ROLE OF RIBAVIRIN, AN ANTI-VIRAL AGENT, IN EUKARYOTIC INITIATION FACTOR 4E (EIF4E) SUBCELLULAR LOCALIZATION AND FUNCTION

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DEDICATION

I would like to thank the members of my Graduate Committee, my co-workers and my husband Ben for their support and encouragements.

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Expression of eukaryotic initiation factor 4E (eIF4E) is necessary for active cellular growth and catalysis of the rate-limiting step in cap-dependent protein synthesis3-5. Studies in the past decade have shown that eIF4E contributes to malignancy by selectively facilitating the translation of a specific set of mRNAs, those that generally encode key proteins involved in cellular growth, angiogenesis and survival6-9. Key questions remaining in the field are: (1) what is the mechanism by which eIF4E levels are increased in tumor cells? (2) What is the role of eIF4E in regulating the translation of gene products involved in various aspects of malignancy? Finally, (3) how can eIF4E be exploited as a therapeutic target for human cancer progression? Recently, Kentsis and colleagues10 demonstrated that ribavirin disrupts subcellular organization of eIF4E and suppresses eIF4E-mediated oncogenic transformation of murine cells and tumor growth of a mouse model of eIF4E-dependent human squamous cell carcinoma. These findings

suggest a specific and novel mechanism by which ribavirin affects cellular distribution and function of eIF4E, a topic that will be explored in the current proposal. This investigation may facilitate understanding in the regulation of transcription and thus provide a potential strategy for interrupting oncogenic cascades.

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Introduction

Control of mRNA translation is essential for cell growth and survival. In eukaryotes, most mRNAs are translated in a cap-dependent manner11. The cap structure m7GppN (m7G), where N is any nucleotide, is found at the 5' terminus of most eukaryotic cellular mRNAs11. A key player in the regulation and control of translation is the mRNA 5' cap-binding protein eIF4E, the rate-limiting member of the eIF4F pre-initiation complex5. Other members of the eIF4F complex include eIF4A, an ATP-dependent helicase, and eIF4G, a scaffolding protein that serves as a docking site for other proteins. In mammalian cells, the functions of eIF4E are dependent upon its subcellular localization. In the cytoplasm, eIF4E recruits ribosomes and other initiation factors to form active pre-initiation complexes, a process that is crucial for m7G cap-dependent translation11. Data suggest that up-regulation of eIF4E increases translation of a subset of transcripts that are post-transcriptionally regulated by eIF4E12. In the nucleus, eIF4E forms multiprotein structures, also known as nuclear bodies, the functions of which remain unknown. However, it has been hypothesized that nuclear eIF4E has a role in the nucleocytoplasmic transport of a particular set of transcripts13. mRNAs that utilize eIF4E are regulated in a variety of gene-specific manners. For instance, some genes are regulated at the level of transport (cyclin D1), some at the level of translation (VEGF), others at both levels (ODC1) or neither (glyceraldehydes-3-phosphate dehydrogenase, GAPDH) Thus, overexpression of eIF4E promotes transport of a specific set of transcripts such as cyclin D1 but not of housekeeping genes such as β-actin and GAPDH14-17. However, binding of eIF4E to the 5' mRNA cap is essential for mRNA

transport and translation, both of which contribute to eIF4E's ability to transform cells[12], [13].

Subcellular fractionation and IF studies demonstrated that between 33 and 68% of total eIF4E is localized to the nucleus of mammalian cells18, 19. This observation raised questions concerning the role of eIF4E in the nucleus and the mechanism by which eIF4E translocates across the nuclear membrane. While the first question is still unanswered, Dostie and colleagues2 have successfully addressed the second question regarding the transport mechanism of eIF4E by characterizing an eIF4E-binding protein, eIF4E-transporter (4E-T). Co-immunoprecipitation experiments from cell extracts demonstrated interaction between eIF4E and 4E-T2. Figure 1 illustrates a mutational study involving the over-expression of WT 4E-T or Y30A 4E-T, a mutant that does not bind eIF4E, in cells treated with leptomycin B (LMB), an export blocker. LMB inhibits export of leucine-rich NES-containing proteins by binding to the export receptor, CRM1 and preventing its association with the NES substrate20. Co-expression of WT 4E-T and HA-eIF4E caused nuclear accumulation of eIF4E in cells treated with LMB. In contrast, co-expression of HA-eIF4E and 4E-T Y30A, which accumulated in the nucleus in the presence of LMB, failed to cause import of eIF4E into the nucleus2. These data strongly suggest that nuclear import of eIF4E is mediated through its binding/association with 4E-T. Furthermore, GST pull-down assays demonstrated that eIF4E interacted with importin α and β only in the presence of 4E-T2. Taken together, data suggest that eIF4E is imported into the nucleus by the importin α/β pathway via a "piggy-back" mechanism through its interaction with 4E-T. Figure 2 depicts a potential model in which eIF4E is shuttled across the nuclear membrane by 4E-T.

