be a co-chaperone. $P58^{IPK}$ was originally characterized as an influenza virus activated protein that inhibits the protein kinase, PKR (Lee et al. 1990; Lee et al. 1992). The inhibition of PKR by $P58^{IPK}$ requires direct protein-protein interaction, which is mediated by one of $P58^{IPK}$'s nine tandemly arranged TPR domains (Lee et al. 1994b; Tang et al. 1996). $P58^{IPK}$ also contains a J-domain homology region to DnaJ at its C-terminus. Importantly, the J-domain is required for the *in vivo* inhibition of PKR (Tang et al. 1996). PKR is a serine/threonine kinase that is activated upon binding dsRNA (Galabru and Hovanessian 1987). The kinase has roles in signal transduction (Mundschau and Faller 1995; Maran et al. 1994), cell growth and differentiation (Dever et al. 1993; Li and Petryshyn 1991), tumor suppression (Barber et al. 1995; Koromilas et al. 1992; Meurs et al. 1993), and apoptosis (Lee and Esteban 1994; Srivastava et al. 1998). The best-understood role of PKR, however, is its ability to down-regulate mRNA

translation via phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF-2α) (Gale, Jr. and Katze 1998b). This has been most extensively characterized as a host-defense against viral infection, but recent studies revealed that eIF-2α phosphorylation by PKR may also be a general response to cellular stress (Clemens and Elia 1997). Indeed, PKR was activated by heat stress (Murtha-Riel et al. 1993; Saito 1990), as well as chemical stressors, such as sodium arsenite (Brostrom et al. 1996) or calcium ionophore (Prostko et al. 1995; Srivastava et al. 1995). Work from our laboratory also supported a connection between PKR and the stress response, as we have found that $P58^{IPK}$ directly interacted with Hsp40, and was negatively regulated by the molecular chaperone (Melville et al. 1997). Furthermore, we recently identified a novel Hsp90-related protein, $P52^{rIPK}$, which is also a negative regulator of $P58^{IPK}$, suggesting that $P58^{IPK}$ is probably regulated in response to different stress signals (Gale, Jr. et al. 1998). We therefore reasoned that $P58^{IPK}$ activity might also be modulated by stress, perhaps to down-regulate PKR activity once the stress was relieved, and cells begin to recover.

In this chapter, we further define the molecular details and regulation of the Hsp40 – $P58^{IPK}$ interaction, and for the first time describe a role for Hsp70 in this increasing complex regulatory pathway. By establishing a role for these molecular chaperones in the regulation of the kinase, we provide a mechanism by which cellular stress can regulate the PKR pathway. Moreover, these

findings highlight the molecular interplay between the pathways that regulate the cellular stress response, viral defense, and growth control.

Materials and Methods

Cells and Bacterial Strains. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum / 2mM L-glutamine / 100U/ml penicillin G / 100μg/ml streptomycin sulfate in monolayer cultures at 37°C in a CO_2 incubator. Cell lysates were prepared from 150-cm^2 flasks in 400μl Buffer A (50mM HEPES / 50mM KCl / 2mM $MgCl_2$ / .5% Triton X-100 / 1mM dithiothreitol. / 2mM phenylmethylsulfonyl fluoride / 10μg/ml Aprotinin). The WSN strain of influenza virus was grown and titered as previously described (Etkind and Krug 1975). Interferon-treated extracts were prepared as described (Katze et al. 1988). For heat shock experiments, the media for HeLa cells was replaced with fresh media at 42°C. The cultures were incubated at 42°C for 30 minutes and then returned to 37°C. Plasmids expressing GST-$P58^{IPK}$ or GST alone were carried in the *E. coli* strain BL-21, as described (Lee et al. 1994b).

Purified Proteins and Antibodies. Glutathione-S-Transferase (GST) tagged $P58^{IPK}$ and GST alone were prepared as described earlier (Lee et al. 1994b). Human Hsp40 was expressed as full-length protein in *E. coli*, and purified as described (Zylicz et al. 1985). The stress-inducible form of Hsp70 (Hsp72) was also expressed in *E. coli*, and purified as described (Freeman et

al. 1995). The ATPase domain of the constitutive form of Hsp70 (Hsc70), referred to as 1-386, was purified as described (Freeman et al. 1995). Polyclonal antiserum to Hsp40 was kindly provided by W. Welch (University of California - San Francisco, San Francisco, CA). The anti-Hsp70 mouse monoclonal antibody, N27F3-4, was purchased from StressGen (Canada), and used to detect full length Hsp70. The anti-Hsp70 mouse monoclonal antibody, 5A5, was purchased from Affinity BioReagents (Golden, CO), and used to detect the 1-386 protein fragment. Mouse monoclonal antisera to the Gal4 activation domain and Gal4 binding domain were purchased from Clonetech (Palo Alto, CA). Mouse monoclonal antiserum to actin was purchased from ICN (Costa Mesa, CA).

GST-Pulldown Assay. We utilized three different GST-pulldown assays for these experiments. The first used GST-P58^{IPK} as a substrate for binding Hsp40 translated *in vitro*, as described (Polyak et al. 1996). The second was used to co-purify Hsp40 and Hsp70 from whole cell extract with GST-P58^{IPK} or GST as binding substrate. For these experiments, 100 µl of *E. coli* extract prepared from cells expressing GST-P58^{IPK} or GST alone was incubated with 50 µl of glutathione agarose beads for 1 hour at 4°C. The beads were washed twice in PBS containing 1% Triton X-100 / .2mM phenylmethylsulfonyl fluoride / 10µg/ml Aprotinin, and once in Buffer A (25mM HEPES pH=7.5 / 5mM $MgCl_2$ / 50mM KCl / 10mM dithiothreitol). 100-250 µg of HeLa cell extract prepared

from untreated, influenza virus infected, or heat stressed cells was added. The reactions were incubated at room temperature for 1 hour with agitation. Beads were washed twice in Buffer A + 1mM ATP. Elution of bound proteins was achieved by addition of 30 µl of 50mM Tris-HCl pH = 8.8 / 50mM free glutathione. Eluted proteins were separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane, according to the procedure of Towbin et al. (Towbin et al. 1979). The filter was blocked in 5% non-fat dry milk and Hsp40 and Hsp70 were detected by Western blot analysis, as described earlier (Melville et al. 1997). Autoradiograms of the Western analyses were scanned using an Epson scanner, and the signal was quantified using ImageQuant software.

The third variation of the GST-pulldown used purified proteins, rather than cell extracts, as a source for Hsp40 and Hsp70. Reactions were carried out in 20 µl of Buffer A with proteins at the following concentrations: GST or GST-P58^{IPK} = 2.5µM, Hsp40 = 1.75µM, and Hsp70 or 1-386 = .75µM. Some reactions also contained ATP at a concentration of 1mM. Reactions were incubated 15 minutes at 30°C. Glutathione-agarose beads were added (20µl), and reactions incubated 30 minutes at 4°C. Beads were washed three times in Buffer A ± 1mM ATP, resuspended in 20µl elution buffer, and incubated 10 minutes at room temperature. Eluted proteins were separated and analyzed as above.

Glycerol gradient sedimentation. Monolayer cultures of HeLa cells in T-150 culture bottles were harvested by scraping in Hanks balanced salt solution (HBSS). Cells were washed twice with ice cold HBSS, scraped into 2 ml HBSS, and pelleted for 10' at 4C at 2000 rpm in a Beckman tabletop centrifuge. Cell pellets were washed once in five volumes hypotonic buffer (10mM HEPES, pH = 7.5, 1.5mM $MgCl_2$, 10mM KCl, .2mM PMSF, .5mM DTT) and resuspended in three volumes of hypotonic buffer. The cells were incubated for 10 minutes on ice to allow them to swell and disrupted by 10 strokes in a Dounce homogenizer. Nuclei were pelleted for 10 minutes at 10,000 x g at 4C, cytoplasmic extracts were collected and dialyzed against gradient buffer (20mM HEPES, pH= 7.5 / 100mM KCl / .2mM EDTA / .2mM PMSF / 5% glycerol) for 45 minutes in a Microdialyzer (GibcoBRL). 500µg of the extract was layered onto a 12.5ml 10-30% glycerol gradient prepared in gradient buffer. Gradients were spun at 41,000 rpm in a SW41 rotor in a Beckman ultracentrifuge for 21 hours at 4°C. Molecular weight standards were run in parallel, and consisted of ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (68 kDa). Fractions were collected (.5ml) and samples separated by SDS-PAGE. Proteins were transferred to nitrocellulose and analyzed by Western blotting for the presence of $P58^{IPK}$, Hsp40, and Hsp70.

Size-exclusion fast protein liquid chromatography. Mock infected and influenza virus infected extracts were prepared as described for glycerol

gradient sedimentation analysis. .5mg of cell extract was applied to a Superose-200 (S-200) 10/30 FPLC column prewashed with dialysis buffer (20mM HEPES, pH = 7.5 / 100mM KCl / .2mM EDTA / .2mM PMSF / 5% glycerol). Samples were run at a rate of 1ml/mn at room temperature. Fractions of .5ml were collected and .1ml examined by Western blotting analysis. Fractions were probed for the presence of Hsp40 and P58^{IPK}. Native protein markers (ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; and bovine serum albumin, 68 kDa) were chromatographed under identical conditions and K_{av} calculated using the formula: $K_{av} = (V_e-V_o)/(V_t-V_o)$; V_e = retention volume, V_o = void volume, V_t = bed volume. The retention volumes of fractions containing peak amounts of Hsp40 or P58^{IPK} were used to calculate the K_{av} values Hsp40 and P58^{IPK}. Values were compared to protein markers and used to approximate the molecular mass of protein complexes. These values are approximate as the K_{av} is a reflection of the Stokes radius of the each protein, rather than molecular weight. Therefore, we have made the assumption that each protein is essentially the same shape (i.e. globular).

Yeast two-hybrid plus assay. The Hsp40 yeast expression plasmid was generated by inserting the whole Hsp40 coding sequence from an *Eco*RI digest of Hsp40 in pBluescript (Stratagene) into the *Eco*RI site of pYX222 (Novagen). The ATPase domain of Hsp70 was expressed as a Gal4 binding domain fusion protein in the pGBT10 vector (BD-1-386) (Irmer and Hohfeld 1997). P58^{IPK} was expressed as a Gal4 activation domain fusion protein, as

described previously (Gale, Jr. et al. 1996). To create pYX-PP1$_C$, an *Eco*RI – *Sal*I fragment containing the whole PP1$_C$ coding sequence was released from pYES2-PP1$_C$ (Tan and Katze, unpublished observations) and cloned into the corresponding sites of pYX222 (Novagen). hSRP1 was expressed as a Gal4 activation domain fusion in the pGAD424 vector (Seki et al. 1997). The two-hybrid plasmids pGBT9 and pGAD424 (Clontech) were used for Gal4 DNA binding domain (BD) and Gal4 transcriptional activation domain (AD) fusions, respectively. pGBT9 and pGAD424 contain the selectable auxotrophic markers, *TRP1* and *LEU2*, respectively. BD-P53 was purchased from Clontech Laboratories.

