INTRODUCTION & OBJECTIVES
This laboratory is involved in studying the regulation of translational initiation in plant, mammalian and insect systems. Attention is mainly focused on the a) regulation of protein synthesis chiefly through the phosphorylation of translational initiation factor 2 (eIF2) in wheat germ lysates, a model for translational system for plants; b) expression of human eIF2 subunits (α, β and γ) in insect cells to obtain purified recombinant trimeric eIF2, to understand the structure and function of each of these subunits and to determine the interaction of eIF2 with other proteins, particularly when the alpha-subunit is phosphorylated; c) to understand the importance of eIF2α phosphorylation and the kinase cascade in apoptosis in the ovarian cells of Spodoptera frugiperda, a lepidopteran insect which is used as a model system to study the expression of heterologous proteins and apoptosis and d) the translational regulation in ageing rat brain.

Although the basic protein synthesis framework is functionally similar between plants and animal systems, very little is known or understood regarding the translational regulation in plants. For example mammalian eIF2α is phosphorylated in response to several stress conditions such as double-stranded RNA virus infection, amino acid starvation, unfolded protein accumulation, heat shock, heavy metal, oxidative stress, heme-deficiency and other conditions as listed (Sudhakar et al, 1999). A family of eIF2α kinases are cloned and characterized from animal systems as has been reviewed in the chapter 1. Previous studies from Roth’s lab has shown a) phosphorylation of plant eIF2α by eukaryotic eIF2α kinases in vitro (Chang et al, 1999), b) that plants encode a dsRNA-dependent kinase (pPKR) (Langland et al, 1995) that is analogous to mammalian pPKR and that addition of high concentrations of poly IC impairs the translation of BMV RNA in wheat germ lysates presumably through the activation of PKR (Langland et al, 1996). The main difficulty, however with their results is that no data were presented to correlate translational inhibition to increased eIF2α phosphorylation or kinase activity in wheat germ lysates treated with dsRNA or purified plant PKR (Langland et al 1996). Infact, we could not obtain dsRNA-mediated translational inhibition in wheat germ lysates (Janaki et al, 1995) like other laboratories (Reijnders et al, 1975; Shaikin et al, 1992). This apparent difficulty in observing dsRNA-stimulated eIF2α phosphorylation has been attributed to the varying levels of a glycosylated protein called p67 that is shown to inhibit PKR-catalyzed eIF2α phosphorylation in plants (Langland et al, 1997; Gil et al 2000b). p67 protein, originally discovered in mammalian systems, has been shown to
inhibit the kinase-catalyzed elf2α phosphorylation but it does not inhibit the activation or autophosphorylation of the kinase (Datta et al, 1989). Further, wheat germ eIF2 affinity for GDP is only 10-20 fold higher than GTP (Shaikhon et al, 1992). The small difference in the relative binding affinities for GDP and GTP for wheat germ eIF2 and non-identification of eIF2B-like protein in wheat germ so far led to the speculation that the GDP/GTP exchange on eIF2 may occur without eIF2Blike protein and phosphorylation of wheat germ eIF2α may not necessarily regulate protein synthesis in plants (Krishna et al, 1997).

Wheat germ eIF2 appears to be heterotetrameric (Shaikhon et al, 1992) containing 4 polypeptides (p36, p41, p42 and p50), where as, mammalian eIF2 is heterotrimeric and has p38, p51 and p52. Since the p41-42 subunits are not in 1:1 ratio, and are phosphorylated by CKII and also by mammalian eIF2α kinases, we considered them as p41-42 doublet subunit (Janaki et al, 1995). Later studies (Metz and Browning, 1997) have shown that it is equivalent to mammalian eIF2α by sequencing studies. Where as p36 and p50 subunits are found to be equivalent to mammalian eIF2β and γ respectively (Metz and Browning, 1997).

Earlier studies from this laboratory have shown that CKII phosphorylates both the p36 and p41-42 doublet subunits of wheat germ eIF2 (Janaki et al, 1995; Vattem Krishna Ph. D. thesis). Interestingly N-ethylmaleimide (NEM) that is shown to inhibit translation and stimulate eIF2α phosphorylation in translating reticulocyte lysates, is also found to inhibit wheat germ translation and stimulate the phosphorylation of p36 (the β-subunit) and p41-42 (α-subunit) subunits of wheat germ eIF2 in lysates. But, the phosphorylation does not inhibit the dissociation of labeled GDP or exchange of labeled GDP for unlabeled GDP (present in the reaction mixtures) in the preformed labeled eIF2.GDP binary complex in translating lysates (Janaki et al, 1995). These findings thus suggest that NEM-mediated translational inhibition may not be due to phosphorylation of wheat germ eIF2α.

Further experiments from this laboratory have shown that NEM-induced phosphorylation of wheat germ eIF2 and of several other proteins in lysates vary with the translational ability of the lysates. Lysates that are translationally active have
shown higher phosphorylation and addition of NEM-did not make significant
difference in these lysates. Lysates that are translationally less active have shown
reduced phosphorylation and addition of NEM significantly enhances the
phosphorylation of several proteins including eIF2. Heat shocked lysates decrease the
phosphorylation of proteins and addition of NEM in such heat-shocked lysates
enhances phosphorylation of several proteins (Fig. 2, from Narahari Janaki Ph. D.
thesis, 1996 with permission). NEM-induced phosphorylation was analyzed by radio-
active labeling and autoradiogram. These studies suggest that NEM activates a
kinase(s). It is not known however if it activates a typical eIF2α kinase that can
stimulate phosphorylation of serine 51 residue in eIF2α. Unlike NEM, heat treatment
of wheat germ lysates decreases phosphorylation of several proteins. Where as in
reticulocyte lysates, heat treatment activates HRI kinase activity and stimulates eIF2α
phosphorylation (Chen and London, 1995). Further, one of my senior colleagues
demonstrated that p41-42 doublet subunit of purified wheat germ eIF2 is
phosphorylated by CKII and reticulocyte eIF2α kinases. The phosphopeptide analysis
of CKII-mediated phosphorylation is different from eIF2α kinase-mediated
phosphorylation (Fig. 3, from Ph. D. thesis of Vattem Krishna, 1997 with permission).

