The Cleavage of Protein Synthesis Initiation Factor eIF4G-1 by Picornaviral 2A Protease and its Role in Viral Infection

A Dissertation

Submitted to the Graduate Faculty of the Health Sciences Center of
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

In

The Department of Biochemistry and Molecular Biology

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February 2004

ACKNOWLEDGEMENTS

My heartfelt thanks and gratitude go first to my mentor Dr. Robert E. Rhoads, who has guided me all these years with his patience, encouragement, support, and understanding. He is not only my scientific advisor but also my English mentor. If I have ever made any progress, there is his effort in it.

I would like to thank my committee, Drs. Eric First, Dennis O'Callaghan, Arrigo De Benedetti, and B. Jill Williams, for their guidance and helpful suggestions during my whole graduate study. I especially want to thank Dr. Peter Sarnow, Stanford University, who took time away from his busy schedule and guided me through my preliminary exam.

My very special thanks and gratitude are devoted to Dr. Robert J. Schneider, New York University, who has been my inspiration during the last year of my graduate study. It is his words "Doing a good piece of work is more important." encouraged me finishing my study and achieving my Ph.D goal.

Special thanks go to my rotation mentors, Drs. Robert E. Rhoads, Eric First, Peter Good, and Barry Lamphear, who led me into this magic molecular world and let me realize what I really want to do in my life.

I am grateful to Drs. Kirk Knowlton and Marlyn Langford for allowing me to work in their labs and teaching me how to handle viruses and more.

I would like to thank Dr. Marjan Trutschl for letting me audit his computer courses and providing me powerful tools to process data quickly and easily.

I appreciate all the advice and help from former and present lab members: Drs. Robert Smith, Aili Cai, Brett D. Keiper, Nadejda Korneeva, Chris Bradley, Tzvetanka

Dinkova, Kyoung Moo Choi, and Sergey Slepenkov; Mr. Michael LaCelle, Dan Duzan, William C. Waddell, Tolvert E. Fowler, Mike Wheeler, and Aaron LeFebvre. Without their help, things might have been completely different.

I take this opportunity to express my deep gratitude to my parents, Mr. Chaolian Zhao and Mrs. Xioulan Wang, who are always my spiritual pillars. Their unconditional love has helped me get through those stressful times in my life. I also want to thank my brother Bin Zhao and my sister Bifang Zhao for their love, support, and encouragement.

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SUMMARY

eIF4G was discovered as a result of its proteolysis coincident with the loss of cap-dependent initiation during poliovirus infection. Cleavage of eIF4G-1 by 2A protease was initially thought to be the major mechanism utilized by entero- and rhinoviruses to shut down host protein synthesis and allow viral protein synthesis to continue by IRES-driven translation, but subsequent evidence has suggested that eIF4G-1 cleavage is not responsible, or is only partially responsible, for the shutoff of host protein synthesis. To understand the relative importance of the integrity of eIF4G in picornavirus infection, we created amino acid sequence variants of eIF4G-1 that are 1000- to 10,000-fold resistant to cleavage by 2A protease *in vitro* (eIF4G-1^{DM}) compared to eIF4G-1^{WT}.

Full functional activity of eIF4G-1^{DM} was demonstrated *in vitro* by its ability to restore cap-dependent translation to a 2A protease-pretreated rabbit reticulocyte system. An isoform containing the binding site for PABP, eIF4G-$1e^{DM}$, was more active in this assay than an isoform lacking it, eIF4G-$1a^{DM}$, but only with polyadenylated mRNA. In addition, the translational activity of eIF4G-$1e^{DM}$ but not eIF4G-$1a^{DM}$ was found to be inhibited by poly(A) and oligo$(dT)_{15}$. Functional activity was also demonstrated *in vivo* with stably transfected HeLa cells expressing eIF4G-1^{DM} from a tetracycline-regulated promoter. In transient cotransfection experiments, cap-dependent GFP synthesis was inhibited by 2A protease expression and partially restored by either eIF4G-$1a^{DM}$ or eIF4G-$1e^{DM}$, whereas IRES-dependent GFP synthesis was stimulated by 2A protease and suppressed by eIF4G-$1e^{DM}$ but not eIF4G-$1a^{DM}$.

To investigate the importance of eIF4G-1 integrity for maintaining host protein synthesis and the relative importance of host protein synthesis for inhibiting viral

production, we infected a cell line expressing eIF4G-1e^{DM} with various picornaviruses. For poliovirus type 1 strains Mahoney, MMd, and Sabin, and poliovirus type 3 strains Saukett and Leon, both the viral infectivity and the viral production were substantially decreased in cells expressing eIF4G-1e^{DM} compared to control cells. When cells were infected with MMd at 10 pfu/cell, expression of eIF4G-1e^{DM} extended host protein synthesis and delayed viral protein synthesis 1-2 h. Intracellular levels of virus were significantly different at 5 h postinfection, which correlated with the difference of viral protein synthesis. By 8 h postinfection, extracellular virus levels were five-fold lower for eIF4G-1e^{DM}-expressing cells compared with control HeLa cells. These results suggest that low levels of intact eIF4G-1 restrict virus replication, perhaps by allowing synthesis of interfering host proteins.

eIF4G-1a^{DM} and eIF4G-1e^{DM} were expressed in sf9 cells infected with recombinant baculovirus. Full-length pure eIF4G-1a^{DM} and eIF4G-1e^{DM} could be obtained through Ni-NTA agarose affinity chromatography followed by Mono Q ion exchange chromatography.