To develop a yeast two-hybrid plus assay, we modified the yeast two-hybrid system by introducing a third protein into the system using the plasmid pYX222. pYX222 contains a *HIS3*$^+$ selectable marker and is compatible with the two-hybrid plasmids, pGBT10 and pGAD424. Yeast strains SFY526 (Clontech) and Y187 (obtained from Dr. S.J. Elledge, Baylor College of Medicine) were used as hosts. MATa strain SFY526 (*ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,-112, can*^{r}, *gal4-542, gal80-538, +URA3::GAL1→lacZ*) was cotransformed with BD-1-386, BD-P53 or pGBT9, and with AD-P58^{IPK}, AD-hSRP1 or pGAD424. MATα strainY187 (*ura3-52, his3, ade2-101, trp1-901, leu2-3, -112, gal4, gal80, +URA3::GAL4→lacZ*) was transformed with pYX-Hsp40, pYX-PP1$_C$ or pYX222. A mating procedure was used to combine all three plasmids, and diploids were streaked on SD plates

lacking Trp, Leu and His for plasmid selection. The *lacZ* reporters integrated in the genomes of both strains were used to assess protein-protein interactions based on a β-galactosidase assay as described by Clontech Laboratories. Yeast cell extracts were prepared by a glass bead method as described previously (Gale, Jr. et al. 1996).

ATPase Assay. ATPase assays were performed as described earlier (Freeman et al. 1995). GST-P58^{IPK} and GST were prepared as above, with the exception that they were dialyzed against 25mM Tris-HCl, pH = 7.5 prior to use. 1-386 was used at a final concentration of .45μM, Hsp40 was used at .9μM, GST and GST-P58^{IPK} was used at 3.2μM.

Refolding Assay. Refolding assays were performed as described in (Freeman and Morimoto 1996). Recombinant Hsc70 was used at a final concentration of 1.6μM, recombinant Hsp40 at 3.2μM. GST-P58^{IPK} was prepared as for the ATPase assays, and used at the concentrations indicated.

Results

P58^{IPK} interacts with Hsp40 *in vitro* and *in vivo*.

Since we originally hypothesized that the inhibition of P58^{IPK} occurred via a direct interaction, we carried out an *in vitro* analysis of P58^{IPK}-Hsp40 binding utilizing a GST pull-down experiment. Hsp40 was translated *in vitro*, in the presence of [^{35}S]-methionine, and reacted with crude extracts from *E. coli* expressing GST (as a control) or GST-P58^{IPK}. Analysis of the bound proteins,

which remained after glutathione-agarose selection and washing steps, revealed that the full length Hsp40 translation product formed a stable, titratable complex with GST-P58^{IPK}, but not significantly with GST alone (Fig. 3.1). It should be noted that this assay was also used to map the PKR - P58^{IPK} interactive domains, and that these experiments show that GST-P58^{IPK} displays specificity in binding (Barber et al. 1995).

The experiments described above demonstrated that P58^{IPK} and Hsp40 bind, but did not rule out the possibility that other factors were mediating the association. We therefore examined whether purified Hsp40 interacted with purified GST-P58^{IPK}. Using the GST-pulldown approach, we found that Hsp40 was retained on beads with GST-P58^{IPK}, but not GST or buffer alone (Fig. 3.2). These data indicate that the binding of Hsp40 to P58^{IPK} is specific, and are consistent with the idea that complex formation is required for the Hsp40 inhibition of P58^{IPK}.

To demonstrate that the interaction between Hsp40 and P58^{IPK} occurred *in vivo* we performed a co-immunoprecipitation analysis of HeLa cell extracts (Fig. 3.3). Extracts from HeLa cells were chemically cross-linked prior to immunoprecipitation with antiserum to P58^{IPK}. The crosslinked immunoprecipitates were cleaved and analyzed by Western blotting to detect Hsp40. Hsp40 was only immunoprecipitated only with antiserum to P58^{IPK}, and not by normal mouse serum or an irrelevant antibody.

Figure 3.1. GST-P58^{IPK} binds to in vitro translated Hsp40. *In vitro* transcribed and translated Hsp40 was incubated with *E. coli* lysate expressing tagged P58^{IPK} (GST-P58^{IPK}), or tag alone (GST-Ctl). The indicated amounts of glutathione-coupled agarose beads were added, and the bound proteins were analyzed by SDS-PAGE. Input Hsp40 translated product is shown at right.

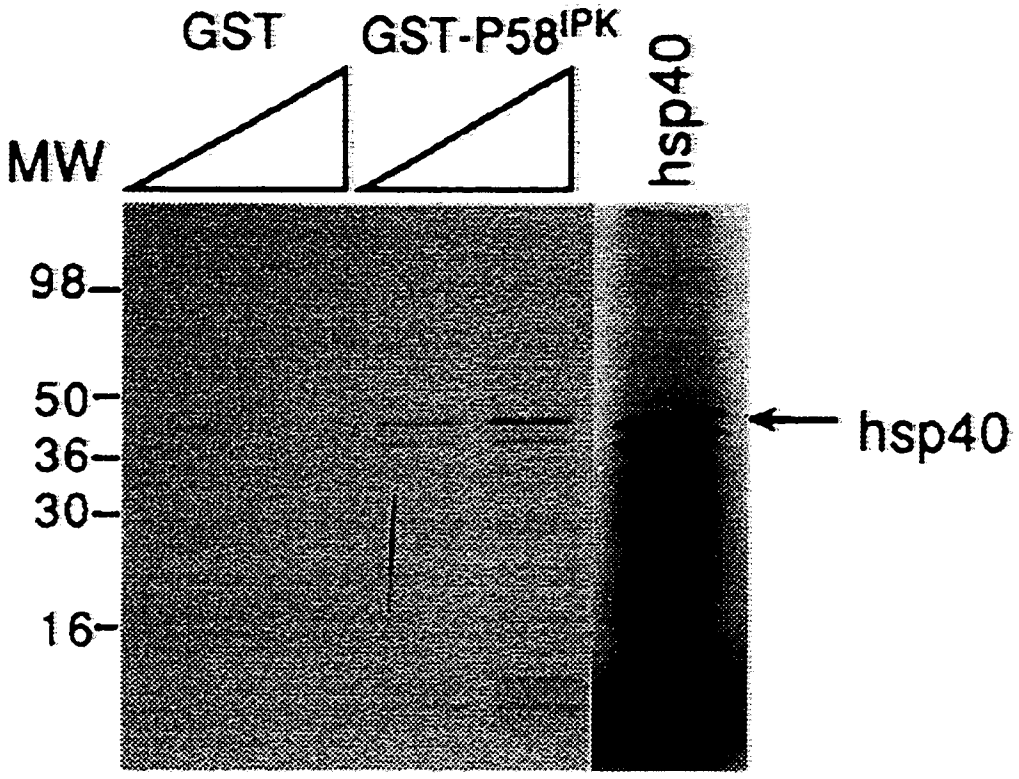

GST
GST-P58IPK
hsp40
MW
98
50
36
30
16
hsp40

Figure 3.2. GST-P58^{IPK} binds directly to Hsp40. Purified Hsp40 was mixed with buffer alone (lane 1), GST-P58^{IPK} (lane 2), or GST (lane 3). Following a brief incubation, glutathione-agarose beads were added, incubated, and then washed with binding buffer. Proteins were eluted in free glutathione, separated by SDS-PAGE, and Hsp40 was detected by Western blot analysis.

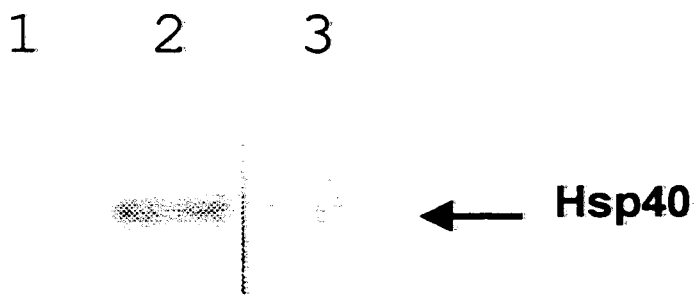
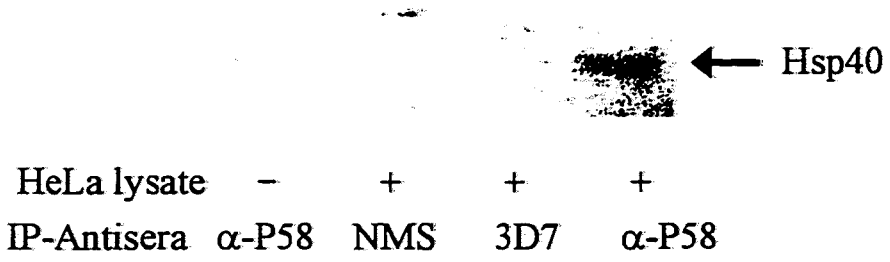

1
2
3
Hsp40

Figure 3.3. P58^{IPK} co-immunoprecipitates with Hsp40 *in vivo*. HeLa cell lysates were chemically cross-linked followed by immunoprecipitation with normal mouse (NMS), and irrelevant antibody (3D7), and antiserum to P58^{IPK} (α-P58^{IPK}) under stringent conditions. A control experimentImmunoprecipitates were cleaved and separated by SDS-PAGE. As indicated, Hsp40 was co-immunoprecipitated only in the lane containing antiserum to P58^{IPK}. As a control, HeLa cell extract was excluded from the first reaction to confirm that the signal did not come from P58^{IPK} antiserum alone.