Based on these observations, in the present study we have

a) characterized the NEM-induced eIF2 phosphorylation in wheat
germ lysates (Chapter I).

b) Studied the importance of serine 51 phosphorylation on
translational inhibition in wheat germ lysates (Chapter II).

Apart from these two major objectives, we have also studied

a) the translational ability of interferon γ-mRNA and mutants in
wheat germ lysates and their interaction with wheat germ eIF2 (Chapter III) and

b) identified caspase-like activity in wheat germ lysates that inhibits
wheat germ translation (Chaper IV).
Fig. 2. Phosphoprotein profiles of different lysates in the presence of NEM and or heat treatment.

Three batches of wheat germ lysates with different translational abilities (WG lysate I: moderate; WG lysate II: strong; WG lysate III: weak) were used for the experiment. Protein synthesis was carried out in standard 25 ml reaction mixtures at 25°C and 40°C (heat treatment) for 10 min, with or with out NEM (1mM) as shown. 10μl aliquots of protein synthesis reactions were then supplemented with 5 μl of Tris-HCl buffer (20 mM, pH 7.8) containing [γ-32P] ATP (10 μCi). The final reaction mixtures containing 2.5 mM Mg2+ were incubated at 25°C for 5 min. Aliquots of 7.5 μl were withdrawn and separated by 10% SDS-PAGE. (A) A coomassie-stained gel and (B) an autoradiogram (from Narahari Janaki Ph. D. thesis, 1996 with permission).

Fig. 3. Cleveland partial peptide digestion of phosphorylated wheat germ eIF2 subunits.

The phosphorylated bands of wheat germ eIF2 subunits were obtained from a 10% SDS-PAGE. The bands were identified by superimposing the developed X-ray film on the dried gel and were then cut out through the X-ray film. The dried gel pieces containing the phosphorylated subunits were equilibrated and made ready for SV8 protease as described under “Materials and Methods” The protease-treated labeled subunit was then separated on 15% SDS-PAGE. An autoradiogram is shown. All lanes represent the SV8 protease-digested radio labeled products.

Lanes. 1, SV8 digest of p41-42 subunit of wheat germ eIF2 phosphorylated by CKII, 2, SV8 digest of p36 subunit of wheat germ eIF2 phosphorylated by CKII, 3, SV8 digest of p41-42 subunit of wheat germ eIF2 phosphorylated by HRI (from Ph. D. thesis of Vattem Krishna, 1997 with permission)
Fig. 2

A

B


NEM - + - + - + + - + - + - + + - + - + + + + + + + +

Lysate I Lysate II Lysate III Lysate I Lysate II Lysate III

Fig. 3

Mr 1 2 3

66-

45-

31-

21-

14-

WG, elf-2 of R1

WG, elf-2 of R1

CK-II

HR1
MATERIALS AND METHODS

1. METHODS

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2. CHEMICALS AND MATERIALS
1.1. Preparation of wheat germ lysate

Wheat germ lysate was prepared as described (Roberts and Paterson, 1973; Ramaiah and Davies, 1985). All the necessary glassware and solutions were autoclaved (except Hepes and DTT which were prepared in autoclaved double distilled water). About 20 g of wheat germ was floated on carbon tetrachloride and cyclohexane mixture in the ratio of 2.5:1. The floated wheat germ was vacuum dried (1 hr in a hood) before processing further. The floated and dried wheat germ (3 g) was removed with the help of a spatula, powdered in liquid nitrogen and made into a paste with extraction buffer (40 mM Hepes-KOH, pH 7.6, 100 mM KOAc, 1 mM Mg(OAc)$_2$, 2 mM CaCl$_2$ and 1 mM DTT) on ice. Extraction and subsequent steps were done as quickly as possible at 3º C. The paste was spun in a high speed refrigerated centrifuge at 15,000 rpm for 15 min. The top 3/4th supernatant was collected and clarified again at 15,000 rpm for 15 min. The above material was then loaded on a 50 x 2.5 cm Sephadex G-25 column that was pre-equilibrated with the column buffer containing 40 mM Hepes-KOH, pH 7.6, 100 mM KOAc, 5 mM Mg(OAc)$_2$. Elution was also carried out using column buffer and 2 ml fractions were collected. Highly turbid fractions were pooled and spun at 15,000 rpm for 20 min. The top 3 4th supernatant was collected and stored as 0.1 ml aliquots in liquid nitrogen.

1.2. Wheat germ lysate protein synthesis

Wheat germ lysate protein synthesis was performed as described (Janaki et al 1995) with the following modifications. Since the endogenous message (mRNA) in wheat germ lysate is almost absent, an exogenous message, brome mosaic virus (BMV) mRNA (20 µg/ml) is used as template RNA in all the translation experiments. Typically, the reaction mixture, in a 25 µl volume, contained amino acid mix without methionine (25 µM each), 1 mM ATP, 100 µM GTP, 8 mM creatine phosphate, 30 µg/ml spermine, 14 mM Heps KOH, pH 7.6, 60 µg/ml of creatine phospho kinase, 0.1mM KOAc, 1 mM Mg(OAc)$_2$, 1 µCi of [³¹S] methionine (1000 Ci/m mole), 0.5 µg of BMV RNA and 30º of wheat germ lysate. 1 mM DTT was added in some experiments as mentioned in the respective figure legends.
Fig. 4. Protein synthesis in different batches (I, II and III) of wheat germ lysates.