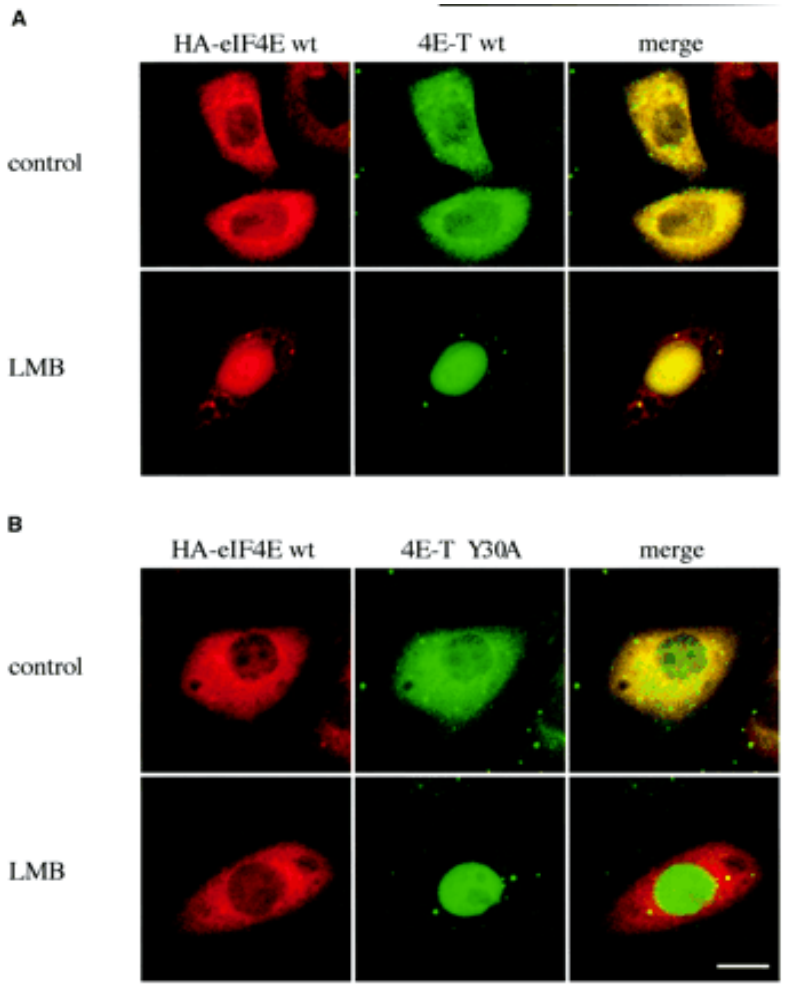

Figure 1. 4E-T imports HA-eIF4E into the nucleus. Hela cells were co-transfected with WT 4E and 4E-T or WT 4E and 4E-T Y30A. After transfection, cells were given either fresh media (control) or media containing LMB. Localization of 4E was determined by indirect IF with anti-HA 12CA5 and Texas red-conjugated anti-mouse IgG (left) and that of 4E-T with anti-4ET and fluorescein-conjugated anti-rabbit IgG (middle). The colocalization of 4E and 4E-T appears yellow (right){Dostie, 2000 #14}