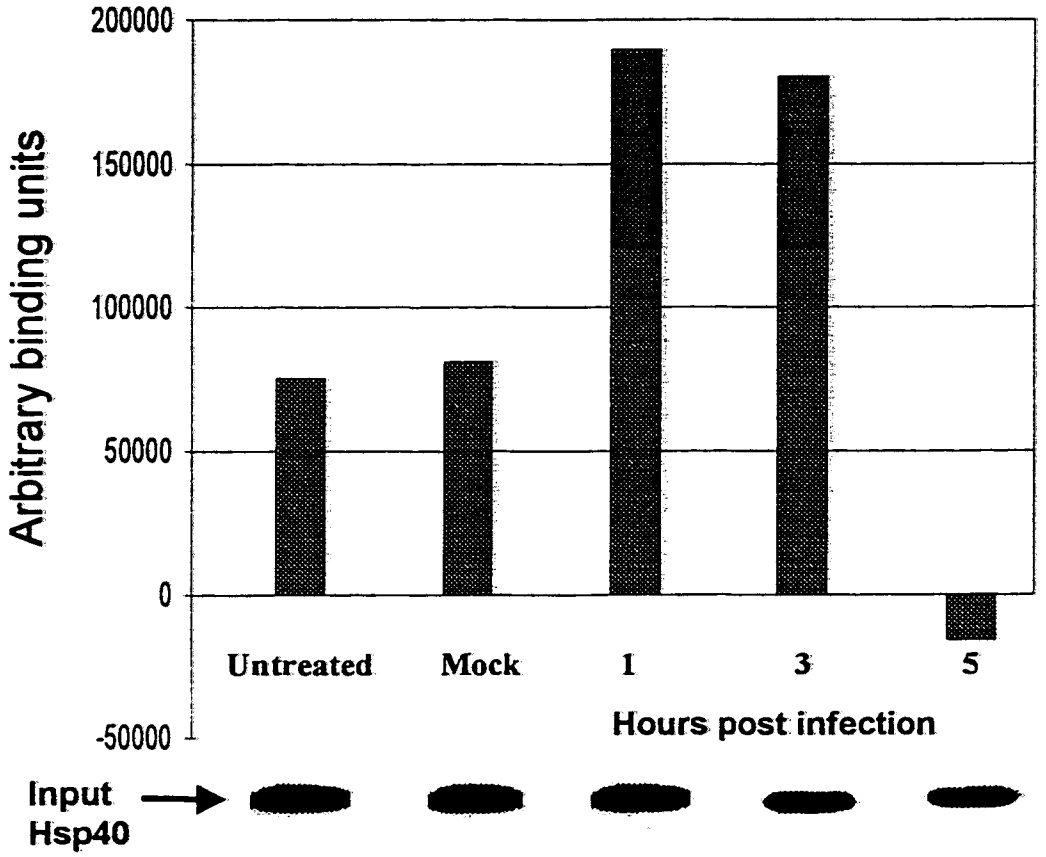

Hsp40
HeLa lysate − + + +
IP-Antisera α-P58 NMS 3D7 α-P58

P58^{IPK} is activated in influenza virus infected cells by dissociation from Hsp40

Previously, we found that P58^{IPK} was present in an inactive form in uninfected cells, but was activated upon infection with influenza virus (Lee et al. 1990; Lee et al. 1992). As presented above, we identified the molecular chaperone, Hsp40, as a negative regulator of P58^{IPK}, and found that these proteins stably interacted in rabbit reticulocyte lysate (Melville et al. 1997). We hypothesized that influenza virus activates P58^{IPK} by dissociating it from Hsp40, allowing P58^{IPK} to inhibit the antiviral activity of PKR. We therefore evaluated the ability of P58^{IPK} to bind to Hsp40 in cell extracts prepared from influenza virus-infected or mock-infected HeLa cells. Using a GST-pulldown approach, we analyzed the binding of recombinant GST-tagged P58^{IPK} to endogenous Hsp40.

HeLa cells were mock infected, or infected with influenza virus, and cell extracts were prepared at various time points post infection. Glutathione-agarose beads, pre-coated with GST-P58^{IPK}, were then added to each extract. The beads were washed and bound proteins were eluted with free glutathione and subjected to SDS-PAGE. Hsp40 was detected by Western blotting, and the signal was quantified. We found that Hsp40 present in untreated or mock infected cell extract co-purified with GST-P58^{IPK} (Fig. 3.4). In a parallel experiment using agarose beads pre-coated with GST alone, Hsp40 was not detected (data not shown). In contrast, when GST-P58^{IPK} was reacted with

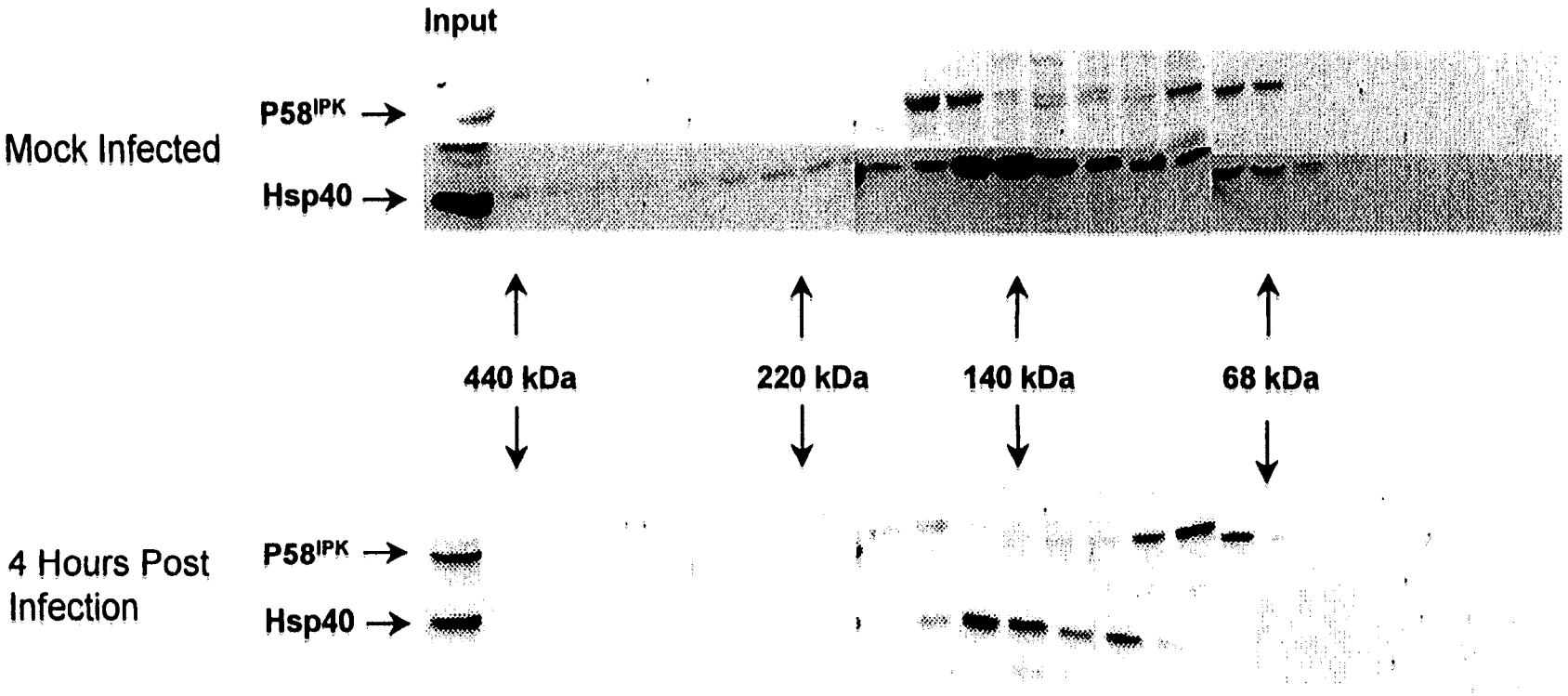
Figure 3.4. P58^{IPK} binding to Hsp40 disrupted in influenza virus infected cells. Glutathione-agarose beads (50µl) precoated with GST-P58^{IPK} were mixed with 300µg HeLa cell extract from uninfected, mock infected, and cells infected with influenza virus for 1, 3, or 5 hours. Bound proteins were separated by SDS-PAGE, as described in Materials and Methods. Hsp40 was detected by Western blotting. The Western blot signal was quantified using ImageQuant software, background binding was subtracted, and the signals were expressed as arbitrary binding units. At the bottom is a Western blot analysis showing the input level of Hsp40 in each extract.


200000
150000
100000
50000
0
-50000
Arbitrary binding units
Untreated
Mock
1
3
5
Hours post infection
Input
Hsp40

influenza virus infected extracts there was a transient increase in the amount of Hsp40 binding to $P58^{IPK}$, followed by a dramatic reduction by 5 hours post infection. Western blot analysis of the input cell extract confirmed that the loss of Hsp40 binding was not due to a reduction in Hsp40 protein level (Fig. 3.4). This finding correlates with our earlier work, which showed that $P58^{IPK}$ was activated in influenza virus infected cells at 4-5 hours post infection (Lee et al. 1992). Furthermore, these results strongly support our hypothesis that $P58^{IPK}$ is activated in influenza virus infected cells through the dissociation of Hsp40.

This evidence is correlated by *in vitro* analyses of native protein complexes using glycerol gradient sedimentation and gel filtration chromatography. Protein complexes were separated based upon sedimentation rate using glycerol gradients, or molecular size using gel filtration. Mock infected cultures and cultures harvested at 4 hours post infection were subjected to glycerol gradient sedimentation analysis, and the resulting fractions were probed for $P58^{IPK}$, Hsp40, and Hsp70 by Western blotting (Fig 3.5). Native protein standards were run in parallel, and are shown between the Western results. $P58^{IPK}$ sedimented in approximately the same fractions in mock and influenza virus infected cells, although it did have a slightly higher rate (i.e. larger complex) in influenza virus infected cells. What is significant about this analysis is that Hsp40 can be found in the same fraction with $P58^{IPK}$ in uninfected cells, but not in extracts prepared from influenza virus infected cells.

Figure 3.5. Glycerol gradient sedimentation of mock and influenza virus infected cell extracts. Extracts were prepared from HeLa cells mock infected or infected with influenza virus at 4 hours post infection. The extracts were fractionated by sedimentation through a 10-30% glycerol gradient cushion. Western blotting analysis was performed on the fractions collected from the gradients probing for HSP40 and $P58^{IPK}$. Protein markers were run in parallel, and the location of these within the gradients are indicated between the protein profiles. Hsp40 and $P58^{IPK}$ were found within the same fractions in uninfected cells, but were separated following influenza virus infection.


Input
Mock Infected
P58IPK
Hsp40
440 kDa
220 kDa
140 kDa
68 kDa
4 Hours Post Infection
P58IPK
Hsp40

We obtained similar results in the gel filtration analysis. In this experiment we used an FPLC Superdex-200 (S-200) gel filtration column to separate proteins in extracts prepared from mock infected or influenza virus infected cells. Native protein standards were examined to establish a standard curve for the S-200 column, based on the K_{AV} of each protein. Hsp40 and $P58^{IPK}$ were present in the same fractions, from MW 118 – 163 kDa, in mock infected cell extracts (Table 3.1). In contrast, Hsp40 and $P58^{IPK}$ localized to different fractions in extracts prepared from influenza virus infected cells. We cannot conclude from these experiments that $P58^{IPK}$ and Hsp40 were directly bound in the uninfected cell extracts, although our findings support that hypothesis. We can conclude, however, that $P58^{IPK}$ and Hsp40 were not in a stable complex in extracts from influenza virus infected cells, and this is consistent with our hypothesis that Hsp40 dissociates from $P58^{IPK}$ in influenza virus infected cells.