Standard lysate protein synthesis assays (25 µl) were carried out as described in Materials and Methods in the presence of BMV RNA (20 µg/ml) at 25°C for 45 minutes. Protein synthesis was assessed by the incorporation of [15S] methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45 min), as described in ‘Materials and Methods’. A control reaction without the addition of BMV RNA was also carried out to assess the endogenous lysate protein synthesis.
Fig. 5. Protein products of BMV RNA translated in two different batches of wheat germ lysate.

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Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (20 µg/ml) at 25°C for 45 minutes. The reaction was terminated by addition of 100 µl of 1X sample buffer and the labeled proteins were separated on 10% SDS-PAGE. The gels were coomassie stained, dried and analyzed by phosphorimage.

The figure is a phosphorimage showing $^{35}$S labeled protein products of BMV RNA translated in two different batches of wheat germ lysates that reflect the ability of lysates to carry out protein synthesis.

Lanes: Mr, protein molecular weight marker (in kD); 1, wheat germ lysate 1 with out BMV RNA; 2, wheat germ lysate 1 + BMV RNA; 3, wheat germ lysate 2 with out BMV RNA; 4, wheat germ lysate 2 + BMV RNA; 5, Masses of the major protein products of BMV RNA (in kD).
The reactions were incubated at 25° C to carry out protein synthesis. The performance of lysates in 5 μl aliquots was assessed with time by their ability to incorporate labeled methionine into acid precipitable protein. Samples of 5 μl were removed at different time intervals and spotted on a Whatman #1 filter paper disc. Proteins in the samples were precipitated by immersing the filters in 10% cold TCA for 1 hour. Afterwards, the filters were washed with 5% hot TCA (3-5) min. and 5% TCA at room temperature (3-5 min) to remove any non-specific radioactivity. Later, the filters were washed with ethanol and acetone and finally air-dried. Radioactivity of the filters was determined in a liquid scintillation counter.

Performance of the various lysates prepared from different batches of wheat germ is shown in Fig. 4. The incorporation of labeled amino acid into protein was not very significant without added BMV RNA. Addition of small amount (0.5μg) of BMV RNA in a 25 μl assay stimulated the incorporation of the labeled amino acid into protein quite significantly. In addition to monitoring the incorporation of [35S] methionine into protein using a scintillation counter (Wallac, Model: 409), the translational products were separated on 10% SDS–PAGE and analyzed by phosphorimager (Molecular Dynamics, Storm 840 Model) as shown in the Fig. 5. The translation of BMV RNA yields prominently four proteins of the following sizes: 110 kD, 97 kD, 35 kD and 20 kD respectively.

1.3. Preparation of heme-deficient reticulocyte lysate

Heme-deficient rabbit reticulocyte lysates that can respond to added hemin in vitro, have been prepared from New Zealand white male anemic rabbits as described (Hunt et al., 1972). Rabbits (approximately 2.5 kg in weight) were made anemic by injecting subcutaneously 2 ml of 1% acetyl phenyl hydrazine consecutively for five days. On the 9th day, the rabbits were bled through the ear vein. Blood was collected into a beaker rinsed with heparin solution. 300 units of heparin were added for every 50 ml of blood. Red blood cells were harvested by centrifugation at 2,000 rpm for 10 min in a refrigerated centrifuge. The buffy coat containing white blood cells was removed and the cells were washed 3 times with buffered saline solution (containing 5 mM Hepes-KOH, pH 7.2, 5 mM glucose, 0.14 M NaCl, 5 mM KCl and 5 mM Mg(OAc)2). While removing
Fig. 6. Protein synthesis in different batches (I, II and III) of rabbit reticuloocyte lysates.

Standard reticuloocyte lysate protein synthesis assays (25μl) were carried out at 30°C for 45 minutes as described in ‘Materials and Methods’ in the presence and absence of 20 μM hemin.

Protein synthesis was assessed by the incorporation of [14C] leucine into acid precipitable protein in a 5μl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45 minutes), as described in ‘Materials and Methods’.
the coat, care was taken to avoid drawing the red blood cells. Cells were then lysed by the addition of an equal volume of ice-cold double distilled water. The stroma was then removed by centrifugation at 10,000 rpm for 15 min. The supernatant was decanted, and stored in liquid nitrogen as 0.1 ml aliquots.

1.4. Measuring reticulocyte lysate protein synthesis

Unlike wheat germ lysates, reticulocyte lysates were not gel filtered. A standard incubation mixture for reticulocyte protein synthesis contains the following: 60% reticulocyte lysate, 4 μM creatine phosphate (CP), 250 μg creatine phosphate kinase (CPK), 80 mM KCl, 1 mM Mg(OAc)₂, 200 μM GTP, 33 μM amino acid mix without leucine and 33 μM [¹⁴C] leucine (340 μCi/mnmole) (Ramaiah et al, 1997; Krishnamoorthy et al, 1998). The components of the incubation mixture were mixed together at 0° C and the protein synthesis reaction was carried out at 30° C. The performance of lysates in 5 μl aliquots was assessed with time by their ability to incorporate labeled leucine into acid precipitable protein. Samples of 5 μl were removed at different time intervals and spotted on a Whatman No.1 filter paper discs. The proteins were precipitated by placing the filter discs in ice-cold 10% trichloroacetic acid (TCA) for 1 hour. The filters were then washed with 5% boiling TCA (3-5 min) and 5% TCA at room temperature (3-5 min). The filters were then washed with ethanol and acetone. After wards, these were air dried and transferred to H₂O₂ solution (1:1 diluted with water) for 10 min. to bleach the red color present on the filters. This is to avoid any quenching effects while reading the filters in a scintillation counter. The filters were again washed in ethanol and acetone before drying. The air dried filters were read in a liquid scintillation counter to determine the protein synthesizing capacity of the extracts.

Performance of the heme-deficient reticulocyte lysates in the presence and absence of added hemin is shown in Fig. 6.