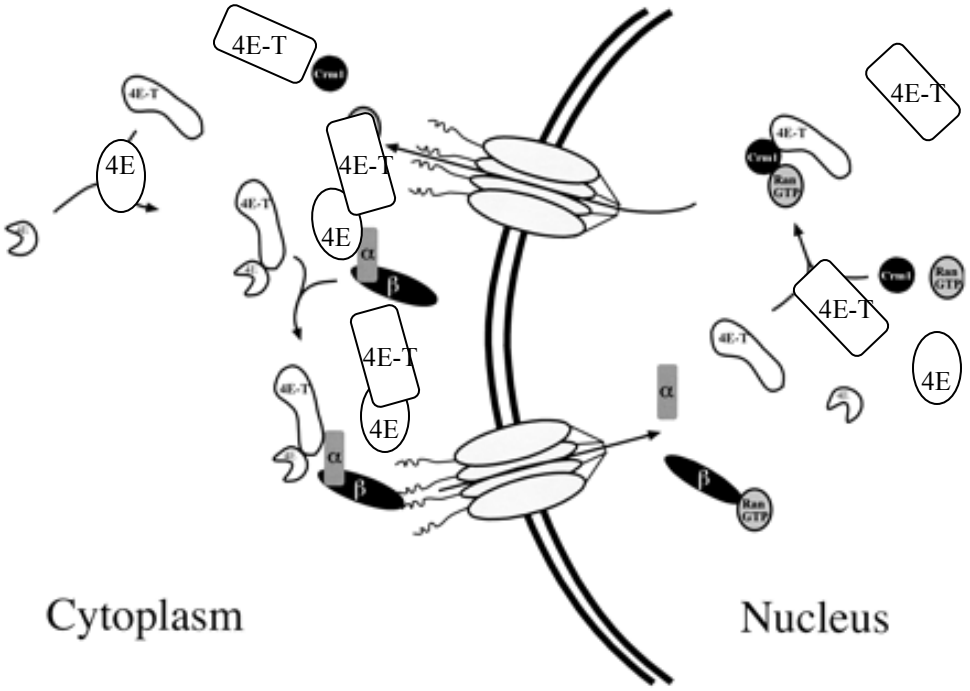

Figure 2**.** Model showing that eIF4E is imported into the nucleus via the importin α/β pathway by a piggy-back mechanism through its association with 4E-T{Dostie, 2000 #14}.

Generally, nucleocytoplasmic transport of macromolecules occurs through the nuclear pore complex (NPC) and is usually an energy-dependent, signal-mediated process{Mattaj, 1998 #44; Nakielny, 1999 #45}. Various types of nuclear localization signals (NLS), nuclear export signals (NES) and their receptors have been described. The best characterized NLSs are those of SV40 large T and nucleoplasmin, which consist of either a single or a bipartite cluster of basic amino acids, respectively{Kalderon, 1984 #43; Robbins, 1991 #46}. Basic NLSs are recognized in the cytoplasm by importin α, an adaptor protein that is associated with the receptor protein, importin β. Interaction of the import cargo with the importin αβ complex triggers docking of the hetero-trimeric complex to the NPC via importin β{Adam, 1994 #38; Gorlich, 1995 #41}. In contrast, the

best characterized nuclear export signal is the leucine-rich NES, which was first identified in the human immunodeficiency type 1 Rev protein{Fischer, 1995 #39} and in the cellular protein kinase A inhibitor, PKI{Wen, 1995 #48}. Leucine-rich NESs are recognized directly by the nuclear export receptor CRM1/exportin 1{Fornerod, 1997 #40; Stade, 1997 #47}. Translocation of substrates through the NPC requires, in addition to receptors, several other proteins including the GTPase Ran/TC4 and p10/NTF2{Mattaj, 1998 #44}. Ran/TC4 cycles between a GDP- and GTP-bound state, with Ran-GDP being predominantly cytoplasmic and Ran-GTP nuclear. Ran-GTP interacts directly with importins and exportins, and promotes directionality of transport by triggering the assembly and disassembly of export and import complexes, respectively{Gorlich, 1996 #42}.

Ribavirin, also known as Virazole, is a guanosine ribonucleotide analogue with a broad spectrum of antiviral activity against RNA and DNA viruses{De Clercq, 1993 #16; Prusiner, 1973 #17}. Upon entering cells, ribavirin is converted into mono, di-, or triphosphorylated (RTP) forms. The classical mechanism of action for ribavirin mono-phosphate involves the depletion of cellular GTP pools by inhibition of inosine monophosphate dehydrogenase{Streeter, 1973 #18}. In recent years, Crotty and colleagues{Crotty, 2000 #19; Crotty, 2001 #20} reported an additional role for ribavirin. Due to the chemical and structural similarity between ribavirin and guanosine, as seen in Figure 3, it is a substrate for the viral RNA-dependent polymerase. Incorporation of ribavirin in place of guanosine and alanine results in fatal mutagenesis of the viral genome.

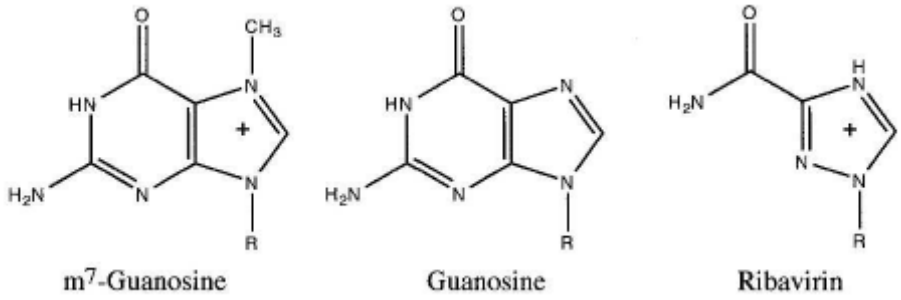

Figure 3. Chemical structures of m^7G, guanosine and ribavirin{Westman, 2005 #37}.