Heat shock mimics influenza virus infection: $P58^{IPK}$ – Hsp40 binding is disrupted

We next analyzed the effects of heat shock on the ability of $P58^{IPK}$ to bind to Hsp40. We used the GST-pulldown approach to examine the ability of $P58^{IPK}$ to bind to Hsp40 in extracts prepared from cells subjected to heat shock. Extracts were prepared from HeLa cells that were subjected to a mild heat shock (30 minutes at 42°C) and allowed to recover at 37°C for 0 to 5 hours, or from untreated cells as a control. GST-pulldowns and quantification of bound

Table 3.1. S-200 analysis of mock and influenza virus infected HeLa cells.

	K_{av}[†]	MW equivalent (kDa)[‡]
Mock infected		
Hsp40	.25 - .31	118 – 234
P58^{IPK}	.28 - .34	88 – 163
Influenza virus infected		
Hsp40	.69 - .74	110-175
P58^{IPK}	.66	84

[†]$K_{av} = (V_e-V_o)/(V_t-V_o)$; V_e = retention volume, V_o = void volume, V_t = bed volume

[‡]MW is approximate; extrapolated from K_{av} of protein standards

Hsp40 were carried out as described above. We found that the amount of Hsp40 bound to $P58^{IPK}$ remained unchanged until 5 hours into the recovery period (Fig. 3.6). At 5 hours, there was little Hsp40 bound to GST-$P58^{IPK}$. Again, we confirmed that the changes in Hsp40 – $P58^{IPK}$ binding were not due to a loss of Hsp40 present in the extract (Fig. 3.6). These results support the idea that the PKR – $P58^{IPK}$ pathway is regulated similarly in heat-stressed cells and cells infected with influenza virus. Furthermore, these experiments provide evidence that the stress-regulated functions of PKR may be controlled in part by $P58^{IPK}$.

$P58^{IPK}$ binds to Hsp70 via the ATPase domain

$P58^{IPK}$ shares structural similarities with Hsp70 – interactive proteins, such as the co-chaperones Hip, Hop, and Cyp40, all of which contain TPR domains that are required for stable interaction with Hsp70 (reviewed in (Smith 1998)). We reasoned that due to these similarities with Hsp70-interactive proteins, $P58^{IPK}$ might interact with Hsp70. We therefore examined whether $P58^{IPK}$ was able to form a stable complex with Hsp70, in the absence or presence of Hsp40. Because ATP is known to affect the ability of Hsp70 to stably interact with other proteins, we also examined its effect in this assay.

We had evidence that $P58^{IPK}$ interacted with Hsp40 in rabbit reticulocyte lysate (RRL). Therefore, we used the same approach to determine if $P58^{IPK}$ could bind endogenous Hsp70 in the lysate (Fig. 3.7). The experiment was performed as above, with the exception that instead of translating protein *in*

Figure 3.6. P58^{IPK} – Hsp40 binding blocked during recovery from heat shock. HeLa cells were subjected to a mild heat stress, as described in Materials and Methods, and allowed to recover for the indicated times. GST-P58^{IPK} pulldowns were performed as in Fig. 3.4 and Materials and Methods, and the Western blot signal of Hsp40 was quantified using ImageQuant software.

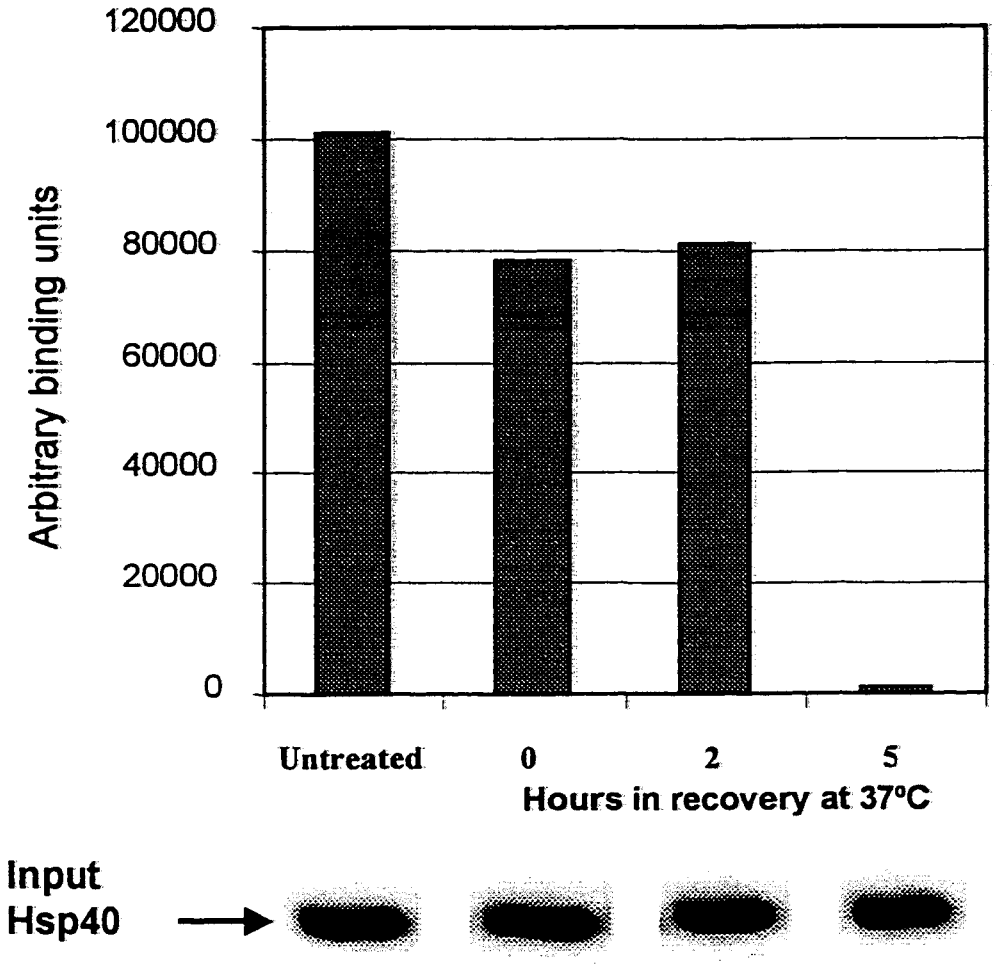
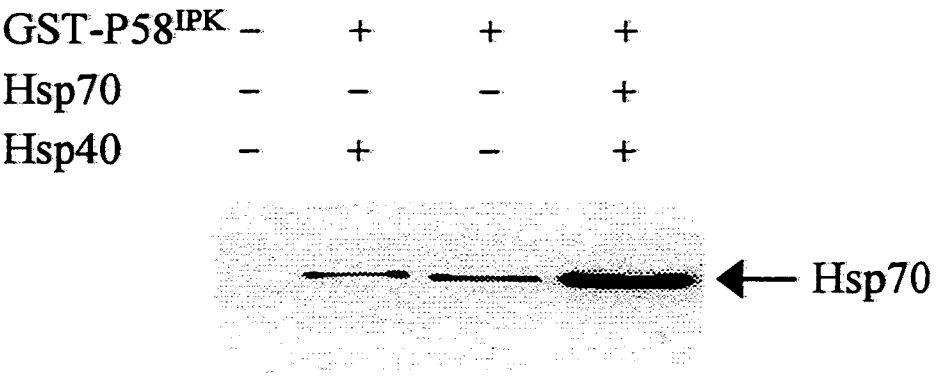

120000
100000
80000
60000
40000
20000
0
Arbitrary binding units
Untreated
0
2
5
Hours in recovery at 37°C
Input
Hsp40

Figure 3.7. GST-P58^{IPK} pulldown from rabbit reticulocyte lysate (RRL). RRL was incubated with GST alone (first lane), or GST-P58^{IPK} with or without purified Hsp40 or Hsc70. Reactions were purified on Glutathione-agarose beads, as described. Purified proteins were eluted from the beads, separated by SDS-PAGE, and probed with antiserum for Hsc70.

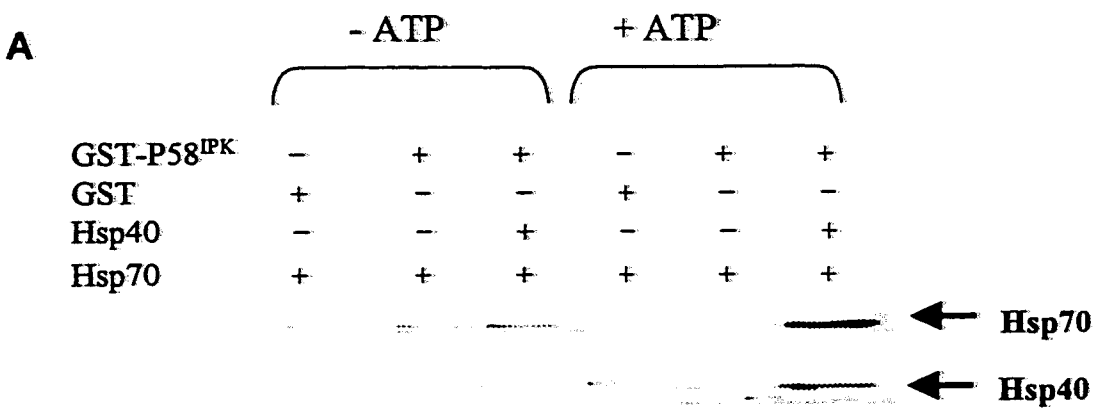

GST-P58IPK – + + +
Hsp70 – – – +
Hsp40 – + – +
Hsp70

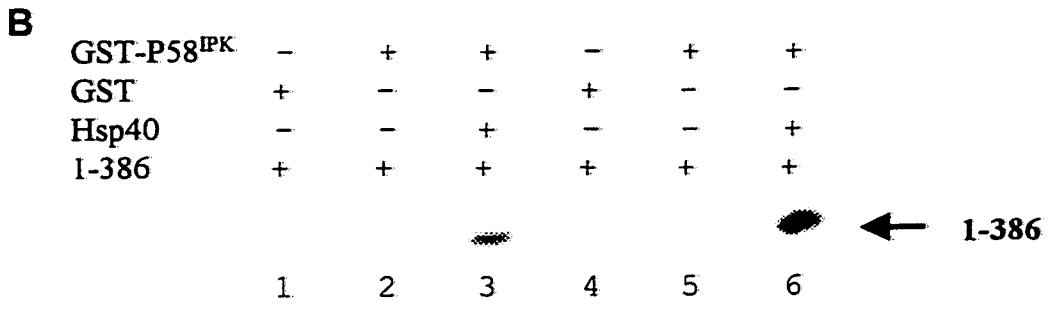
vitro, purified Hsp40 and Hsp70 were used to supplement the endogenous levels, as indicated (Fig. 3.7). We found that P58^{IPK} did bind to endogenous Hsp70 in RRL, and that additional Hsp40 had no effect on the efficiency of binding. To further characterize this interaction we repeated the GST-pulldown assay using only purified proteins.