1.5. Protein phosphorylation in translating wheat germ and reticulocyte lysates

Protein synthesis in wheat germ and reticulocyte lysates was carried out (in 25 μl) as described in the earlier section, by supplementing unlabeled amino acids.
Protein synthesizing lysates were treated with various agents (as mentioned in the figure legends) added at the start of the protein synthesis incubation, for 10 min. at 25°C (wheat germ lysates) or at 30°C (reticulocyte lysates). The lysates were then pulsed with 20 μCi of [γ-32P]ATP (3000 Ci/mmole) for 10 minutes. The reaction mixture was concentrated by pH 5.0 precipitation as described (Janaki et al, 1995; Babu and Ramaiah, 1996) by the addition 0.8 ml of NaF and EDTA (50 mM and 5 mM final respectively) followed by the addition of 12 μl of 0.5 M glacial acetic acid. The reaction mixtures were then left on ice for 1 hour for the proteins to precipitate and then centrifuged at 10,000 rpm for 15 minutes. The supernatant obtained after centrifugation was discarded. The pellet was suspended in 50 μl of 1X SDS – sample buffer (62.5 mM Tris-HCl, pH 6.8, 40 % glycerol, 5 % SDS, β-mercaptoethanol and bromophenol blue of 0.1 %) and heated in boiling water bath for 3 min. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gels as described (Ramaiah et al., 1997) and the phosphoproteins were analyzed by autoradiography or phosphorimage. Details and modifications (if any) are mentioned in the figure legends.

1.6. Autoradiography

The labeled proteins were separated on SDS-PAGE. The gel was then dried in a bio-rad gel drier equipment and the dried gel was exposed to an X-ray film (Kodak) at -70°C. Alternatively, the labeled proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was then exposed to X-ray film at -70°C. The film, after exposure for the required time, was developed by a set of photographic solutions obtained commercially and as per the manufacturer's instructions. Alternatively, phosphorimagery is used to scan the labeled dried gels or the dried nitrocellulose membranes.

1.7. Western blotting

After separation of proteins on SDS-PAGE, the proteins were transferred eletrophoretically on to nitrocellulose membrane. Transfer of proteins was carried at 70 volts for 4hrs at 4°C in transfer buffer (25 mM Tris buffer and 195 mM Glycine in 40% methanol) Afterwards, the membrane was removed and stained with Ponceau S
red solution. Marker proteins were marked with a ball point pen and the stain was removed with excess double distilled water. Regions of nitrocellulose membrane, free of proteins were blocked with TBST (Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 5% milk powder for 1 hr at room temperature. The blocking solution was decanted and the membrane was rinsed once with TBST solution. The membrane was then incubated with a primary antibody for overnight at 4°C with gentle shaking. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound antibodies. The nitrocellulose membrane was then incubated with alkaline phosphatase conjugated secondary antibody. Incubation was done for 1 hr at room temperature. Primary and secondary antibodies were stored for reuse. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound secondary antibodies. Then membrane was then treated with the color developing solution (66 μl of NBT, 33 μl of BCIP in 10 ml of AP buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM MgCl₂). The solution was removed soon after the development of bands and the blot was thoroughly washed with water, properly dried and scanned.

1.8. Protein Estimation

Protein estimation was done according to standard Bio-Rad method and as per the instructions of the manufacturer.

1.9. Preparation of wheat germ eIF2

Wheat germ eIF2 was prepared as described earlier by Lax et al., (1986) with some minor modification to enhance the purity of the preparation. All the steps in the procedure were carried out at 4°C unless otherwise indicated. The scheme of eIF2 purification from wheat germ is shown in Fig. 7 and various steps in the purification procedure are as follows.

Preparation of post-ribosomal supernatant 500 g of wheat germ was divided into four batches, ground into a fine powder in liquid nitrogen and mixed with buffer A containing 20 mM Hepes-KOH, pH 7.6, 120 mM KCl, 1 mM Mg(OAc)₂, 2 mM CaCl₂, 6 mM β-mercaptoethanol and with protease inhibitors like soya bean trypsin inhibitor (0.1 mg/ml) and PMSF (0.5 mM). For every gram of wheat germ 1.2 ml of buffer A was used. The paste was centrifuged at 12,000 rpm for 20 min. The top
yellowish fatty layer was removed with spatula and top 3/4th content was drawn out and passed through 1 litre G-25 column. The protein elute after the void volume was collected and spun at 15000 rpm for 20 min. The 15 K supernatant was centrifuged at 40 K rpm for 4.5 hr in a Ti70 beckman rotor. The supernatant thus obtained is referred as post-ribosomal supernatant (PRS).

Ammonium sulphate fractionation of the post-ribosomal supernatant (PRS): The entire PRS (1 litre) containing 120 mM KCl obtained as described above was brought to 40\% saturation by the gradual addition of 226 g of ammonium sulphate. The contents were stirred for 2 hrs at 4°C and centrifuged at 12 K rpm for 30 min. The 12 K supernatant was brought to 70\% saturation by the gradual addition of 200 g of ammonium sulphate. The contents were stirred and centrifuged at 12 K rpm again. The 12 K pellet obtained in this step was resuspended in about 50 ml of buffer B containing 40 mM KCl (buffer B containing 20 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1 mM EDTA and 10\% glycerol). The suspension was then dialyzed against 100 volumes of buffer B in 40 mM KCl and clarified by centrifugation at 10 K rpm for 10 min prior to storage.

Separation of 40-70\% ammonium sulphate fraction of PRS on DEAE - 52: The 40-70\% ammonium sulphate fraction of PRS, about 100 ml containing 12 g of total protein, was diluted with buffer B containing 40 mM KCl (in the ratio of 1:4 and was applied to a 200 ml DEAE - 52 column which is equilibrated with above buffer B. The column was washed with same buffer until the absorbance of the washed fraction at 280 nm was less than 0.1. The proteins of the column were then eluted with 120 and 250 mM KCl 10 ml fractions, collected and those fractions whose absorbance was 0.4 and above were pooled and concentrated by 0-80\% ammonium sulphate cut.