CHAPTER 1

INTRODUCTION: THE REGULATION OF TRANSLATION

A. Translational Regulation in Eukaryotic Cells

1. Regulation of protein synthesis

Protein synthesis (translation) is one of the most important metabolic processes of eukaryotic cells. It allows the information stored in DNA to be deliveried from mRNA to proteins, the basic structural and functional components of the cell. The translational machinery is composed of three major components: ribosomes, mRNA, and translation factors, each of which can become the targets for regulation.

a. Ribosomes

The eukaryotic ribosome consists of a 40S and a 60S subunit. Even though the ribosome is the major site of protein synthesis, it is not the rate-limiting factor under normal conditions. The cellular levels of ribosomes may become rate-limiting under certain circumstances, such as in cells very active in protein synthesis. For example, liver cells from fed rats utilize 90-95% of their ribosomes in protein synthesis (Henshaw and others 1971), suggesting that still higher rates of protein synthesis might have been possible were there a greater number of ribosomes. On the other hand, in translationally repressed cells, such as liver cells from fasted rats (Henshaw and others 1971) or quiescent cells in culture (Duncan and McConkey 1982; Meyuhas and others 1987), fewer than half of the ribosomes are actively translating mRNAs. Phosphorylation of ribosomal proteins may also affect translational initiation. Ribosomal protein S6 is a well-studied example. Its phosphorylation promotes the initiation of translation of mRNAs encoding ribosomal proteins and elongation factors, which possess an oligopyrimidine tract in the 5'-untranslated region (UTR). The study of S6 has shed light

on the signal transduction pathways that link growth-promoting stimuli to S6 phosphorylation (Meyuhas 2000; Amaldi and Pierandrei-Amaldi 1997).

b. mRNA

Cis-regulatory elements of mRNAs are involved in translational control. Both the 5'-terminal m^7GpppN cap structure, present in all cellular (*i.e.*, non-organeller) mRNAs, and the poly(A) tract, present in all mRNAs except those encoding histones, stimulate translational initiation (Rhoads 1988; Tarun and Sachs 1995). Some special *cis*-regulatory elements exist in the 5'- or 3'-UTRs of certain mRNAs to regulate translation by interacting with *trans*-acting factors (Rouault and others 1990; Laroia and others 1999).

c. Translation factors

Translation in eukaryotes is a complex, multistep process (Hershey and Merrick 2000) and can be divided into three stages: initiation, elongation, and termination. Translational factors are involved in all three stages. Translation is regulated by both the activities and the concentrations of these factors.

a) Initiation factors

The effects of the various *cis*-acting elements in the 5'-UTR of mRNA are modulated through the activity of initiation factors and other *trans*-acting factors. Eleven initiation factors have been identified, comprising over 25 polypeptide chains (Table 1-1) (Hershey and Merrick 2000). These initiation factors are regulated both in protein levels and activities.

Table 1-1. Initiation factors from mammalian, plant, and yeast cells

	Mammals[a]		Plants[a]		Yeast[a]				
Name	mass (kD)	acc. no.	mass (kD)	acc. no.	gene	mass (kD)	acc. no.	%ID[b]	Function
eIF1	12.6	L26247	12.6	AC005287	*SUI1*	12.3	M77514	58	AUG recognition
eIF1A	16.5	L18960	16.6	AC006951	*TIF11*	17.4	U11585	65	Met-tRNA$_i$ binding to 40S subunit
eIF2α	36.2	J02646	41.6	AF085279	*SUI2*	34.7	M25552	58	affects eIF2B binding by phosphorylation
eIF2β	39.0	M29536	26.6	AL162351	*SUI3*	31.6	M21813	42	binds to eIF2B, eIF5
eIF2γ	51.8	L19161	50.9	AC002411	*GCD11*	57.9	L04268	71	binds GTP, Met-tRNA$_i$; GTPase
eIF2Bα	33.7	U05821	39.8	AC016529	*GCN3*	34.0	M23356	42	nonessential; helps recognize P-eIF2
eIF2Bβ	39.0	U31880	43.6	AC012395	*GCD7*	42.6	L07116	36	binds GTP, helps recognizes P-eIF2
eIF2Bγ	50.4	U38253			*GCD1*	65.7	X07846		GEF activity
eIF2Bδ	57.8	Z48225	29.4	AC016041	*GCD2*	70.9	X15658	36	binds ATP, helps recognizes P-eIF2
eIF2Bε	80.2	U19511	81.9	AC004238	*GCD6*	81.2	L07115	30	GEF activity
eIF3	***See Table 1-2***								
eIF4AI	44.4	X03039.1	46.7	AB019229	*TIF1*	45.1	X12813	65	ATPase, RNA helicase
eIF4AII	46.3	X12507.1	46.8	AC005287	*TIF2*	44.6	X12814		ATPase, RNA helicase
eIF4B	69.2	S12566	57.6	AF021805	*TIF3*	48.5	X71996	26	binds RNA; stimulates helicase
eIF4E	25.1	M15353	26.5	AL110123	*CDC33*	24.3	M21620	33	binds m^{7}G-cap of mRNA
		(eIFiso4E)	22.5	AB013393					
eIF4GI	171.6	AF104913	153.2		*TIF4631*	107.1	L16923	22	binds eIF4E, 4A, 3, PABP•RNA
eIF4GII	176.5	AF012072	176.5		*TIF4632*	103.9	L16924	21	binds eIF4E, 4A, 3, PABP•RNA
		(eIFiso4G)	87.0	AB013396					
eIF5	48.9	L11651	48.6	AC007576	*TIF5*	45.2	L10840	39	stimulates eIF2 GTPase
eIF5B	139.0	AF078035			*FUN12*	97.0	L29389	70	GTPase; promotes junction reaction

[a] The masses (kD) and accession numbers pertain to human or rat, *Arabidopsis thaliana,* and *S. cerevisiae* proteins. Only one of the numerous isoforms of the plant proteins was included arbitrarily. A complete listing of *A. thaliana* initiation factors is found in Browning (1996).