GST-P58^{IPK} was incubated with purified recombinant Hsp70 and Hsp40, separately or in combination, and the proteins were purified and examined. Western blot analysis demonstrated that in the absence of ATP, there was a weak interaction between Hsp70 and P58^{IPK}, which was neither enhanced nor diminished with the addition of Hsp40 (Fig. 3.8*A*; lanes 2 & 3). In the presence of both Hsp40 and ATP, however, significantly more Hsp70 was bound by GST-P58^{IPK} (lane 6). In addition, Hsp40 also co-purified in the complex. Control experiments confirmed that Hsp40 and Hsp70 were not similarly purified on GST alone (lanes 1 & 4; data not shown).

As an initial step to determine if the P58^{IPK} – Hsp70 interaction was limited to the C-terminal refolding domain of Hsp70, we repeated the binding experiments using the amino terminal 44-kDa ATPase domain of Hsc70 (referred to as 1-386). We found that P58^{IPK} formed a stable complex with 1-386 (Fig. 3.8*B*), and that this interaction was enhanced by ATP and dependent upon Hsp40 (lane 6). The ability of ATP to stabilize the complex between 1-386, Hsp40, and GST-P58^{IPK} is analogous to the TPR protein Hip, where ATP

Figure 3.8. ATP and Hsp40 required for P58^{IPK} binding to Hsp70. Purified GST alone, or GST-P58^{IPK}, or binding buffer was used as a binding substrate. In all panels, the first three lanes were reactions without ATP, and the last three were plus ATP. *A*) Full length Hsp70 was mixed with GST or GST-P58^{IPK}, with or without Hsp40, and purified on glutathione-agarose beads. Hsp40 and Hsp70 were detected by Western blotting. Panel *B*) is a similar experiment, using the 1-386 fragment of Hsp70.


A
- ATP
+ ATP
GST-P58IPK
GST
Hsp40
Hsp70
Hsp70
Hsp40

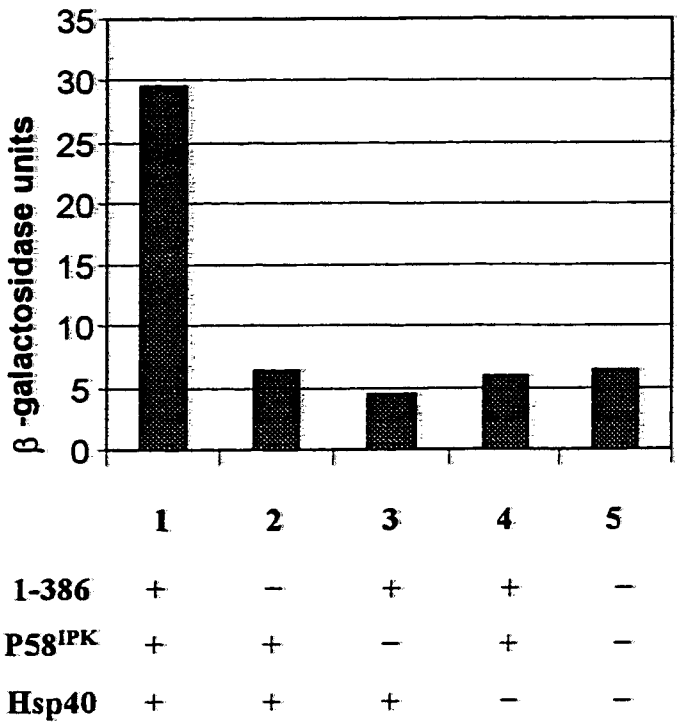

B
GST-P58IPK
GST
Hsp40
1-386
1-386
1 2 3 4 5 6

also enhanced Hip – Hsp40 – 1-386 binding (Hohfeld et al. 1995). Similarly, ATP stimulated binding between auxilin and Hsc70 (Jiang et al. 1998).

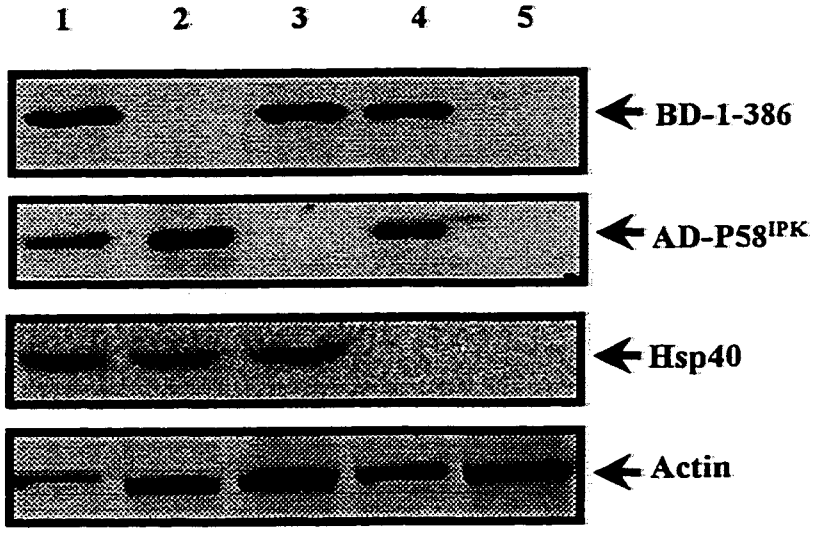
As an independent confirmation of our *in vitro* binding results, we examined the formation of this trimeric complex in an *in vivo* environment using the yeast two-hybrid plus system. This is a method to analyze the interaction of three proteins *in vivo*, and quantitatively compares different binding conditions. We expressed $P58^{IPK}$ as a fusion protein with the GAL4 activation domain (AD-$P58^{IPK}$) and Hsp40 as a full-length protein on a different plasmid (pYX-Hsp40). The yeast strain SFY526 was co-transformed with both plasmids, and grown on selective media. The ATPase domain of Hsc70 was expressed as a GAL4 binding domain fusion (BD-1-386) in the yeast strain Y187. The yeast were mated, and progeny were grown on selective media for maintenance of the three plasmids. These yeast contain a β-galactosidase reporter gene under control of the GAL4 promoter, and the strength of the hybrid protein interaction is measured by β-galactosidase activity. Western blotting analysis confirmed expression of the transformed plasmids (Fig. 3.9*B*). We found that the $P58^{IPK}$ – 1-386 interaction was stimulated approximately 6-fold in the presence of Hsp40 (compare lanes 1 and 4 of Fig. 3.9*A*). Moreover, the interaction was specific to the ATPase domain of Hsc70, which supports the results of our *in vitro* binding experiments. It is interesting to note that the amount of $P58^{IPK}$ – 1-386 binding in the absence of Hsp40 was the same as background (compare lane 4 to lanes 2, 3 and 5). This suggests that the endogenous yeast DnaJ protein, Ydj-1 did

Figure 3.9. $P58^{IPK}$ – Hsc70 interaction specific to the ATPase domain of Hsp70 in yeast two-hybrid+ assay. Yeast expressing AD-$P58^{IPK}$ with pYX-Hsp40 or vector alone were mated with yeast expressing BD-1-386. The resulting yeast were grown on triple-selective media, and the $P58^{IPK}$ – 1-386 interaction measured by β-galactosidase assay. The chart in panel *A*) shows β-galactosidase units from yeast expressing: AD-$P58^{IPK}$, pYX-Hsp40, and BD-1-386 (lane 1), AD-$P58^{IPK}$, pYX-Hsp40, BD-P53 (lane 2), AD-hSRP1, pYX-Hsp40, BD-1-386 (lane 3), AD-$P58^{IPK}$, pYX-PPIc, BD-1-386 (lane 4), and pGBT9, pYX223, pGAD424 (lane 5). Western blotting was performed to confirm expression of the proteins is shown in *B*). The actin probe, shown at the bottom, was to confirm that each lane contained approximately the same amount of protein.

A


35
30
25
20
15
10
5
0
β -galactosidase units
1 2 3 4 5
1-386 + − + + −
P58IPK + + − + −
Hsp40 + + + − −

B

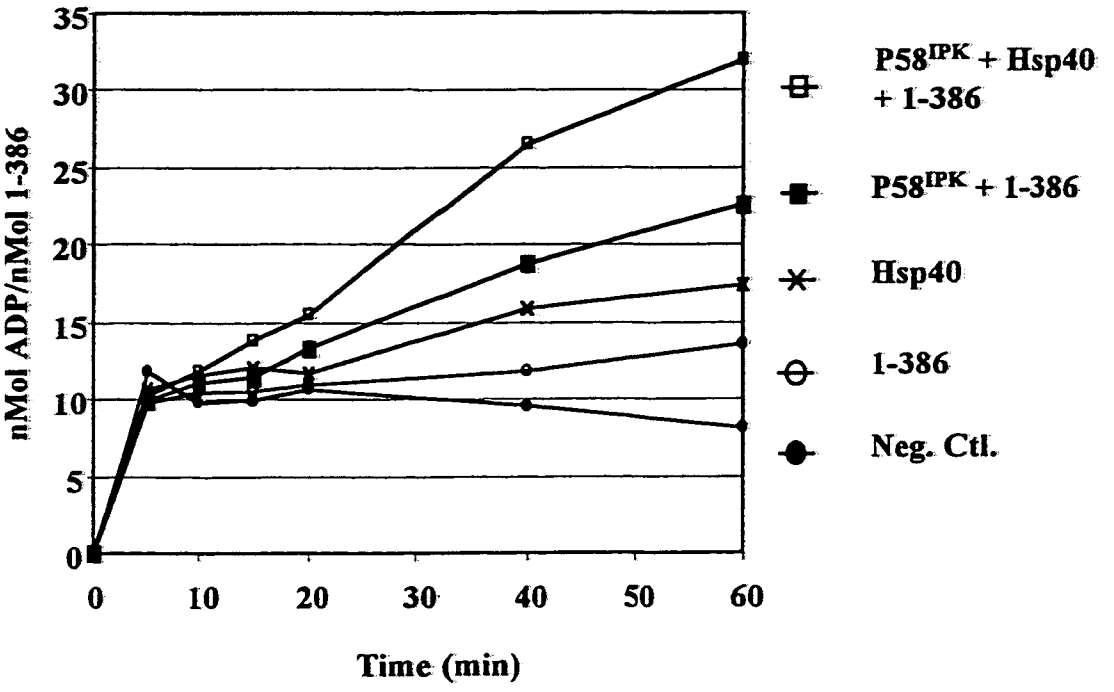

1 2 3 4 5
BD-1-386
AD-P58IPK
Hsp40
Actin

not substitute for Hsp40 in this interaction, further corroborating the specificity of the $P58^{IPK}$ – Hsp40 – 1-386 interaction.