Purification of eIF2 on phosphocellulose and CM sephadex C-50 column: The 120 mM KCl concentrated fraction of DEAE-52 was fractionated and concentrated by 0-50\% and 50-80\% ammonium sulphate. The fractions were dialyzed with buffer B containing 100 mM KCl. The dialyzed fraction of 50-80\% ammonium sulphate cut (25 ml containing 10 g of total protein) was applied to a 50 ml phosphocellulose column equilibrated in the above buffer B. The protein in the column was then eluted with buffer B, containing 350, 500 and 700 mM KCl. 3 ml
SCHEME FOR WHEAT GERM eIF2 PURIFICATION

Wheat germ (500 g)

Lysed in liquid nitrogen and extracted with buffer

Passed through G 25 column sephadex column

Spun at 40,000 rpm for 4 hrs in T; 70 rotor

Ribosomal pellet

post ribosomal supernatant

(fractionated by ammonium sulphate)

0-40%

40-70%

passed through DEAE - 52 column

flow through (40 mM KCl) 120 mM KCl 250 mM KCl

fractionated by ammonium sulphate

0-50%

50-80%

passed through phosphocellulose

flow-through 350 mM KCl 500 mM KCl 700 mM KCl

0-80% ammonium sulphate cut

passed through CM sephadex column

flow through (100 mM KCl) 350 mM KCl

concentrated against 50% glycerol in buffer B.

Fig. 7. Scheme for wheat germ eIF2 purification.

eIF2 was purified from post ribosomal supernatant by ion-exchange chromatography as described in "Materials and Methods".
Fig. 8A. Purification profile of wheat germ eIF2.

Different fractions that were obtained during the purification of wheat germ eIF2 were separated on 10% SDS-PAGE as described in ‘Materials and Methods’. The figure is a coomassie stained gel.

Lanes: Mr, molecular weight marker (kD); 1, Post ribosomal supernatant (PRS); 2, PRS 40-70% ammonium sulphate cut; 3, Diethyl amino ethyl cellulose (DEAE), 120 mM KCl elute; 4, 50-80% ammonium sulphate cut of DEAE-120; 5, Phosphocellulose (P11), 500 mM KCl elute; 6, CM sephadex, 350 mM elute.

Fig. 8B. Identification of purified wheat germ eIF2 by a polyclonal antibody.

Wheat germ eIF2, eluted in the 350 M KCl fraction of CM sephadex, was separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a polyclonal anti wheat germ eIF2α antibody raised by Vattem Krishna (Ph.D. thesis, 1997) from this laboratory. The figure is a western blot.

Lanes: Mr, Molecular weight marker (kD), 1, purified WG eIF2 - CM sephadex, 350mM KCl elute (1μg), 2, purified WG eIF2 - CM sephadex, 350mM KCl elute (2μg)
fractions were collected, pooled and concentrated by 0-80% ammonium sulphate fractionation.

The fractions were dialyzed against buffer B containing 100 mM KCl. The concentrated 500 mM KCl elute of phosphocellulose column (3 ml containing 6 mg of protein) was further chromatographed on a CM sephadex column. The column was pre-equilibrated with 100 mM KCl in buffer B. After washing the column with 100 mM KCl, the bound proteins were eluted with 350 mM KCl. The eIF2 fractions of the CM sephadex column were dialyzed against buffer B containing 50 mM KCl and concentrated against buffer B containing 50% glycerol. Purification profile of different fractions is shown in Fig. 8, Panel A.

The purified eIF2 was tested by

1) a polyclonal antibody that was raised in this laboratory (Vattem Krisha Ph. D. thesis, 1996) as shown in Fig 8, Panel B.

2) the protein was further characterized by its ability to serve as a substrate for eIF2α kinases. Phosphorylated form of WG eIF2α was identified by an autoradiogram or by a phosphospecific antibody that recognizes specifically serine 51 phosphorylation in eIF2α.

1.10. Recombinant mutant mammalian PKR-GST over expression and purification

Competent cell preparation A primary culture of E coli BL-21 cells was prepared by inoculating a single colony into 3 ml of LB broth, pH 7.5 (for 1 lit : 10 g NaCl, 5 g yeast extract and 10 g bacto tryptone) and incubated at 37°C for 10-12 hrs. 1 ml of primary culture was inoculated into 100 ml of LB and incubated at 37°C in an orbital shaker at 300 rpm for 2 hrs. Turbidity was measured using spectrophotometer and an O D of 0.4 to 0.6 at 595 nm indicated the growth to be in log phase. Then the culture was harvested by centrifuging at 4000 rpm for 10 min. and the pellet was suspended in 40 ml of chilled 0.1 M MgCl2 and spun at 4000 rpm for 10 min. The pellet was resuspended in 40 ml of 0.1 M CaCl2 and incubated on ice for 30 min and was then spun at 4000 rpm for 10 min. The pellet was resuspended in 10 ml of chilled 0.1 M CaCl2 and incubated on ice for 60 min. To this, chilled glycerol 15% was added and incubated on ice overnight in cold room. It was aliquoted and stored at -70°C.
Fig. 9. Purification of mammalian recombinant PKR-GST fusion protein.

PKR-GST, fusion protein was over expressed in BL21 E. coli cells supplemented with IPTG as described in ‘Materials and Methods’. The cells were harvested, sonicated and the crude lysate was passed through glutathione sepharose 4B column. The bound protein (PKR-GST) was eluted with 10 mM GSH in 20 mM Tris- HCl, pH 8.0.

Panel A: Coomassie stained gel
Panel B: Western blot probed with anti-PKR monoclonal antibody, which gives single band of 97 kD indicating PKR-GST fusion protein.