[b] %ID, percent sequence identity shared by yeast and human proteins.

(Ref. Hershey and Merrick 2000)

Table 1-2. eIF3 subunits from mammalian, plant, and yeast cells

Human			*Arabidopsis*			Yeast				
name	mass	acc. no.	name	mass	acc. no.	name	gene	mass	acc. no.	Comments
p170	166.5	D50929	eIF3a	114.3	AL050399	p110	*TIF32*	111.1	AF004912	(*RPG1*) binds RNA, PCI family
p116	98.9	U78525	eIF3b	81.9	AC007478	p93	*NIP1*	88.1	J02674	binds eIF1, eIF5
p110	105.3	U46025	eIF3c	103.0	AF040102	p90	*PRT1*	93.4	L02899	PCI family
p66	64.0	U54558	eIF3d	66.2	AB001475					binds RNA
p48	52.2	U54562	eIF3e	51.8	A1137080					PCI family, Int-6
p47	37.6	U94855	eIF3f	31.9	AF002109					MDN motif
p44	35.4	U96074	eIF3g	32.7	AC008153	p36	*TIF35*	30.5	AF004913	binds RNA, eIF4B
p40	39.9	U54559	eIF3h	38.4	AC007354					MDN motif
p36	36.5	U39067	eIF3i	36.4	AC005397	p39	*TIF34*	38.8	U56937	WD repeats, TRIP1
p35	29.0	U97670					*HCR1*	29.6	U14913	
p28	25.1	N/A	eIF3k	25.7	AL61583					
						p135	*TIF31*	145.2	AF004911	(also called *CLU1*)

(Ref. Hershey and Merrick 2000)

b) Elongation factors

There are two elongation factors in all eukaryotes, eEF1 and eEF2. eEF1 is required for recruitment of aminoacyl-tRNA to the ribosome and binds both aminoacyl-tRNA and GTP. eEF1B catalyzes guanine nucleotide exchange on eEF1A. Mammalian eEF1B consists of three subunits (α,β, and γ), two of which (α and γ) have nucleotide-exchange activity, at least *in vitro*. The activities of both eEF1A and eEF1B are regulated by phosphorylation (Janssen and others 1988; Venema and others 1991a; Venema and others 1991b; Mulner-Lorillon and others 1994; Kielbassa and others 1995; Chang and Traugh 1998; Sheu and Traugh 1999). eEF2 is involved in the subsequent translocation event in which the ribosome moves along the mRNA. eEF2 has a bacterial homologue, EF2. eEF2 activity is inhibited by ADP ribosylation of diphthamide at His-715 and regulated by phosphorylation (Nygard and others 1991; Redpath and others 1993; Ryazanov and Spirin 1990; Diggle and others 1998; Hovland and others 1999).

c) Termination factors

Termination factors include eRF1, eRF2, and eRF3. eRF1 recognizes UAA, UAG, and UGA termination codons and promotes peptide hydrolysis from tRNA. eRF2 is GTPase and stimulates the activity of eRF1. eRF3 stimulates eRF1 activity in a GTP-dependent fashion. The regulation of translation by termination has received less attention than initiation or elongation, but recent studies suggest that factors involved in translation termination are also critical for positioning ribosomes for subsequent rounds of translation and regulating translational efficiency (Hoshino and others 1999; Vincent and others 1994; Andjelkovic and others 1996).

2. Translation initiation

a. Modes of translational initiation

a) Cap-dependent initiation

The 5'-end of all nuclear-encoded mRNAs in eukaryotes contains a cap structure (m^7GpppN). The involvement of the cap structure in mRNA translation was suggested soon after its discovery in the early 1970s (Shatkin 1976). The cap was found to stimulate translation in an *in vitro* system (Both and others 1975; Muthukrishnan and others 1975). Later, eIF4E was shown to bind the cap structure (Sonenberg and others 1978; Hellmann and others 1982). A current model for cap-dependent translation initiation is that eIF4E, in concert with other eIF4 factors, interacts with m^7GpppN to recruit mRNA to the 43S complex and to form the 48S translation initiation complex. The 48S complex then scans along the 5'-UTR of the mRNA until it encounters the initiation AUG codon, whereupon the 60S ribosomal subunit joins and the 80S initiation complex is formed (Hershey and Merrick 2000).

b) Internal initiation

The 5′-UTRs of picornaviral RNAs and some of the cellular mRNAs are unusually long and highly structured, with numerous AUG triplets that are not used as translation initiation sites. These RNAs are initiated by an unusual mechanism that involves well-defined RNA structures called internal ribosome entry sites (IRESes). IRES-mediated internal initiation allows recognition of the start codon by the ribosome in a cap-independent way. Internal initiation on the two main IRES classes of picornavirus requires all the canonical initiation factors for cap-dependent initiation except eIF4E. Also, the integrity of eIF4G is not required. Initiation complex formation requires eIF2,

eIF3, eIF4A, and either the complete eIF4F complex or fragments of eIF4G that include the central domain containing the eIF3-binding site and one of the eIF4A-binding sites (Lamphear and others 1995; Imataka and Sonenberg 1997). IRES structures are discussed in more detail in section C.1.c of this Chapter.