$P58^{IPK}$ stimulates the ATPase activity of Hsp70

Our results showing that $P58^{IPK}$ interacted with the ATPase domain of Hsp70 suggested that the interaction might have a functional consequence. As is evident with most J-domain proteins, which interact with the ATPase domain of Hsp70 in order to regulate ATPase activity. This has been demonstrated for Hsp40, Hdj-2, Hsj-1, auxilin, and most other J-domain proteins (reviewed in (Cheetham and Caplan 1998)). We thus hypothesized that $P58^{IPK}$ could also stimulate the ATPase activity of Hsp70. For these experiments, we utilized the ATPase fragment of Hsc70 alone (1-386), which retains ATPase activity even in the absence of the C-terminal refolding domain (Freeman et al. 1995). $P58^{IPK}$ stimulated this activity in a dose dependent manner, and to the same degree as observed for Hsp40 (Fig. 3.10). Furthermore, when combined in the same reaction, Hsp40 and $P58^{IPK}$ displayed an additive effect, which suggested that the proteins do not work synergistically. Control experiments using GST alone confirmed that the stimulation was not due to contaminating *E. coli* proteins present in the purified preparation (data not shown). As a control, we verified that the GST-$P58^{IPK}$ preparation contained no significant endogenous ATPase activity (data not shown). These experiments demonstrate that $P58^{IPK}$, like other J-domain proteins, is able to specifically interact with and stimulate the ATPase activity of Hsp70.

Figure 3.10. P58^{IPK} stimulates ATPase activity of Hsp70. Purified GST-P58^{IPK} was mixed with purified 44 kDa ATPase domain of Hsc70 (1-386), in the absence or presence of Hsp40. $^{[32]}$P-α-ATP was added as a substrate for 1-386 ATPase activity, and reactions were carried out for 1 hour, as described in Materials and Methods. The amount of ADP was calculated and normalized to the amount of 1-386 present in the assay. (●) = Negative control (no P58^{IPK} or chaperones), (O) = 1-386 alone, (✖) = Hsp40, (■) = P58^{IPK} + 1-386, (□) = P58^{IPK} + Hsp40 + 1-386.

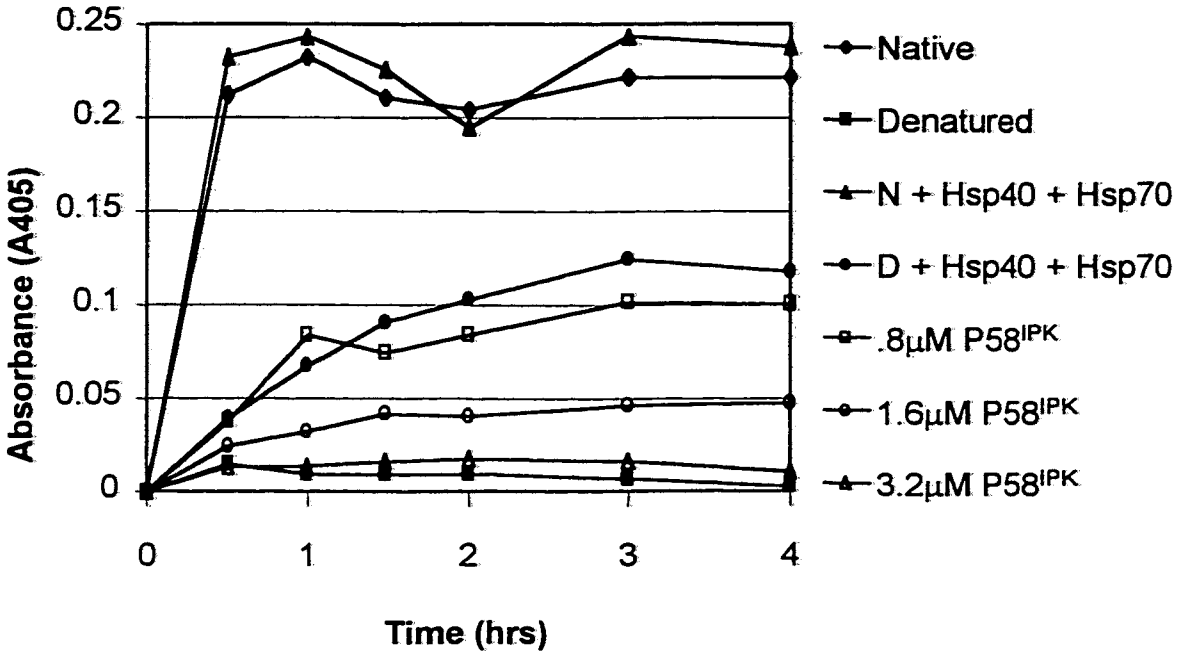

35
30
25
20
15
10
5
0
nMol ADP/nMol 1-386
0
10
20
30
40
50
60
Time (min)
P58IPK + Hsp40 + 1-386
P58IPK + 1-386
Hsp40
1-386
Neg. Ctl.

P58^{IPK} inhibits refolding of denatured β-galactosidase by Hsp70

The data presented above indicate that P58^{IPK} may be a molecular co-chaperone, or a molecular chaperone, itself. To distinguish between the two, we tested the effects of P58^{IPK} on Hsp70 – mediated refolding of a chemically denatured substrate. The ATPase activity of Hsp70 is strongly correlated with the refolding activity of the chaperone (Beissinger and Buchner 1998). Thus, proteins that stimulate Hsp70 ATPase activity usually stimulate the refolding activity of the chaperone, as well. The refolding assay measures the ability of Hsp70 to renature chemically denatured β-galactosidase into an enzymatically active form (Freeman and Morimoto 1996). Many DnaJ proteins tested, including Hsp40, stimulate Hsp70 to refold and renature β-galactosidase (Cheetham and Caplan 1998).

We therefore tested whether P58^{IPK} could also stimulate the refolding activity of Hsp70. To our surprise, we found that not only was P58^{IPK} unable to stimulate refolding by Hsp70, but that it was inhibitory to the Hsp40 – Hsp70 refolding reaction (Fig. 3.11). Control experiments confirmed that this inhibition was specific to GST-P58^{IPK}, and not a contaminant from the purification (data not shown). There are two possible conclusions that can be drawn from these results. First, P58^{IPK} may be a true inhibitor of Hsp70. While it is uncommon for a protein that stimulates Hsp70 ATPase activity to inhibit refolding, P58^{IPK} would not be unique. The anti-apoptotic protein, BAG-1 stimulated the ATPase activity of Hsp70, inhibited the refolding reaction by forming a complex with Hsp70 and

Figure 3.11. P58^{IPK} inhibits refolding by Hsp70 and Hsp40. Chemically denatured b-galactosidase was the substrate for the refolding reaction using Hsp40 and Hsp70. The restoration of b-galactosidase activity was monitored at the indicated times by measuring the ability of b-galactosidase to cleave the substrate ONPG. The samples are as follows: (◆) native β-galactosidase (positive control); (▲) native β-galactosidase + Hsp40 + Hsp70 (positive control); (●)denatured β-galactosidase + Hsp40 + Hsp70; (□)denatured β-galactosidase + Hsp40 + Hsp70 + .8μM P58^{IPK}; (○)denatured β-galactosidase + Hsp40 + Hsp70 + 1.6μM P58^{IPK}; (△)denatured β-galactosidase + Hsp40 + Hsp70 + 3.2μM P58^{IPK}; (■)denatured β-galactosidase (negative control).


0.25
0.2
0.15
0.1
0.05
0
Absorbance (A405)
0
1
2
3
4
Time (hrs)
Native
Denatured
N + Hsp40 + Hsp70
D + Hsp40 + Hsp70
.8μM P58IPK
1.6μM P58IPK
3.2μM P58IPK

the substrate protein (Takayama et al. 1997). A second explanation is that P58^{IPK} may actually stimulate Hsp70 to refold a substrate, but only for a specific substrate, or set of substrates. Therefore, in this assay P58^{IPK} would compete with Hsp40 for Hsp70, but P58^{IPK} would not target Hsp70 to β-galactosidase because P58^{IPK} has no affinity for the enzyme. This is analogous to what is seen with the auxilin – Hsc70 system in the presence of the yeast DnaJ protein, Ydj-1. Ydj-1 inhibits the auxilin stimulated uncoating of clathrin baskets by Hsc70 by apparently sequestering the chaperone away from auxilin (King et al. 1997). Future studies will be needed in order to distinguish between a general inhibition of Hsp70 by P58^{IPK}, and a substrate-targeting defect.

Discussion

We showed in this chapter that Hsp40 and P58^{IPK} bind *in vivo* and *in vitro*, and that other proteins were not necessary for the association (Figs. 3.1-3.3). Furthermore, the Hsp40 – P58^{IPK} protein complex was disrupted by influenza virus infection at 4-5 hours post infection (Figs. 3.4 & 3.5, Table 3.1). This was consistent with the hypothesis that Hsp40 is the negative regulator of P58^{IPK} that is dissociated from P58^{IPK} following infection with influenza virus infection. However, it is not known how influenza virus infection releases P58^{IPK} from the negative regulation of Hsp40. It may be that the activation of P58^{IPK} is a normal response to cellular stress. This is supported by our observation that

$P58^{IPK}$ was released from Hsp40 several hours into the recovery period following a mild heat shock (Fig. 3.6).

In addition of Hsp40, $P58^{IPK}$ binds to the molecular chaperone, Hsp70 (Fig. 3.7). The interaction is stimulated by Hsp40, which forms a trimeric complex with $P58^{IPK}$ and Hsp70 (Fig. 3.8*A*). The requirement of ATP for complex formation is interesting because it suggests that Hsp70 likely undergoes a conformational change before being able to bind to $P58^{IPK}$. As discussed earlier, the hydrolysis of ATP by Hsp70 stimulates the chaperone to adopt an 'open conformation'. Perhaps this exposes a $P58^{IPK}$-interactive site in the ATPase domain that is normally concealed. The observation that $P58^{IPK}$ binds specifically to the ATPase domain of Hsp70 supports this hypothesis (Figs 3.8*B* & 3.9).

$P58^{IPK}$ stimulated the ATPase activity of Hsp70 to the same degree as Hsp40 (Fig. 3.10). This suggests that $P58^{IPK}$ may be a positive regulator of Hsp70 activity. However, $P58^{IPK}$ inhibited Hsp70-mediated refolding of denatured β-galactosidase (Fig. 3.11). These findings are not necessarily discrepant if one considers that $P58^{IPK}$ may act as a targeting mechanism for Hsp70. That is, $P58^{IPK}$ directs the molecular chaperone to PKR, or possibly other proteins with which $P58^{IPK}$ interacts, and stimulates Hsp70 to refold those proteins. If this is the case, then $P58^{IPK}$ might inhibit the interaction between Hsp70 and other proteins, such as β-galactosidase, because $P58^{IPK}$ does not bind to β-galactosidase.