Lanes: Mr, protein molecular weight marker (kD); 1, crude lysate of BL21 E. coli cells supplemented with IPTG (~10 μg); 2, protein eluted with buffer containing 10 μM GSH on Glutathione sepharose 4B column.

Fig. 10. Phosphorylation of wheat germ and reticulocyte eIF2 in the presence of purified PKR-GST protein.

Wheat germ or reticulocyte eIF2 (~ 500 ng) was phosphorylated by the addition of mammalian recombinant PKR-GST (~ 40ng) (purified as described in legend of Fig 1B) in the absence or presence of poly IC, in a reaction mixture containing 20 mM Tris-HCl (pH 7.8), 25 mM Magnesium acetate, 1 μM DTT, 80 mM KCl and 50 μM ATP at 25°C for 10 minutes. Reactions were separated on 10% SDS-PAGE and analyzed by western blotting using a phosphospecific anti-eIF2α antibody as described in ‘Materials and Methods’. The figure is a western blot.

Lanes: 1, wheat germ eIF2 without added PKR-GST; 2, wheat germ eIF2 + PKR-GST; 3, wheat germ eIF2 + PKR-GST + poly IC 0.5 μg/ml; 4, wheat germ eIF2 + PKR-GST + poly IC 1 μg/ml; 5, wheat germ eIF2 + PKR-GST + poly IC 2.5 μg/ml; 6, reticulocyte eIF2 without added PKR-GST; 7, eIF2α; 8, eIF2α + eIF2 + PKR-GST; 9, eIF2α + eIF2 + PKR-GST + poly IC 0.25 μg/ml.
Transformation: The plasmid DNA (10 ng) was added to *E. coli* BL21 (DE3) competent cells (100 µl) and incubated on ice for 30 min followed by heat shock which was given for 45 sec at 42°C and transferred immediately on to ice. To the cells, 500 µl of LB broth containing ampicillin (100 µg/ml) was added and incubated at 37°C in an orbital shaker at 150 rpm for 45 min. The suspension was later spun at 4000 rpm for 1-2 min. Most of the supernatant was discarded and the cell pellet was suspended in 50 µl of supernatant. The cell suspension was added to LB agar plate containing ampicillin (100 µg/ml) and was spread evenly by a spreader. The plates were then incubated at 37°C for 10-12 hrs.

Over expression and purification of PKR-GST: 5ml culture of LB containing ampicillin (100 µg/ml) was inoculated with transformed *E. coli* BL21 (DE3) cells for overnight growth at 37°C. Next day it was diluted to 50 ml with fresh LB containing ampicillin in 250 ml flask. Culture was grown to 0.8 OD at A 600 nm. Then IPTG was added to 2mM and incubated for 3-4 hr at 37°C. The culture was harvested by centrifuging at 6,500 rpm at 4°C for 15 min. The pellet was resuspended in NETN (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0 and 1% NP-40) with protease inhibitors PMSF (0.5 mM and pepstatin 1 µg/ml). Pellet from the 50 ml culture was resuspended in 1.6 ml of NETN buffer and sonicated 4 x 20 s on ice. Lysate was centrifuged at 4°C for 15 min and the supernatant was filtered through 0.45 µm filter. The filtered sample was loaded on GST column, which was prewashed with NETN. After loading the filtered lysate extract, the column was washed with NETN until the O.D came down to 0.01 and the PKR-GST was eluted with Tris-HCl, pH 8.0 containing 5 mM reduced glutathione (Fig. 9, Panel A). Purified PKR-GST was subjected to 4hr dialysis to eliminate the reduced glutathione.

The purified mutant recombinant PKR is identified using anti-PKR monoclonal antibody (Fig 9, Panel B) The PKR-GST does not require polyIC for its activation as suggested by Prof. Bryan G. Williams (Personal communication). Accordingly, herein we observed that recombinant PKR-GST (as shown in Fig 10) phosphorylates wheat germ eIF2 in the absence of added polyIC (lane 2) and also in the presence of increasing concentrations of polyIC (lanes 3, 4 and 5) without any significant difference as analyzed by a phosphospecific anti-eIF2α antibody. Similarly, the kinase phosphorylates also purified reticulocyte eIF2 (lane 7) and the
presence of polyIC does not make any difference (lane 8). Absence of added kinase
does not result in any significant phosphorylation of purified wheat germ eIF2 (lane
1) or reticulocyte eIF2 (lane 6).

1.11. **In vitro transcription**

*In vitro* transcription kit was (Promega Corporation, USA) utilized to
transcribe the above constructs to synthesize the respective chimaeric mRNAs
as shown in Fig. 11. Standard 100 µl assay mixtures consists of 500 ng of DNA,
20 µl of 5X Transcription buffer (Promega), 10 mM DTT, 0.5 mM rATP, 0.5 mM
rUTP, 0.5 mM rGTP, 0.5 mM rCTP, 2µl of RNASIN (RNAse inhibitor, Promega)
and 3 µl of T7 RNA Polymerase or SP 6 RNA Polymerase. The final volume was
adjusted with nuclease-free water. The reaction mixtures were incubated for 1hr at
37°C. To the reaction mixture, further 3µl of T7 RNA Polymerase or SP6 RNA
Polymerase was added followed by 1hr incubation at 37° C. Then 10 µl of Dnase
(1unit/µl) was added and incubated 20 min at 37° C. The reaction mixture was then
passed through 1ml of G-50 sephadex column that was pre-equilibrated with TE
buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA). To the filtrate, 10
µl of 10 M ammonium acetate and 1ml of absolute ethanol were added and incubated
for 20 min. The reaction mixture was centrifuged at 14 K rpm for 30 min at 4° C. To
the pellet 500 µl of 70% ethanol was added followed by centrifugation at 14 K rpm
for 10 min at 4°C. The pellet was resuspended in 30 µl of DEPC treated redistilled
water.