Moreov ›erty of ribavirin; data demonstrated that ribavirin suppresses eIF4E-mediated oncogenic transformation by physically mimicking the 5' mRNA cap. The affinity of eIF4E for its nucleoside ligand was measured *in vitro* by tryptophan fluorescence emission spectroscopy. Ribavirin binds eIF4E with a similar affinity as m^7GTP (K_D~0.13 μM and 0.19 μM respectively){Kentsis, 2004 #1}. In NIH 3T3 fibroblasts treated with 1 μM ribavirin, the subcellular organization of eIF4E was disrupted and eIF4E was re-distributed to the cytoplasm. Furthermore, the association between eIF4E and ribavirin affected transport and translation of cyclin D1 mRNA, an eIF4E-dependent transcript; at 100 μM ribavirin, nearly all of cyclin D1 mRNA was restricted to the nucleus{Kentsis, 2004 #1}. In a mouse model of human squamous cell carcinoma, treatment with ribavirin caused a marked suppression of tumor growth such that animals treated with 40 μg/kg of ribavirin each day, for 20 days, had six fold less tumor volume than control animals treated with saline{Kentsis, 2004 #1}. The exact mechanism by which ribavirin suppresses transformation and tumorigenesis is unknown.

The aims in this study are:

1. Determine whether ribavirin blocks nuclear import of eIF4E by disrupting the interaction between eIF4E and eIF4E-T

1a. Determine the effect of ribavirin on the subcellular localization of

eIF4E in Hela cells by subcellular fractionation

1b. Assess import of eIF4E in ribavirin-treated cells by measuring nuclear import directly in digitonin-permeabilized Hela cells

1c. Examine the effects of ribavirin on the association of recombinant eIF4E and eIF4E-T through *in vitro* binding assays, co-immunoprecipitation, immunofluorescence and fluorescence resonance energy transfer

2. Determine the biological significance of ribavirin on eIF4E-dependent Transcripts

2a. Assess binding between eIF4E and ribavirin by equilibrium dialysis

2b. Test whether ribavirin disrupts binding between eIF4E and m^7G cap by m^7G cap-sepharose affinity chromatography

2c. Evaluate impacts of ribavirin on eIF4E-dependent mRNAs by real-time PCR and western blotting.

EXPERIMENTS & RESULTS

I. Determine whether ribavirin blocks nuclear import of eIF4E by disrupting the interaction between eIF4E and eIF4E-T

As stated in Background and Significance, ribavirin influences the subcellular distribution of eIF4E, possibly by preventing its transport to the nucleus. In this aim I will test the hypothesis that it does so by inhibiting the interaction of eIF4E with 4E-T, which is essential for this translocation. To establish the appropriate experimental conditions, I nmust first confirm the data from the cell fractionation experiment, following the protocol from Kentsis et al{Kentsis, 2004 #1}. Next, I will use an *in vitro* nuclear import assay involving digitonin-permeabilized cells to assess the effects of ribavirin on import of eIF4E. Finally, effects of ribavirin on the association of eIF4E with 4E-T will be evaluated by an *in vitro* binding and Co-IP. In addition, indirect IF will be used to monitor 4E-T mediated import of eIF4E in ribavirin-treated cells.

Experiment 1: Cell fractionation

Cell fractionation will be conducted as previously described by Kentsis et al{Kentsis, 2004 #1}. For cell treatments, ribavirin (Calbiochem) will be dissolved in PBS and filter-sterilized, whereas untreated cells will receive filter-sterilized PBS. Cells will be fractionated and relative abundance of protein in nuclear and cytoplasmic fractions will be estimated by immunoblotting. Drug-treated and control cells will be washed twice in PBS and lysed by slow pipeting in buffer containing 0.5% NP-40. Lysed suspensions will be centrifuged at 1,000g for 3 min and the supernatant will be saved as the cytoplasmic fraction. The nuclear pellet will be resuspended in lysis buffer supplemented with 0.33% sodium deoxycholate and 0.66% Tween 40 and incubated for 5 min. Intact nuclei will be sedimented by centrifugation at 1000g for 3 min and the supernatant will

be saved as the postnuclear fraction. The extent of nuclear and cytoplasmic contamination in the respective fraction will be verified by evaluating the content of U6 snRNA, tRNA and β-actin. The purpose of this experiment is to confirm the observation published by Kentsis et al{Kentsis, 2004 #1} and to establish the conditions for the experiments described hereafter. I anticipate that western blots of protein extracts of nuclear (N) and cytoplasmic (C) fractions of ribavirin-treated cells, will display cytoplasmic distribution of eIF4E. The relative protein abundance of eIF4E in both fractions will be quantified by scanning desitometry, using purified, recombinant eIF4E to generate a standard curve.