CHAPTER 4: SUMMARY

When this study began, we knew that $P58^{IPK}$ inhibited PKR through direct protein-protein interaction, and that this interaction was promoted in influenza virus infected cells because $P58^{IPK}$ was released from negative regulation by $I\text{-}P58^{IPK}$. We now show that $I\text{-}P58^{IPK}$ is the molecular chaperone Hsp40. Moreover, our data indicate that Hsp70, the cognate partner of Hsp40, is also a component of the PKR – $P58^{IPK}$ pathway. The central feature of this molecular interplay is the PKR inhibitory protein, $P58^{IPK}$. This has implications for the PKR field because it directly links regulation of the kinase to the stress response. This work is also an example of an emerging theme in the molecular chaperone field. That is, chaperone proteins are recruited into molecular pathways, such as the PKR pathway, by a group of proteins referred to as co-chaperones. Co-chaperones are proteins that interact with molecular chaperones to modulate their catalytic activity or target them to specific pathways or substrates.

$P58^{IPK}$ is a co-chaperone protein

The ability of $P58^{IPK}$ to bind to Hsp70 and stimulate the ATPase activity indicates that $P58^{IPK}$ is a novel member of the co-chaperones. Furthermore, we propose that $P58^{IPK}$ also targets Hsp70 to PKR, and stimulates the molecular chaperone to refold, thus inactivating, the kinase. Although we have not yet

determined if Hsp70 plays a role in the inhibition of PKR, recent studies of two other proteins provide examples of J-domain co-chaperones that recruit Hsp70 to specific protein targets. First is the clathrin-uncoating factor, auxilin, which contains a C-terminal J-domain homology region. The J-domain is required for the clathrin uncoating activity of auxilin, as well as interaction with Hsc70 (Jiang et al. 1998; King et al. 1997). The current hypothesis is that auxilin targets Hsc70 to specific sites on the clathrin basket, and stimulates the chaperone to refold the clathrin molecule, and thus uncoat the vesicle (Ungewickell et al. 1997). Indeed, deletion of the J-domain of auxilin abrogates clathrin uncoating (Ungewickell et al. 1995). Furthermore, the clathrin-uncoating activity can be blocked by other J-domain proteins, such as yeast Ydj-1 and human Hsj-1 (King et al. 1997; Cheetham et al. 1996). Presumably, these general chaperones do not contain a clathrin-binding domain, and thus compete with auxilin for Hsc70.

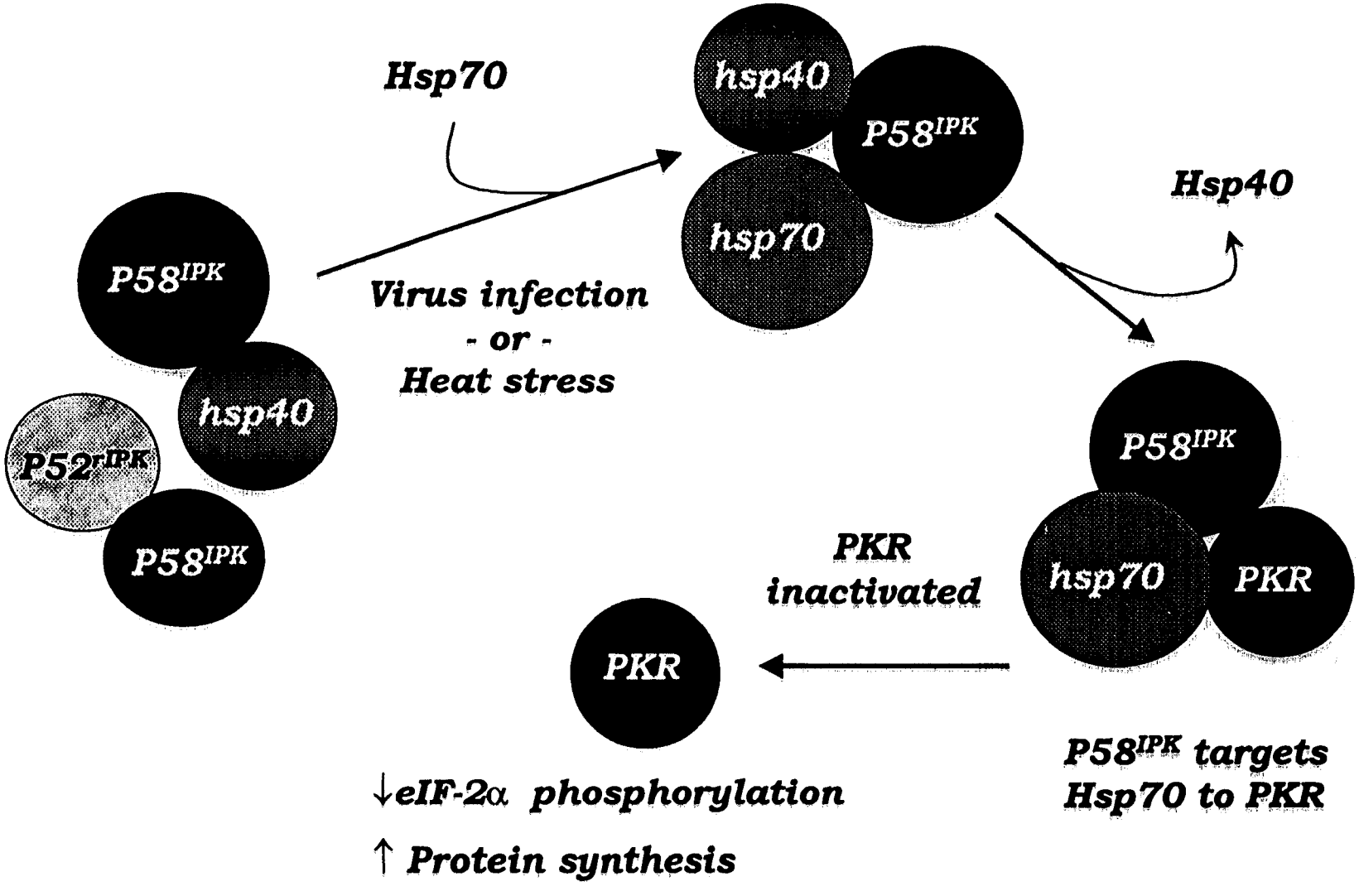
The second co-chaperone is the tumor antigen (T antigen) of the polyomavirus, simian virus 40 (SV40), which has a J-domain at its N-terminus. Work by Kelley and Georgopoulos showed that the T antigen J-domain could functionally substitute for the J-domain in DnaJ of *E. coli* (Kelley and Georgopoulos 1997). More recently, a reciprocal experiment found that the J-domain from Hsj-1 could functionally substitute for the T antigen J-domain (Zalvide et al. 1998). The T antigen also contains a binding site for the retinoblastoma tumor suppressor protein (pRb), which the T antigen may inactivate through direct protein-protein interaction. However, recent reports

demonstrate that the J-domain of the T antigen is critical for the inactivation of pRb and malignant transformation (Harris et al. 1998). Srinivasan and colleagues proposed that the T-antigen targets Hsc70 to pRb, and stimulates the chaperone to alter the conformation of pRB (Srinivasan et al. 1997). This tenet is supported by a report showing that a functional J-domain in the T antigen is critical for the accumulation of free E2F and activation of E2F promoter sequences (Harris et al. 1998). Thus, the T antigen and auxilin systems highlight the potential of J-domain co-chaperone proteins to target specific proteins for refolding by Hsp70. The function of the J-domains in these co-chaperones is central to our revised model for the PKR pathway.

Revised model of PKR – P58^{IPK} pathway

We propose in our revised model that P58^{IPK} is inactive prior to heat shock or influenza virus infection because it is bound to its own inhibitor, Hsp40 (Fig. 4.1). In response to a stress event, such as heat shock or virus infection, P58^{IPK} then binds to Hsp70. The trigger might also be an influenza virus protein that elicits a stress response. Indeed, influenza virus infection of lung tissue mimics cellular stress induced by oxidative stress (Choi et al. 1996). Our *in vitro* binding results indicate that Hsp40 may be required for the association of Hsp70, and there might exist a trimeric Hsp40 – P58^{IPK} – Hsp70 complex. However, given our earlier results showing that Hsp40 impairs the ability of P58^{IPK} to inhibit PKR, Hsp40 likely dissociates before P58^{IPK} binds to PKR. The

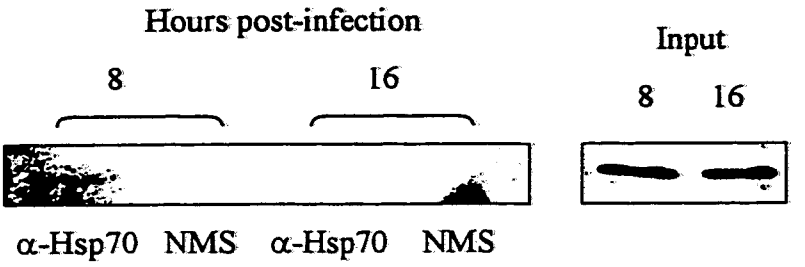
Figure 4.1. Revised model of PKR pathway. See text for details


Hsp70
hsp40
P58IPK
hsp70
Hsp40
P58IPK
hsp40
P52rIPK
P58IPK
Virus infection
- or -
Heat stress
P58IPK
hsp70
PKR
PKR inactivated
PKR
P58IPK targets
Hsp70 to PKR
↓eIF-2α phosphorylation
↑ Protein synthesis

mechanism of the release of $P58^{IPK}$ by Hsp40 is unclear. It is possible that the cellular stress response triggered by heat shock or influenza virus infection recruits Hsp40, along with the other stress response proteins, to participate in the general protection of the cell, and thus liberate sufficient $P58^{IPK}$ to downregulate PKR. Alternatively, the chaperone is modified in some way, so that it no longer is able to bind to $P58^{IPK}$. Regardless of the cause of the release of $P58^{IPK}$, the final step in the PKR pathway is the inhibition of PKR. We speculate that $P58^{IPK}$ brings Hsp70 to PKR, and stimulates the chaperone to refold, and thus inactivate the kinase. Preliminary evidence from our laboratory shows that Hsp70 may be bound to PKR late during influenza virus infection (Fig. 4.2). This hypothesis is supported by the observation that $P58^{IPK}$ stimulates the ATPase activity of Hsp70. The inhibition of PKR ensures that translation will not be impaired, in the case of influenza virus infection, or that it will be restored, in the case of recovery from heat stress.