*Preparation of $^{32}$P-RNA 203γ IFN:* Uniformly labeled T7 transcripts were
synthesized using [α-$^{32}$P] UTP (0.8 Ci/mmol) and 10 nM UTP for labeling (as shown
in Fig. 11, lane 5).

1.12. **Electrophoretic Mobility Shift Assay**

Uniformly labeled T7 5'-terminal 203 nt IFN-γ mRNA transcript was
synthesized using [α-$^{32}$P] UTP (0.8 Ci/mmol) and 10 nM UTP for labeling. Wheat
eIF2 was purified as described in the earlier section. Complex formation between
Fig. 11. Synthesis of chimaeric RNAs and labeled IFN-γ mRNA, 203 nt by *in vitro* transcription.

![Image](image)

RNA products are transcribed *in vitro* using SP6 RNA polymerase (for chimeric mRNAs) and T7 RNA polymerase (for IFN-γ labeled mRNA, 203 nt), separated on 1.3% agarose gel and viewed under UV as described in ‘Materials and Methods’.

Lanes:
1. pHST 106 RNA (Alpha mosaic virus (AMV) leader and rabbit α-globin mRNA);
2. pHST 215 (Alpha mosaic virus leader sequence with Interleukin1 mRNA);
3. pHST 301 (plant α amylase leader and α amylase RNA);
4. pHST 400 (AMV viral leader and AMV RNA);
5. IFN-γ labeled RNA, 203nt;
6. RNA mass ladder (Novogen Co.);
Mr, RNA marker (Novogen Co.).
wheat germ eIF2 and labeled 203nt mRNA was assayed by electrophoretic mobility shift assay. The reaction mixture (20μl) contained [32P] mRNA (2.2 X 10^6 c.p.m./pmol), unlabeled competitor RNA as shown and wheat germ eIF2 in binding buffer (50 mM KCl, 20 mM Tris- HCl, pH 7.8, 2 mM Mg acetate, 1 mM dithiothreitol). After incubation for 15 min at 30°C followed by incubation for 10 min on ice, samples were run for 5 h at 100 V and 4°C through 4% native polyacrylamide gels in 90 mM boric acid, 25 mM EDTA, 90 mM Tris base. The native gel was dried and subjected to autoradiography.

1.13. In vitro caspase assay

100 μl of wheat germ cell free system was diluted to 1 ml with 20 mM Hepes-KOH, pH 7.5. To this 4 μl of 50 mM solution of Ac-DEVD-AFC was added to obtain a final concentration of 200 μM and incubated in a quartz 1 cm square cell. Fluorescence was measured in a spectrofluorometer (excitation: 400 nm, and emission: 505 nm) as described in Bhuyan et al 2001.

1.14. Isolation of rat liver nuclei

Preparation and purification interphase mouse liver nuclei was carried out as described (Blobel and Potter, 1966). Rat liver was obtained from the male albino rats. The livers were removed quickly and chilled immediately in several volumes of ice-cold 0.25 M sucrose in TKM (50 mM Tris-HCl, pH 7.5, 25 mM KCl and 5 mM MgCl2). All subsequent operations were performed at temperatures near 0-3°C. Livers were blotted and minced with scissors in two volumes of ice-cold 25mM sucrose in TKM. They were homogenized in a Potter-Elethjem homogenizer with a motor-driven Teflon pestle (clearance 0.025 cm) with 10 to 15 strokes at 1700 rev/min. The homogenate was centrifuged at 2000 rpm for 10 min to obtain loose pellet consisting of crude nuclei. Crude nuclei were then resuspended in TKM buffer.

1.0 ml of crude nuclei, prepared as described above, was pipetted into polyallomer tube of the SW 28 rotor; 2.0 ml of 2.3M sucrose in TKM was then added by means of a syringe with a 13-guage needle (rather than with a pipette, because of the high viscosity of the solution) and thoroughly mixed with the 250 mM sucrose homogenate by inversion. Sucrose concentration of the homogenate was thereby
raised to approximately 1.62 M, the density of which is just sufficient to float mitochondria and rough endoplasmic reticulum. The mixture was then under laid by 1.0 ml 2.3 M sucrose in TKM with a syringe and 13-gauge needle: tip of the needle was placed at the bottom of the tube and the heavy sucrose solution was introduced, forcing the lighter homogenate upward. After centrifugation for 45 minutes at 28,000 rpm in a Beckman SW 28 rotor at 0° to 3°C, the supernatant was removed. Material adhering to the wall of the tube was removed with a spatula and added to the supernatant; the tube wall was then wiped dry with tissue paper wrapped around a spatula. The white nuclear pellet was taken up in TKM buffer. The nuclei were suspended in nuclei storage buffer (10 mM PIPES, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, 50% glycerol) stored in a freezer at -70°C.

1.15. DNA laddering

About $1 \times 10^5$ rat liver nuclei were introduced into 50 µl of wheat germ cell-free extract and the system was incubated at 25°C for 8 to 10 hrs. Later, the samples were suspended in 10 volumes of buffer D (100 mM Tris-HCl, pH 8.0, 5mM EDTA, 0.2 mM NaCl, 0.4% SDS, 0.2 mg/ml proteinase K), incubated overnight at 37°C, then extracted with 1:1 phenol:chloroform, and precipitated by 0.6 volumes of isopropanol and followed by precipitation by 2 volumes of ethanol. DNA extracts were electrophoresed in 2% agarose gels in TBE (0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA). DNA was visualized by ethidium bromide staining (Zhao et al, 2001).