c) Ribosome shunting initiation

Ribosome shunting is a form of discontinuous scanning. The 40S ribosome does not simply scan along the 5'-UTR but rather "hops" from one region to another. At any one time, some ribosomal subunits may be engaged in strictly linear scanning while others may be shunting. The ratio of these two subpopulations of ribosomes may be affected by variables such as initiation factor concentrations (Yueh and Schneider 1996).

b. Stepwise ribosome recruitment

Translation initiation can be divided into four steps (Fig. 1-1). A different class of initiation factors catalyzes each of the discrete steps of initiation. First, the 40S ribosomal subunit interacts with eIF3 to form the native 40S complex ($40S_N$) during step 1. A ternary complex of eIF2•GTP•Met-tRNAi binds to the $40S_N$ complex to form the 43S complex during step 2. In step 3, eIF4F recruits mRNA to form the 48S initiation complex, which is rate-limiting for initiation under normal conditions. After the 48S initiation complex is formed, it begins to scan the 5'-UTR of mRNA until it reaches the first AUG codon in good sequence context (Kozak 1986). During step 4, the GTPase-activating protein eIF5, together with eIF5B, stimulates GTP hydrolysis by eIF2. The initiation factors are then replaced by the 60S ribosomal subunit to form the 80S initiation complex and the elongation phase begins.

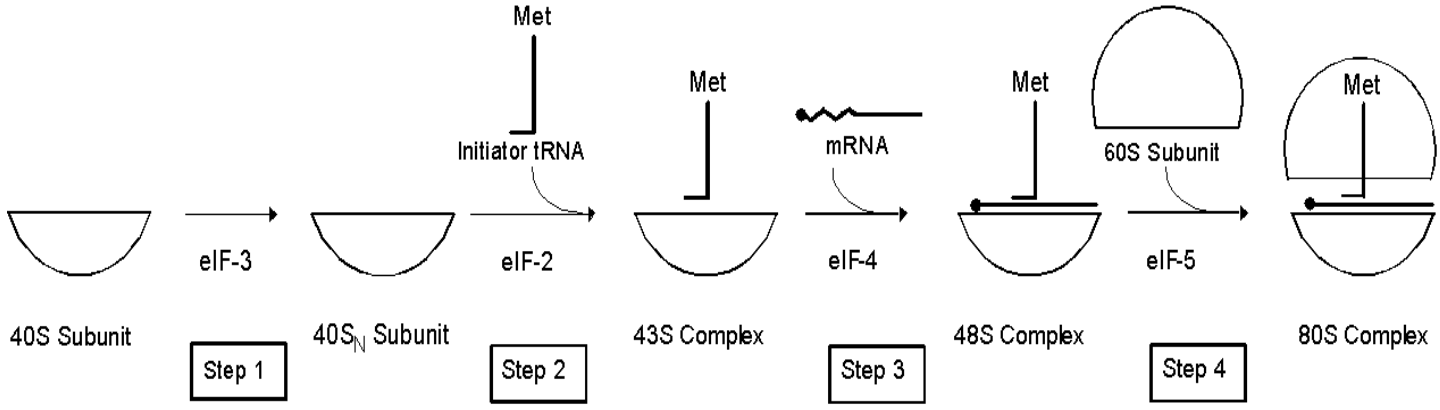

Met
Initiator tRNA
Met
mRNA
Met
60S Subunit
Met
eIF-3
eIF-2
eIF-4
eIF-5
40S Subunit
40S$_N$ Subunit
43S Complex
48S Complex
80S Complex
Step 1
Step 2
Step 3
Step 4

Fig. 1-1. Stepwise recruitment of initiation complex components and major sites of action for various initiation factor groups (reproduced from Rhoads, 1993). This is a simplified diagram to illustrate how the 80S initiation complex is formed during translation initiation. Step 1, the 40S ribosomal subunit interacts with eIF3 to form the native 40S complex ($40S_N$); step 2, eIF2 brings Met-charged initiator tRNA (tRNAi) to form the 43S complex; step 3, eIF4 factors recruit mRNA to form the 48S complex, which then begins to scan the 5'-UTR of mRNA until it reaches the AUG codon; step 4, eIF5 promotes GTP hydrolysis and initiation factor release from the 48S complex, after which the 60S ribosomal subunit joins to form the 80S initiation complex, followed by formation of the first peptide bond.

c. Regulation sites during ribosomal recruitment

Regulation of translation initiation involves modulation of either the levels or activities of the initiation factors (Rhoads 1993; Hershey and others 1996). Two sites have been extensively studied. One of them is at step 2, which regulates the degree of initiator tRNA binding to the $40S_N$ ribosomal subunit and involves phosphorylation of the eIF2α subunit. Phosphorylated eIF2α binds tightly to eIF2B, the guanine nucleotide exchange factor, and prevents GTP recycling (Hershey and Merrick 2000). Four eIF2 kinases, 1) RNA-regulated protein kinase-like endoplasmic reticulum kinase, 2) general control non-derepressible gene 2 kinase, 3) double-stranded RNA-dependent protein kinase, and 4) heme-regulated eIF2α kinase, are found in most somatic cells. They phosphorylate the α-subunit of eIF2 in response to a variety of stresses, such as heat shock, virus infection, altered pH, chemical poisoning, and the deprivation of amino acids, glucose, heme, or iron (Schneider and others 1985; Rhoads 1988; Jackson 1991; Sistonen and others 1992; Favre and others 1996; Schneider 2000b).