We have previously shown that $P58^{IPK}$ inhibited PKR in a stoichiometric manner by direct protein-protein interaction. Our studies also showed that the 6^{th} TPR domain was required for direct interaction with PKR, and inhibition of the kinase. Furthermore, Tan et al. demonstrated that $P58^{IPK}$ binding to PKR prevents dimerization of the kinase, which is a required step for full catalytic activity (Tan et al. 1998). Interestingly, monomerization of the *E. coli* P1 replication initiator RepA requires functional a functional DnaK – DnaJ system (Wickner et al. 1991; Wickner et al. 1992), thus implicating another J-domain

Figure 4.2. PKR coimmunoprecipitates with Hsp70 late in influenza virus infection. MDBK cells were infected with influenza virus and harvested at 8 and 16 hours post-infection. Extracts were immunoprecipitated with antiserum to Hsp70 (α-Hsp70), or normal mouse serum (NMS). Immunoprecipitates were separated by SDS-PAGE, and proteins transferred to nitrocellulose. PKR was detected by Western blotting analysis using enhanced chemiluminescence. Shown at the right is a Western blot analysis of the input material, confirming PKR is present in the extract.


Hours post-infection
8
16
Input
8
16
α-Hsp70
NMS
α-Hsp70
NMS

protein in the disruption of protein multimers. Just as most J-domain proteins require their Hsp70 counterpart, the requirement of $P58^{IPK}$ for its J-domain in order to inhibit PKR *in vivo* supports the notion that Hsp70 may be involved in $P58^{IPK}$ activity. We have identified a second negative regulator of $P58^{IPK}$, called $P52^{rIPK}$, which also binds to and inhibits $P58^{IPK}$ (Gale, Jr. et al. 1998). This is particularly interesting in the context of molecular chaperones, as $P52^{rIPK}$ contains a region of homology to Hsp90. The conditions under which $P52^{rIPK}$ and $P58^{IPK}$ interact within the cell, however, and the consequences of this interaction, remain to be determined.

These findings are significant for the study of $P58^{IPK}$ because they demonstrate the likely mechanism by which influenza virus activates $P58^{IPK}$, namely by disrupting the complex between $P58^{IPK}$ and its negative regulator, Hsp40. Furthermore, this report is the first evidence or a role for $P58^{IPK}$ in the absence of influenza virus infection, as demonstrated by the liberation of $P58^{IPK}$ during recovery from heat shock. We therefore speculate that the cell regulates the ability of PKR to mediate antiviral activity, growth suppression, signal transduction, and translational control by balancing the activities of Hsp40, Hsp70, $P58^{IPK}$, and possibly $P52^{rIPK}$.

The emerging theme in the molecular chaperone field is that there are roles for molecular chaperones in specific pathways. The Cdc37 system is one example of a protein that specifically regulates the activity of protein kinases through its ability to interact with molecular chaperones. The Cdc37 protein

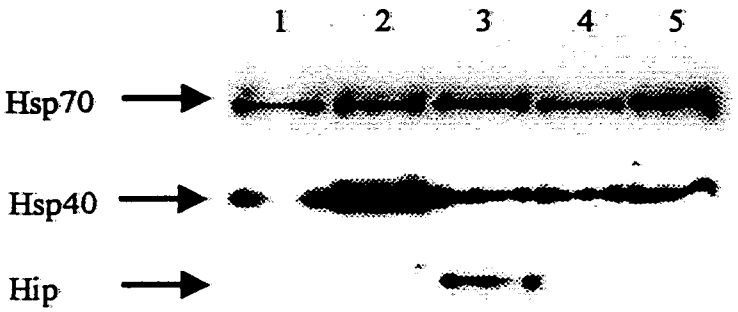
(Cdc37p), was originally identified as a cell cycle control protein in yeast, has recently been shown to function as a specific co-chaperone for a subset of kinases (Hunter 1997). Cdc37p binds to kinases, such as Cdk4, Ras, and v-Src in association with Hsp90, and is required for the maturation of the kinase to a fully functional form (Hunter 1997; Kimura et al. 1997; Stepanova et al. 1997). The functions we propose for Hsp40 and Hsp70 in the PKR pathway are similar to their roles in steroid receptor activation. The molecular chaperones Hsp40, Hsp70, and Hsp90 all participate in steroid receptor regulation (for reviews see (Pratt and Welsh 1994; Pratt 1998)). They are assisted by several co-chaperones, including Hip, Hop, Cdc37, Cyp-40, and the FKBP's (Pratt and Toft 1997; Smith 1998). Hsp40 and Hsp70 shuttle the receptor between different activation states: from the inactive state to the aporeceptor (inactive, but ready to bind ligand), and then to full activation following ligand binding. Thus, as we propose in the PKR pathway, Hsp40 and Hsp70 are regulatory proteins that modulate that activity of a substrate by altering the conformation.

Future directions

Characterize the interaction between P58^{IPK}, Hsp40, and Hsp70

We have begun to identify the interactive sites on P58^{IPK}, Hsp40, and Hsp70 by mapping the P58^{IPK} – interactive site on Hsp70 to the ATPase domain. We also have preliminary evidence that the J-domain and TPR domain 6 are dispensable for interaction with Hsp70 (Fig. 4.3). A detailed

Figure 4.3. Hsp70 binds to P58^{IPK} ΔTPR6 and 8-3 mutants. GST pulldown experiments were performed as described for Fig. 3.8. Reactions 1-3 used GST-P58^{IPK} as binding substrate, lane 4 GST-8-3 (P58^{IPK} mutant deleted for the J-domain, see ref. Lee et al 1994 MCB), and lane 5 GST-ΔTPR6 (P58^{IPK} deleted for TPR domain 6). Hsp40 and Hsp70 were added to each reaction. In addition, reaction 2 contains the Hsp70-binding protein Bag-1, and reaction 3 contains the Hsp70-interactive protein Hip. Hsp40, Hsp70, and Hip were detected by Western blotting analysis. Neither Bag-1 nor Hip decreased the amount of Hsp70 bound to P58^{IPK} (lanes 2 & 3), indicating that P58^{IPK} binds to a different site on Hsp70 than either Bag-1 or Hip. The Hip protein was also purified (lane 3) with GST-P58^{IPK}, but this is likely due to Hsp70-associated Hip that purified with P58^{IPK}-bound Hsp70. Lanes 4 and 5 show no marked reductions in Hsp40 or Hsp70 binding. This is a preliminary indication that the sites required for stable interaction with Hsp40 and Hsp70 do not lie within the J domain or TPR domain 6.


1
2
3
4
5
Hsp70
Hsp40
Hip

mapping study is required to pinpoint the interactive sites on $P58^{IPK}$, Hsp40, and Hsp70. Our laboratory has generated a large number of $P58^{IPK}$ mutants, and fruitful collaborations with other laboratories have given us access to a variety of Hsp40 and Hsp70 deletion mutants and catalytically inactive proteins. By identifying the specific sites and structural determinants required for $P58^{IPK}$ binding to Hsp40 and Hsp70 we will elucidate the molecular mechanisms behind the regulation of these molecules. For example, we do not understand what role Hsp40 plays in the association between $P58^{IPK}$ and Hsp70. Does Hsp40 provide a molecular bridge between $P58^{IPK}$ and Hsp70? Or, does Hsp40 alter the conformation of $P58^{IPK}$ to a form that is able to bind Hsp70?

There are three ways to address the question of how these proteins interact. First, we have already established that the GST-pulldown assay is adequate as a means to detect whether these proteins can interact, as well as carefully control additional factors, such as ATP. We have also developed the yeast two-hybrid+ system as a method to quantify the strength of interaction between binding partners. The in vivo analysis of the interactions between $P58^{IPK}$, Hsp40, and Hsp70 in mammalian cells has been extremely difficult due in most part to the relatively low abundance of $P58^{IPK}$ in the cell. However, our laboratory has recently established the tetracycline-repressor (Tet-off) system as a means for conditionally overexpressing $P58^{IPK}$ and PKR in mammalian tissue culture. This technique will be extremely valuable for analyzing $P58^{IPK}$ – molecular chaperones complexes *in vivo*. Moreover, because this system is in

cultured mammalian cells, the protein complexes could be analyzed under a variety of conditions, including cellular stress, interferon treatment, and influenza virus infection.

Explore the interplay between molecular chaperones and the PKR pathway

As a first step, the role of Hsp70 in the PKR pathway needs to be confirmed. A $P58^{IPK}$ molecule that is mutated in the conserved HPD tripeptide in the J-domain will help to address two important questions. First, this mutant should be tested to see if $P58^{IPK}$ is a *bona fide* regulator of Hsp70. We predict that the HPD mutant will not stimulate the ATPase activity of Hsp70. The second question the HPD mutant will address is the role of Hsp70 in the PKR pathway. Our model predicts that if the $P58^{IPK}$ HPD mutant is unable to stimulate the ATPase activity of Hsp70, then the mutant will fail to inhibit PKR in an *in vivo* assay. In order to prove this model, however, we must examine whether PKR has an altered conformation once it is inhibited. This could be determined by protease digestion mapping PKR that is inactive, activated, or inhibited by $P58^{IPK}$ and Hsp70. If the conformation of PKR is altered, different protease-sensitive sites could be exposed, and sizes of the peptide fragments would change.

Stress regulation of the PKR pathway

As discussed earlier, PKR may be activated in response to cellular stress. Our studies show that $P58^{IPK}$ may be activated in response to stress

during the recovery phase. Using the *in vitro* PKR assay, $P58^{IPK}$ activity can be monitored in extracts prepared from cells subjected to environmental (e.g. heat shock, oxidative stress) or chemical stress (e.g. ethanol, sodium arsenite). This analysis would determine if $P58^{IPK}$ were activated in response to stress, as we predict. However, it would not reveal if regulation of the PKR pathway is important for the cellular stress response. As discussed above, selective translation of mRNA for heat shock proteins may require phosphorylation of eIF-2α, and this may be mediated by PKR.

Is the activation of $P58^{IPK}$ by influenza virus infection specific, or is this part of a general stress response?

This was the central question of these studies, and we have taken the first step in answering it. We showed that Hsp40 likely dissociates from $P58^{IPK}$ during influenza virus infection, thus activating $P58^{IPK}$. However, what stimulates the release of $P58^{IPK}$ from Hsp40? Perhaps once that question is answered, we will know if influenza virus is specifically activating $P58^{IPK}$, or if the activation of $P58^{IPK}$ is part of a general stress response by the cell. It is interesting to note that influenza virus infection and the cellular stress response share many characteristics.

First, both influenza virus infection and cellular stress utilize a mechanism of selective translation through dephosphorylation of eukaryotic initiation factor 4E (eIF-4E). Influenza virus infection of lung tissue also mimics an oxidative stress response. Our previous work showed that both influenza

virus infection and the stress response inhibit PKR activity. Finally, we showed there that Hsp40 and $P58^{IPK}$ binding is disrupted at late times in infection or during recovery from heat shock. Given these similarities, it is tempting to speculate that influenza virus infection triggers a general stress response, and uses this response as a means to activate $P58^{IPK}$, inhibit PKR, and selectively translate viral proteins.

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IMAGE EVALUATION TEST TARGET (QA-3)

150mm

6"

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