1.16. Phosphopeptide mapping in one dimension

Peptide mapping in one dimension by limited proteolysis in SDS-polyacrylamide gels was done as described (Cleveland et al, 1976). p41-42 sub unit of WG eIF2 were phosphorylated by CKII in vitro or in NEM-treated wheat germ lysate. The bands corresponding to the phosphorylated p41-42 subunits of wheat germ eIF2 were cut out of the dried gel through the X-ray film. The dried gel pieces containing the phosphorylated subunits were processed for SV8 (200 ng/lane) protease digestion. Gel slices were equilibrated for at least 60 min in 1 ml of gel slice equilibration buffer.
containing 250 μl of 0.5 M Tris-HCl, pH 6.8, 10 μl of 10% SDS, 100 μl of glycerol, 2 μl of 0.5 M EDTA, 3 μl of β-mercaptoethanol, 630 μl of water and a trace amount of bromophenol blue. Equilibration was repeated with a fresh equilibration buffer so that all the residual acetic acid and contaminants like gel drying filter paper were removed.

The above samples (gel slice containing labeled phosphoproteins) were incubated in the wells of a 15% gel (1.5 mm thick with 3-5 cm long stacking gels) for 15 min in the presence of SV8 protease (200 ng) and gel slice overlaying solution that contains 20% glycerol.

Electrophoresis was carried out at 100 V until the bromophenol blue clears the stacking gel. Afterwards, the voltage was increased to 150 V. To achieve greater proteolysis, the polarity of current was reversed for 3 min just before the bromophenol blue dye enters the resolving gel and then turned again to the normal mode till the end of the run. This ensures maximum digestion of the protein with the protease enzyme. After completion of the run, the gel was dried and subjected to autoradiography.

1.17. Determination of protein sulfhydryls in wheat germ lysates

Protein sulfhydryls were determined in WG lysates obtained from different batches having low to high translational abilities, heat-treated lysates and lysates treated with different SH-reactive agents such as DTT, GSH, GSSH and diamide using 5, 5'-Dithiobis-2-nitrobenzoic acid (DTNB) as described (Habeeb, 1972). The -SH content was estimated in a total reaction mixture of 50 μl containing 45 μl of WGL (3mg/ml) and 5 ml of the respective -SH reactive agent or redistilled water as indicated in the legend to Fig. The mixtures were incubated for 20 min at 25° C or at 40° C wherever indicated. To this reaction, 33 μl of DTNB solution containing 2% SDS, 0.08 M sodium phosphate buffer, pH 8.0 and 0.5 mg/ml EDTA were added. After 15 minutes, the sample was read at 410 nm against lysate mixture in SDS to give apparent absorbance. A reagent blank was subtracted from the apparent absorbance to give the net absorbance. For calculation of sulfhydryl content, the net absorbance is employed with a molar absorptivity value of 13, 600 M⁻¹ cm⁻¹.
2. CHEMICALS AND MATERIALS

ATP, CP, CPK, DTT, poly IC, NEM, SV 8 protease, Casein Kinase II, CM Sephadex C-50 were obtained from Sigma, USA. [14C] Leucine (340 mCi/mmol) was obtained from Amersham Pharmacia, UK. [γ-32P] ATP (3500 Ci/mmol) and [35S] Methionine (1100 Ci/mmol) were obtained from Jonaki Center, BRIT, Hyderabad and BRIT, Mumbai, India respectively. Ion exchange resins DE-52 (DEAE cellulose), P-11 (phosphocellulose) and nitrocellulose membrane were obtained from Whatmann Company, UK. Phosphospecific antibody for eIF2α was obtained from Research Genetics, Huntsville, AL. Hepes buffer, BMV RNA, RNAse inhibitor, In vitro transcription kit, western blot color developing agents NBT and BCIP, secondary antibodies were obtained from Promega Corporation, USA. X-ray films were purchased from Kodak, India. Acrylamide, bis-acrylamide and SDS were purchased from Boehringer and Mannheim, Germany. Ac-DEVD-AFC and Ac-DEVD-CHO were obtained from Pharningen, USA. And all other chemicals required for routine work, were purchased from Sigma, USA.

SP6 transcripts of plant mRNAs as well as chimaeric forms of mammalian and plant mRNA were prepared using DNA vectors provided by Dr. Lee Gehrke at MIT. These are (construct number / 5'-UTR leader / coding region); 106/ AMV / rabbit-α-globin; 416 / rabbit-α-globin / AMV; 215 / AMV / IL-11 301 / α-amylase / α-amylase; 302 / AMV/ α-amylase; 400 /AMV/ AMV (AMV: alfalfa mosaic virus; IL-1, human interleukin-1).

WG eIF2 polyclonal antibody that was raised in this laboratory (Krishna Ph. D. thesis, 1996) was used in this study. pGEX-PKR construct was obtained from Prof. Bryan Williams, Cleveland Clinic Foundation, USA. Chimaeric mRNAs were obtained from Prof. Raymond Kaempfer, Hebrew University, Israel. Purified PKR, K3L were obtained from Dr. Krishna, Prof. R. Wek laboratory, Indiana University School of Medicine, Indianapolis, IN. Recombinant human eIF2α and mutants (as shown in Fig.12) that were over expressed in this laboratory (Sudhakar Ph. D. thesis, 2000) were also used in the present study. Wheat germ was obtained from Sigma, and also locally from Krishna Mills, Bangalore, India. New Zealand white male rabbits were procured locally from National Institute of Nutrition, Hyderabad.
Fig. 12. Recombinant human eIF2α wt, 51A and 51D.

Panel A

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The recombinant human eIF2α wt, 51A and 51D proteins that are used in the present study were over expressed earlier in this laboratory using baculovirus expression system in Sf9 cells and partially purified using Sephacryl S-300 and DE-52 column (Sudhakar et al, 2000).

Panel A is a coomassie stained gel and Panel B is a western blot probed with anti-eIF2α antibody.

Lanes: Mr, protein molecular weight marker, kD; 1, ∼2 μg of recombinant human eIF2α wild type (wt); 2, ∼2 μg of recombinant human eIF2α 51A; 3, ∼2 μg of recombinant human eIF2α 51D.