The second site that is highly regulated is at step 3, in which mRNA is recruited to the 43S initiation complex by eIF4 factors to form the 48S initiation complex (Fig. 1-1). The formation of the 48S initiation complex is rate-limiting under normal conditions and is regulated through both eIF4E and eIF4G.

d. eIF4E

eIF4E is a 25-kDa phosphoprotein (Rychlik and others 1986) that binds to the m^7G-cap structure to recruit mRNA. The phosphorylation site of eIF4E is at Ser-209 (Joshi and others 1995). Several kinases can phosphorylate eIF4E *in vitro*, including protein kinase C (Tuazon and others 1990; Morley and others 1991; Whalen and others

1996), an insulin-stimulated protamine kinase (Makkinje and others 1995), and the MAP-activated serine/threonine kinases Mnk1 and Mnk2 (Fukunaga and Hunter 1997; Waskiewicz and others 1997; Waskiewicz and others 1999). Phosphorylation of Ser-209 reduces the cap affinity of eIF4E (Scheper and others 2002). The level of eIF4E phosphorylation is correlated with the protein synthesis rate (Rhoads 1993; Rhoads and Lamphear 1995).

The availability of eIF4E can be regulated by binding to the eIF4E-binding proteins (4E-BP1, 4E-BP2, and 4E-BP3) (Pause and others 1994; Lin and others 1994b). 4E-BP is also a phosphoprotein and binds eIF4E to sequester it from participation in translational initiation. It releases eIF4E when phosphorylated (Pause and others 1994), and its phosphorylation is correlated with eIF4E phosphorylation (Pause and others 1994; Lin and others 1994a; Hu and others 1994; Mader and Sonenberg 1995; Haghighat and others 1995; Mendez and others 1996). The 4E-BPs and eIF4G bind a common motif on eIF4E (Mader and others 1995). 4E-BPs thus function as competitors with eIF4G for binding eIF4E (Haghighat and others 1995) and thereby inhibit cap-dependent translation (Feigenblum and Schneider 1996; Mader and Sonenberg 1995). Dephosphorylation of 4E-BP leads to a switch from cap-dependent to internal initiation in cells infected with some viruses, *e.g.*, influenza virus (Feigenblum and Schneider 1993), adenovirus (Zhang and others 1994; Schneider 2000a; Schneider and Mohr 2003), and encephalomyocarditis virus (EMCV) (Kleijn and others 1996).

The signaling pathway initiated by insulin is the best understood mechanism for mitogenic stimulation of protein synthesis. Insulin appears to regulate both the initiation and elongation phases of translation (Mendez and others 2001; Kimball and others 1994),

by altering the phosphorylation of translation initiation factors (eIF2, eIF2B, eIF3, eIF4B, eIF4E, and eIF4G) and elongation factors (eEF1 and eEF2). Insulin regulates eIF4E in two ways: 1) it increases phosphorylation of eIF4E (Manzella and others 1991; Mendez and others 1996; Flynn and Proud 1996a; Flynn and Proud 1996b), and 2) it stimulates phosphorylation of 4E-BP to make more eIF4E available for protein synthesis (Lin and others 1995).

eIF4E behaves as an oncoprotein, and changing the levels of eIF4E has a dramatic effect on cell physiology. This is consistent with the fact that eIF4E acts at the rate-limiting step in protein synthesis. Over-expression of eIF4E causes deregulated growth and malignant transformation of rodent and human cell lines in culture (Lazaris-Karatzas and others 1990; De Benedetti and Rhoads 1990), and reduction of eIF4E levels by expression of eIF4E antisense RNA (De Benedetti and Rhoads 1991) results in phenotypic reversal of *ras*-transformed fibroblasts (Rinker-Schaeffer and others 1993; Graff and others 1995).

B. eIF4G

eIF4G was first recognized as a high-molecular-mass protein (formerly called p220 or eIF4γ) found in eluates from m^7G affinity columns in a complex with eIF4E and eIF4A, termed eIF4F (Grifo and others 1983). eIF4G-1 availability can be regulated by hsp27 during heat shock (Cuesta and others 2000a) and modified by cleavage under stress and certain picornaviral infections (Ehrenfeld 1996; Zamora and others 2002).

1. Isoforms of eIF4G

cDNAs for eIF4G have been cloned from numerous sources, including human, rabbit, *D. melanogaster*, wheat, *S. cerevisiae*, and *S. pombe* (reviewed in Keiper and others 1999). There are at least two different genes for eIF4G in yeast, wheat, and mammals (Browning 1996; Goyer and others 1993, Browning and others 1987, Yan and others 1992, Yan and Rhoads 1995, Gradi and others 1998). In humans, the two different genes encode eIF4G-1 (a.k.a., eIF4GI) and eIF4G-2 (a.k.a., eIF4GII), which share 46% identity at the amino acid (aa) level and an overall similarity of 56% (Yan and others 1992, Gradi and others 1998). The human cDNA initially isolated for eIF4G-1 contained an open-reading frame for a protein of 154 kDa (Yan and others 1992), referred to as eIF4G-1a (Bradley and others 2002). Subsequently, two collinear but longer cDNAs were isolated that diverged at the 5' end (Imataka and others 1998, Johannes and Sarnow 1998). These extended the open reading frame by an additional 156 codons to produce a theoretical polypeptide of 172 kDa, termed eIF4G-1e. Recently, an even longer form of 176 kDa, eIF4G-1f, was detected by mass spectrometric analysis and represents the major form, at least in human K562 cells (Bradley and others 2002). eIF4G-1e and eIF4G-1f contain the binding site for PABP but eIF4G-1a does not (Fig. 1-2).