Experiment 2: *In vitro* nuclear import assay

In the early 90s, Adam et al{Adam, 1990 #27} developed an *in vitro* system utilizing digitonin-permeabilized cells to study biochemical processes in the transport of macromolecules across the nuclear envelope. Digitonin preferentially solubilizes cholesterol from membranes, which leaves large holes, allowing entry of macromolecules. The plasma membrane contains higher concentrations of cholesterol than the nuclear envelope, thus it is selectively permeabilized, while the nuclear membrane remains intact and functional for transport and accumulation of proteins containing NLS{Adam, 1990 #27}. However, transport will require the addition of exogenous cytosol (from Hela or xenopus egg extracts) to permeabilized cells, as crucial cytoplasmic factors necessary for import are lost during digitonin treatment. I will follow the protocol described in "*Current Protocols in Cell Biology*[44]" to monitor the effects of ribavirin on the nuclear import of eIF4E. Briefly, Hela cells will be grown on coverslips and rinsed with cold transport buffer [20 mN Hepes, 2mM DTT, 110mM potassium acetate, 5mM magnesium acetate, aprotinin, leupeptin, and pepstatin (PIs)] followed by immersion in cold transport buffer containing 40 μg/ml digitonin. Cells will be allowed to permeabilize for 5 min after which the

digitonin containing buffer will be removed and replaced with cold transport buffer. The coverslips will be inverted over 15 µl of import mixture [transport buffer+ 1mM ATP + 5mM phosphocreatine + 20U/ml creatine phosphokinase+ GFP-eIF4E] +/- Hela cytosolic extracts, which may be purchased from NIH. For the ribavirin inhibition experiment, the coverslips will be incubated with transport buffer containing 100 µM RTP for 45 min at 20°C. Controls for nuclear import include reaction mixes prepared with probes containing 5 µg/ml positive (GFP-GST-NLS) and negative (GFP-GST-mutNLS) NLS sequences. CC3, an inhibitor of importin β-mediated nuclear import, is a positive control for drug treatment. Please refer to Table 1 for a summary of experimental samples; each measurement will be performed in triplicate. Reactions will be covered and incubated at room temperature (RT) for 15 min. Paraformaldehyde will be added to stop the reactions and nuclear import will be assessed by fluorescence microscopy.

	Expected Outcome		
Addition	**GFP-GST-NLS**	**GFP-GST-mutNLS**	**GFP-GST-4E**
Buffer	No import	No import	No import
Cytosol	Import	No import	Import
Cytosol+Ribavirin	Import	No import	No import
Cytosol+CC3	No import	No import	No import

Table1. Permeabilized Hela cells, grown on coverslips, will be inverted onto transport mixture containing the following: buffer, cytosolic extract, extract+ ribavirin or extract+ CC3. For import controls, positive and negative NLS substrates will be added to transport mixture. Cells will be assessed for nuclear import by flourescence microscopy.

For a summary of anticipated results for the transport of known import cargoes, please refer to Table1. If ribavirin blocks the transport of eIF4E into the nucleus, then cytoplasmic enrichment of eIF4E will be observed. This experiment can not rule out the possibility that ribavirin may be affecting nuclear export of eIF4E. In order to test this hypothesis, nuclear import will have to be blocked. This experiment will involve first treating Hela cells with CC3, to block import mediated through importin β1, then, ribavirin.

Experiment 3: Determine the effects of ribavirin on the association of eIF4E with 4E-T *in vitro*

GST-pulldown experiment will be performed following a modified version of the protocol described by Dostie et al{Dostie, 2000 #14}. Baculovirus Flag-4E-T and GST-eIF4E will be purified from Sf9 cells, as described by Haghighat et al{Haghighat, 1995 #25}. Purification of recombinant proteins will be performed in transport buffer 2 [Hepes, potassium acetate, magnesium acetate, DTT, Casamino acids, Tween and PIs]. Approximately 1 μg of Flag-4E-T and 1 μg of GST-eIF4E will be used in the assay as binding has been established at this concentration previously[2]. Proteins will be mixed and preincubated on ice for 30 min in a final volume of 30 μl. Transport buffer, containing various concentrations of ribavirin tri-phosphate (Moravek) (0, 0.1, 0.3, 1, 3 and 10 μM) and glutathione-Sepharose resin (Amersham Pharmacia Biotech) will be added to the samples and incubated for 40 min at RT with rocking and washed with transport buffer. eIF4E:4E-T complexes will be eluted in SDS sample buffer, resolved by SDS-PAGE and analyzed by western blotting. If RTP disrupts the interaction between eIF4E and 4E-T, then a reduction in the pulldown of 4E-T should be observed. 4E-T not bound to GST-eIF4E should be released and detected in the flow through fractions. Actin will be used as a negative control. Dostie et al{Dostie, 2000 #14} characterized 4E-T and demonstrated that it binds

eIF4E through conserved binding sites. However, should this assay fail to demonstrate association between eIF4E and 4E-T, as expression of eIF4E as a GST fusion may occlude the binding site needed for association with 4E-T, two alternative approaches will be employed: (1) Pulldown using GST-4E-T and Flag-eIF4E and (2) Pulldown using untagged eIF4E.