2. Structure of eIF4G

Human eIF4G-1 can be divided into three distinct domains that have similar sizes. The amino-terminal one third (residues 1-683) binds PABP and eIF4E and is required for cap-dependent translation (Lamphear and others 1995; Mader and others 1995; Imataka and others 1998). The picornaviral proteases 2A and L cleave the amino-terminal third

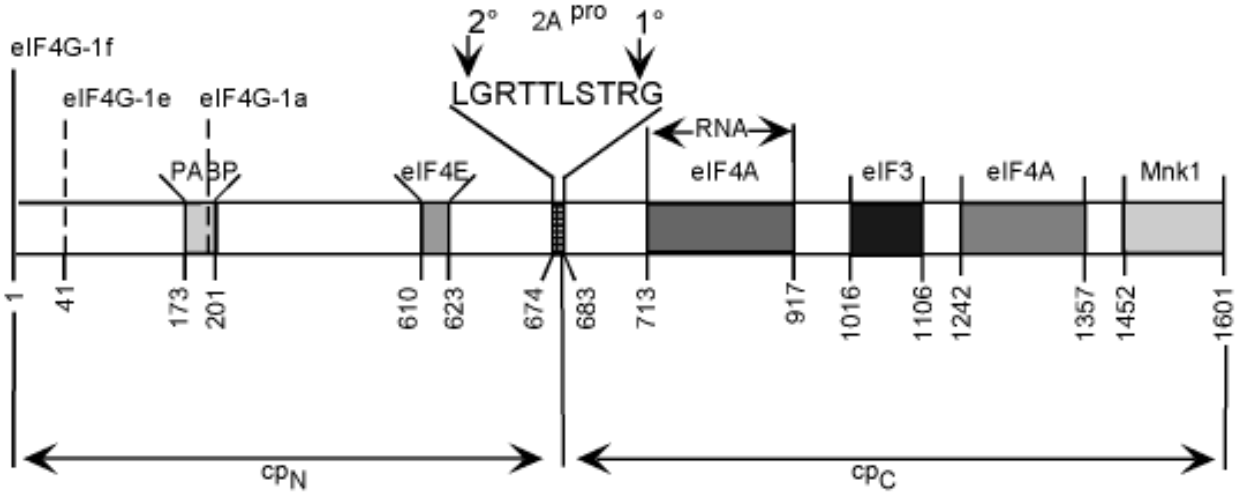

2°
2A pro
1°
eIF4G-1f
LGRTTLSTRG
eIF4G-1e
eIF4G-1a
RNA
PABP
eIF4E
eIF4A
eIF3
eIF4A
Mnk1
1
41
173
201
610
623
674
683
713
917
1016
1106
1242
1357
1452
1601
cpN
cpC

Fig. 1-2. Schematic diagram of the primary structure of human eIF4G-1.

eIF4G-1a, eIF4G-1e, and eIF4G-1f are the 154-kDa (Yan and others 1992), 172-kDa (Imataka and others 1998, Johannes and Sarnow 1998), and 176-kDa (Bradley and others 2002) forms. N-terminal (cp_N) and C-terminal (cp_C) cleavage products are formed during infection of cells with picornaviruses of the entero-, rhino- and aphthovirus genera. The primary (1°) and secondary (2°) cleavage sites of Coxsackievirus serotype 4 (CVB4) 2A protease are shown. Binding sites for other proteins are indicated by shaded boxes.

from the rest of the protein (Lamphear and others 1993), in effect separating the eIF4E-binding site from downstream domains. The central domain (residues 683-1106) associates with eIF3 and eIF4A (Lamphear and others 1995; Imataka and Sonenberg 1997; Korneeva and others 2000) and possesses an RNA-binding site (Pestova and others 1996b). The carboxy-terminal one third contains a second eIF4A-binding site (Lamphear and others 1995) and also binds the eIF4E kinase Mnk1 (Pyronnet and others 1999). The minimal protein sequence of eIF4G-1 required for cap-dependent translation has been mapped to residues 550-1090 (Morino and others 2000), which includes the eIF4E-binding site and the central domain.

3. Function of eIF4G

A constant feature of eIF4G from all sources is its presence in subassemblies of the initiation machinery, ranging in complexity from the ~1 MDa 48S initiation complexes to the simple eIF4G•eIF4E complex. The biochemical basis for this is now apparent: numerous binding sites characterize eIF4G as a linking protein and suggest that its role in initiation is to co-localize mRNA recruitment factors (Keiper and others 1999). It serves as the coordinator to regulate the translation of various mRNAs through binding other translation initiation factors.

a. The level of eIF4G is regulated

eIF4G mRNA is translated by an unusual mechanism involving internal entry of ribosomes rather than the scanning mechanism (Johannes and others 1999). The IRES found in the 5'-UTR of the mRNA for eIF4G suggests that the translation of eIF4G can be by internal initiation (Johannes and Sarnow 1998). This would permit an autoregulatory mechanism allowing continued synthesis of eIF4G in cells infected by

picornaviruses. Internal initiation is also a potential mechanism for up-regulating eIF4G expression in cells undergoing, or recovering from, other stresses that impair cap-dependent initiation (Gan and Rhoads 1996).

eIF4G is sequestered by the chaperone hsp27 to an insoluble complex, and dissociation of eIF4F is enhanced during heat shock (Cuesta and others 2000a). Down-regulation of eIF4G protein levels, observed upon expression of antisense RNA against eIF4E mRNA, suggests that eIF4E and eIF4G are coordinately regulated (De Benedetti and others 1991). The kinetics for loss of eIF4G and the decline of the overall translation rate parallel closely those of eIF4E loss, consistent with the possibility that eIF4G may be destabilized when unable to form a complex with eIF4E. This phenomenon is sometimes observed for proteins that normally exist in higher-order structures (Rechsteiner and Rogers 1996).