Experiment 4: Determine the effects of RTP on the association of eIF4E and 4E-T by Co-IP

To test whether eIF4E and 4E-T associate in cultured cells treated with ribavirin, a Co-IP experiment will be used. Hela cells will be treated with various concentrations of ribavirin (0.1, 1, 10, and100 μM) for 48 hours, harvested and lysed in buffer containing 0.2% NP-40. Afterwards, lysates will be cleared by microcentrifuging at 16,000 g at 4°C, for 15 min. The supernatant will be collected and used to set up immunoprecipitation reactions. Pre-cleared lysates will be incubated with protein G-Sepharose beads and anti-eIF4E for 2 hours, at 4°C with rotation. Samples will be microcentrifuged and washed with lysis buffer. Proteins in the immunoprecipitated complex will be solubilized in SDS-PAGE loading buffer, subjected to SDS-PAGE, then immunoblotted using anti-4E-T. Pre-immune serum will be used as a negative control. If ribavirin treatment disrupts endogenous association between eIF4E and 4E-T, then detection of eIF4E in ribavirin treated cells should be markedly reduced. As an alternative to Co-IP, FRET microscopy may be used to determine the effects of ribavirin on the association of eIF4E with 4E-T. Briefly, FRET occurs when two fluorophores are in sufficient proximity (<100 angstroms) and in optimal relative orientation such that an excited donor fluorophore, CFP, can transfer its energy to a second, longer-wavelength acceptor fluorophore, YFP, in a non-radiative manner. Due to variable expression of the FP-eIF4E/4E-T constructs, it will be difficult to demonstrate FRET unambiguously by sensitized acceptor emission alone.

However, FRET efficiency can also be measured by acceptor photobleaching, which takes advantage of the concept that FRET quenches the donor fluorescence as the excitation energy is transferred to the acceptor. After photobleaching of the acceptor, then quenching no longer occurs, and the donor fluorescence increases. Quantification of this increase, with *Image J* software, is a reliable and robust measure of FRET{Miyawaki, 2000 #52}. Andrei and colleagues{Andrei, 2005 #23} appended YFP onto eIF4E and CFP onto 4E-T and successfully demonstrated the presence of FRET, with an efficiency of 13%. I will follow the protocol described by Andrei et al{Andrei, 2005 #23}, except in drug inhibition experiments, cells will be treated with ribavirin. Cells expressing only YFP and CFP will be used as negative controls.

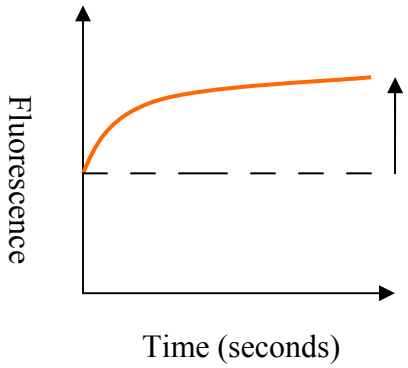

Fig 4(a) Photobleach acceptor flurophore over time and observe an increase in donor fluorescence (orange) which may be used to calculate FRET efficiency

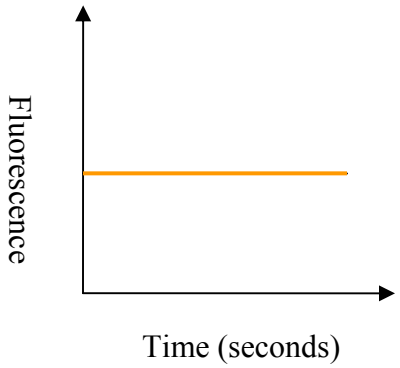

Fig 4(b) If ribavirin treatment disrupts interaction between eIF4E and 4E-T, then acceptor photobleaching should not result in any increase in donor fluorescence

One of the key advantages to FRET is the ability to study direct protein-protein interactions *in vivo*. However, there are many obstacles to this technique: minimal FRET {Dostie, 2000 #14}signal, high background noise and non-reusability of the sensors due to photobleaching.

Experiment 6: Determine the effects of ribavirin on 4E-T mediated import of eIF4E by IF

As discussed in the earlier section, the IF data from Figure 1 support the conclusion that eIF4E is imported into the nucleus by 4E-T. To test the hypothesis that ribavirin inhibits import of eIF4E into the nucleus by disrupting the interaction between eIF4E and 4E-T, it will be necessary to perform the IF experiment, as described by Dostie et al{Dostie, 2000 #14}, with the exception of treating the cells with ribavirin. Briefly, Hela cells will be transfected with 10 μg of either HA-eIF4E and WT 4E-T or HA-eIF4E and 4E-T Y30A plasmid DNAs. Cells will be treated with ribavirin, or Rib4C (negative control) and after 24 hours (h), trypsinized and plated on slides. At 36 h post-transfection, the medium will be replaced with either fresh medium (for Rib4C and ribavirin-treated cells) or medium containing LMB (blocker of Crm-1 mediated export), and incubated for 5 h prior to fixation, after which cells will be permeabilized with formaldehyde/Tween-20. Please refer to Table 2 for an outline of experimental samples. The localization of HA-eIF4E will be determined by indirect IF with anti-HA 12CA5 (BabCo) and Texas red-conjugated anti-mouse IgG and that of 4E-T with anti-4E-T (Dostie et al) and fluorescein-conjugated anti-rabbit IgG.

Expected Outcome

Addition	HA-eIF4E	WT 4E-T	Merge
Rib4C	N+C	N+C	N+C
Ribavirin	C	N+C	C
LMB	N	N	N

Addition	HA-eIF4E	4E-T Y30A	Merge
Rib4C	C	N+C	N+C
Ribavirin	C	N+C	C
LMB	C	N	N/A

Table2. Cells are transfected with either HA-eIF4E and WT 4E-T (top panel) or HA-eIF4E and Y30A 4E-T (bottom panel). Localization of 4E and 4E-T will be determined by indirect IF with fluorescence-conjugated antibodies. N denotes nuclear and C for cytoplasmic.

If anti-HA and anti-4E-T antibodies are non-specific and give rise to excess background, other antibodies (monoclonal anti-eIF4E) will be utilized.

II. Determine the biological significance of the effect of ribavirin on eIF4E-dependent transcripts

High-affinity binding of the m^7G mRNA cap to eIF4E occurs by recognition of the methylated and positively charged quaternary amine m^7G base by two conserved tryptophans, W56 and W102, which form an aromatic stack as a result of cation interactions{Marcotrigiano, 1997 #35; Niedzwiecka, 2002 #36}. Kentsis et al used tryptophan emission fluorescence spectroscopy to study the binding between RTP and eIF4E and found the K_d of the interaction to be 0.13 μM, similar to that of m^7G nucleoside{Kentsis, 2004 #1}. However, a very recent finding published by Westman et al{Westman, 2005 #37} using fluorescence titration experiments reported that the affinity for RTP and eIF4E is at least 100 fold lower. In order to address the hypothesis of the current aim (ribavirin interferes with translation of eIF4E-dependent transcripts by competing with mRNAs for binding to eIF4E), I need to first reconcile this apparent discrepancy. I will evaluate and confirm the association between eIF4E and ribavirin by equilibrium dialysis, a classic, reliable and straightforward technique.

While the first aim studies the impacts of ribavirin on cellular localization of eIF4E, this section of the proposal will examine the biological relevance of ribavirin treatment on the function of eIF4E. In order to determine the role of ribavirin on eIF4E-mediated transformation, two sets of experiments will be conducted. First, I will test whether ribavirin competes with m^7GTP for eIF4E by m^7G-sepharose affinity chromatography, assuming that binding between eIF4E and RTP will be confirmed from above. Second, I will assess the effects of ribavirin on the mRNA and protein expression levels of eIF4E-dependent transcripts by real-time PCR and western blotting. Vascular endothelial growth factor (VEGF), c-*myc*, and matrix metalloprotease 9 (MMP-9) are chosen as candidate genes in this study due to their roles in cell growth, angiogenesis and malignancy{De Benedetti, 2004 #53}.

Experiment 7: Determine binding between eIF4E and ribavirin by equilibrium dialysis

Equilibrium dialysis is performed with two Teflon cells, one containing protein and the other an equal volume of buffer, separated by a size-selective dialysis membrane. The unbound drug is allowed to reach equilibrium between the two compartments. Measurement of the drug concentration in each compartment allows the degree of protein binding to be calculated. I will follow the procedure described by "*Current Protocols in Pharmacology*[45]" to determine the binding between eIF4E and [^{3}H] RTP. In order to determine protein binding parameters, it will be necessary to establish the time required for equilibrium. One compartment of the Teflon cells will be filled with 1 ml of PBS through one of the double filling ports and the other with PBS buffer spiked with RTP, also known as the dosing compartment. The complete cell set will be maintained in a temperature controlled environment (37°C). Following 1, 2, 4, 6 and 20 hr of dialysis, liquid from both compartments of cells will be removed. The concentration of RTP will be determined in both compartments by a liquid scintillation counter (LSC). The duration of dialysis will be plotted against the ratio of RTP in both compartments to determine the time at which equilibrium is achieved. In addition, the percentage of nonspecific binding (NSB) at equilibration time can also be calculated:

NSB (%) = 100-[(dose concentration-amt recovered)/(dose concentration)]x100

Note: Dose concentration is the concentration of RTP and amount recovered is the concentration in the dosing compartment plus the concentration in the buffer compartment