The N-terminal half of mammalian eIF4G has five high-scoring PEST regions (Yan and others 1992), possession of which shows high correlation both with a short physiological half-life and with regulated stability (Rogers and others 1986; Rechsteiner and Rogers 1996). In human eIF4G, PESTs are largely, but not exclusively, located toward the N-terminal end of the protein, in regions of secondary structure predicted to be rich in loops (Morley and others 1997). This suggests that the C-terminal fragment would be more stable metabolically than either the N-terminal fragment or the intact protein. This has in fact been observed when eIF4G is cleaved in *Xenopus* oocytes by microinjection of Coxsackievirus serotype B4 (CVB4) 2A protease; the C-terminal eIF4G fragment exhibits greater stability than the N-terminal fragment (Keiper and Rhoads 1997). The cleavage of eIF4G by calcium-dependent protease and in response to

hemin treatment differs from that by picornavirus infection (Wyckoff and others 1990; Wyckoff and others 1992; Clemens and others 2000), suggesting that the mechanisms of degradation of eIF4G in uninfected cells differ from those seen in cells infected by picornaviruses.

Whether cellular proteolytic mechanisms are responsible for the cleavage of eIF4G in cells infected with picornaviruses is not clear. Early studies suggested that, in infected cells, eIF4G is not cleaved directly by the viral 2A protease but rather by a cellular enzyme activated indirectly, possibly *via* a protease cascade (Lloyd and others 1986; Kräusslich and others 1987). Later studies showed that eIF4G could be cleaved by picornaviral proteases directly in purified eIF4F preparation (Liebig and others 1993; Lamphear and others 1993; Kirchweger and others 1994; Lamphear and others 1995) or in a complex of recombinant eIF4G and eIF4E proteins *in vitro* (Haghighat and others 1996). eIF4G alone is a poorer substrate for cleavage by picornavirus protease than when complexed with eIF4E (Haghighat and others 1996; Ohlmann and others 1997), suggesting that a conformational change of eIF4G upon its interaction with eIF4E is required to expose the cleavage site. However, the fact that cleavage of eIF4G by 2A protease or L protease occurs *in vitro* does not rule out the possibility that cellular proteases also play a role during virus infection. Purification of the eIF4G-cleavage activity from poliovirus-infected cells was attempted, but the cleavage activity did not co-purify with the 2A protease (Bovee and others 1998).

b. Changing eIF4G levels affects cell physiology

Amplification of the gene encoding eIF4G was observed in independent squamous cell lung carcinomas (Brass 1997). Over-expression of eIF4G in NIH3T3 cells

leads to malignant transformation (Fukuschi-Shimogori and others 1997). These data suggest that over-expression of eIF4G may have a central role in cell transformation and loss of growth control, just as described for eIF4E. The possibility that eIF4G concentrations affect cell physiology is further supported by the observation that it is present in *S. cerevisiae* at an extremely low concentration (Altmann and others 1997). This is also consistent with the fact that most of the eIF4G in reticulocyte lysate is ribosome-associated (Rau and others 1996).

A homologue of eIF4G, p97 (also known as DAP-5 and NAT1) exhibits similarity to the central and C-terminal domains of mammalian eIF4G and contains binding sites for eIF3 and eIF4A (Imataka and others 1997; Levy-Strumpf and others 1997; Shaughnessy and others 1997; Yamanaka and others 1997). Interestingly, both NAT1 (Yamanaka and others 1997) and p97 (Imataka and others 1997) were found to inhibit both cap-dependent and cap-independent translation *in vitro* and *in vivo*. This is in contrast to the C-terminal two-thirds of mammalian eIF4G, which stimulates translation of uncapped mRNA *in vitro* (Ohlmann and others 1996) and EMCV IRES-driven translation *in vivo* (Borman and others 1997; Yamanaka and others 1997). Transgenic over-expression of the apolipoprotein B mRNA-editing enzyme (APOBEC-1) causes carcinomas in mouse liver, which is correlated with a decrease of p97 protein levels caused by introducing stop codons into the coding region of p97 mRNA during the mRNA-editing process (Yamanaka and others 1997). This suggests that the reduction of this eIF4G competitor may lead to the transformation of mammalian cells by allowing more eIF4G to participate in translation.

c. Phosphorylation of eIF4G

Little is known about the role of phosphorylation in modulating the activity of eIF4G. In some studies, enhanced phosphorylation has been correlated with increased rates of translation. Increased phosphorylation of eIF4G was found in reticulocytes treated with phorbol esters (Morley and Traugh 1989), in 3T3-L1 cells treated with insulin (Morley and Traugh 1990), in epithelial cells treated with okadaic acid or epidermal growth factor (Donaldson and others 1991; Bu and others 1993), in human cells following influenza infection (Feigenblum and Schneider 1993), in T lymphocytes treated with phorbol ester or concanavalin A (Morley and Pain 1995b), and in *Xenopus* oocytes during meiotic maturation (Morley and Pain 1995a). The phosphorylation state of specific eIF4G residues is altered by serum and mitogens, and the serum-stimulated phosphorylation sites are located at serines 1108, 1148 and 1192 (Raught and others 2000). In many of these cases, phosphorylation of eIF4G is also associated with enhancement of its interaction with eIF4E, reflecting increased eIF4F complex formation and translation initiation. Under these conditions, eIF4G, eIF4E, and the 4E-BPs are all phosphorylated. It is therefore difficult to know the relative contributions of each phosphorylation to the increase in protein synthesis. Conversely, after heat shock, the dephosphorylation of eIF4E and eIF4G is correlated with the inhibition of protein synthesis (Lamphear and Panniers 1991). The inhibition can be relieved by the addition of the eIF4F complex (Lamphear and Panniers 1990).