To determine the binding or RTP to eIF4E, reaction mix containing ribavirin and eIF4E will be added to the dosing compartment and PBS to the buffer compartment. The reaction will be equilibrated for the amount of time as determined from above. Finally, concentration of RTP in both compartments will be determined by LSC. If ribavirin associates with eIF4E, then plotting free RTP concentration against fraction of RTP bound by eIF4E should result in a hyperbolic graph and K_D can be obtained. If binding is not observed between eIF4E and ribavirin in equilibrium dialysis, then the stability of eIF4E will be examined. It is possible that prolonged exposure of the protein at 37°C might have caused significant degradation, in which case supplementing the buffer with protease inhibitors might ameliorate the problem.

Experiment 8: Test whether ribavirin disrupts binding between eIF4E and m^7G by m^7G-sepharose affinity chromatography

In order to determine whether ribavirin disrupts association of eIF4E with m^7G, an mRNA cap analog, an affinity chromatography will be used. Briefly, m^7G-sepharose beads will be incubated with 2 μM purified recombinant eIF4E (eIF4E obtained from Gerhard Wagner, Harvard University, Cambridge, MA) for 30 min at RT. Unbound eIF4E will be removed with washes of buffer containing NaCl/ sodium phosphate/ BSA/ NP-40. eIF4E-bound to m^7G-sepharose will be solubilized and subjected to SDS/PAGE and visualized by Western blotting. For the drug inhibition experiment, m^7G beads will be incubated with eIF4E and RTP (0, 0.1, 1, 10 and 100 μM). If RTP disrupts the interaction between eIF4E and m^7G sepharose beads, then western blot of eIF4E bound to beads upon competition with increasing concentration of RTP should result in a reduction in binding. m^7GTP will be used as a positive control and Rib4C will be used as a negative control.

Experiment 9: Evaluate impacts of ribavirin on eIF4E-dependent mRNAs by real time PCR

To study the effects of ribavirin on the mRNA levels of eIF4E-dependent transcripts, real-time PCR will be used to quantitate c-*myc*, VEGF and MMP-9 gene expression. Briefly, Hela cells will be treated with increasing concentration of RTP (0, 0.1, 1, 10 and 100 μM) and RNA will be extracted from nuclear and cytoplasmic fractions or from whole cell extracts by using Qiagen RNeasy Maxi kit. RNA will be converted to cDNA using a random hexamer-primed reverse transcription reaction. Primers for c-*myc*, VEGF and MMP-9 will be used in the real-time PCR reactions and are all commercially available. Expression will be normalized to β-actin, SYBR green will be used as a fluorescent dye, which will be monitored and calculated at each cycle of the PCR. The increase in fluorescence is proportional to the amount of starting template. Since cyclin D1 is the only transcript that has been tested and shown to be affected by ribavirin treatment, it will be used as a positive control. Kentsis and colleagues demonstrated that 100 μM RTP did not alter total mRNA levels or stability, but did cause near complete nuclear retention of cyclin D1 transcripts{Kentsis, 2004 #1}. This experiment will facilitate understanding of ribavirin's effect on various eIF4E-dependent transcripts. If this effect were a general phenomenon, then drug treatment should result in nuclear distribution of c-*myc*, VEGF and MM9 mRNAs. In the event that the real-time PCR is unsuccessful, the reverse transcriptase reaction will be performed using the three sets of specific primers. This should result in production of those three specific genes as cDNAs, which will be run on an agarose gel and the luminosity will be measured for each gene. While this is harder to quantitate than real-time PCR, it is a viable alternative.

Experiment 10: Evaluate impacts of ribavirin on eIF4E-dependent mRNAs by western blotting

Hela cells will be treated with RTP at increasing concentrations (0, 0.1, 1, 10, and 100 μM) for 48 hours. Proteins from whole cells or nuclear and cytoplasmic fractions will be extracted and quantitated by bicinchoninic acid-copper reduction (Pierce). 20 μg aliquots will be resolved using SDS-PAGE, transferred to Immobilon-P membrane, blocked, and probed by using primary antibodies against eIF4e (Transduction Laboratories), c-*myc* (Covance), VEGF (Invitrogen) and MMP-9 (Invitrogen). Bound antibodies will be chemiluminescently detected by using horseradish peroxidase-conjugated secondary antibodies. Ribavirin treatment of NIH 3T3 cells caused nuclear retention of cyclin D1 mRNAs{Kentsis, 2004 #1} without affecting transcription or mRNA stability. Since the machinery for protein translation is present in the cytosol, cyclin D1 protein production was markedly reduced in ribavirin-treated cells. If ribavirin treatment in Hela cells will result in nuclear retention of c-*myc*, VEGF and MM9 mRNAs, then protein expression should also be reduced.

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Vitae

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