d. The interaction of eIF4G with the 5' and 3' ends of mRNA simultaneously and synergistically increases translational efficiency

The interaction between PABP and eIF4G was first discovered in yeast (Tarun and Sachs 1996). Subsequently, PABP was found to associate with iso-eIF4G and eIF4B in wheat germ (Le and others 1997; Wei and others 1998). Identification of longer isoforms of eIF4G with PABP binding sites (Johannes and Sarnow 1998; Imataka and others 1998; Bradley and others 2002) suggests that this interaction also exists in mammalian cells. The ability of eIF4G to bind eIF4E and PABP simultaneously allows for circularization of the mRNA. Formation of circular mRNA with the minimal components m^7G-mRNA•eIF4E•eIF4G•PABP•poly(A) has been demonstrated and directly visualized by atomic force microscopy (Wells and others 1998). The observation of circular polyribosomal mRNAs provides *in vivo* evidence that this conformation is translationally active (Christensen and Bourne 1999). A circularized mRNA may allow ribosomes to rapidly reload following termination to reinitiate the next round translation.

Another possible mechanism for synergy between the cap and poly(A) tract is that eIF4G binding to eIF4E and PABP increases their affinity for mRNA. The association of eIF4G with eIF4E increases affinity of the latter for the mRNA cap structure (Haghighat and Sonenberg 1997; Ptushkina and others 1998; Ptushkina and others 1999). Similarly, the interaction of wheat germ Pab1p with eIF4F increases the affinity of eIF4F for cap analogs (Wei and others 1998) and the affinity of PABP for poly(A) (Le and others 1997).

Another mechanism to explain this synergy is that the binding of eIF4E or PABP to eIF4G alters eIF4G conformation so as to allow it to bind more efficiently to other

proteins in the translational initiation complex. Evidence for conformational transitions within eIF4G upon binding to eIF4E has been reported (Hershey and others 1999).

e. eIF4G localizes Mnk to regulate the phosphorylation of eIF4E

There exist phosphorylated and nonphosphorylated forms of eIF4E (Rychlik and others 1986). The ratio of these two forms varies in different cells and depends on the protein synthesis rate. The phosphorylation state correlates positively with the rate of translation. *In vivo* phosphorylation of eIF4E is carried out by the protein kinase Mnk1 (Fukunaga and Hunter 1997; Waskiewicz and others 1997), which is associated with the eIF4F complex *via* its interaction with the C-terminal region of eIF4G. Moreover, the phosphorylation of an eIF4E variant lacking eIF4G-binding capability is severely impaired in cells, suggesting that eIF4G provides a docking site for Mnk1 to phosphorylate eIF4E. Mnk1 also interacts with the C-terminal region of the translational inhibitor p97, which does not bind eIF4E, raising the possibility that p97 can block phosphorylation of eIF4E by sequestering Mnk1 and thereby inhibit translation (Pyronnet and others 1999; Waskiewicz and others 1999). The block in phosphorylation of eIF4E caused by adenovirus infection does not involve sequestration or removal of eIF4E from eIF4F by the eIF4E-binding proteins, 4E-BP1 or BP2. In fact, the adenovirus early E1A gene product induces phosphorylation of the 4E-BPs during the early stages of infection, which blocks their binding to eIF4E (Feigenblum and Schneider 1996; Gingras and Sonenberg 1997). Instead, the p100 protein of adenovirus specifically displaces Mnk1 from eIF4F complexes during late infection, concomitant with inhibition of eIF4E phosphorylation and shutoff of host cell mRNA translation (Cuesta and others 2000b; Cuesta and others 2001).

f. eIF4G may be involved in regulation of mRNA stability

Mutations in the genes encoding translation initiation factors were found to increase rates of deadenylation and decapping of mRNAs in *Saccharomyces cerevisiae*, suggesting that some *cis*-acting sequences modulate mRNA decay rates by affecting the translation status of the transcript (Schwartz and Parker 1999). eIF4G was also found to be present in a complex containing heat shock proteins hsc70 and hsp70, PABP, and the AU-rich binding protein AUF1, which mediates rapid degradation of cytokine and proto-oncogene mRNAs through AU-rich elements in the 3'-UTR (Laroia and others 1999).

C. Translation Initiation and Picornaviral Strategies

1. Picornaviruses

a. General information about picornaviruses

The animal picornavirus family comprises many different species that cause a wide range of diseases, *e.g.,* acute paralysis (poliovirus and enterovirus-71), hemorrhagic conjunctivitis (enterovirus-70), viral myocarditis (Coxsackie B viruses), foot-and-mouth disease, hepatitis, and common colds (Semler and Ehrenfeld 1989). The picornaviridae are nonenveloped viruses with a single-stranded RNA genome of positive polarity. The picornaviridae family consists of six genera: aphthovirus, cardiovirus, enterovirus, hepatovirus, parechovirus, and rhinovirus. The enterovirus genus includes poliovirus (3 serotypes), Coxsackievirus (23 serotypes), echovirus (28 serotypes), human enterovirus (4 serotypes), and many nonhuman enteric viruses.

b. The picornaviral genome

The picornavirus genome contains about 8000 nucleotides, with a single long open-reading frame coding for a polyprotein. This polyprotein includes either one or two protease domains that cleave the polyprotein into the various structural and non-structural proteins. The genome of picornaviruses is covalently linked at the 5′ end to a genome-linked viral protein (VPg) by an O4-(5′-uridylyl)-tyrosine linkage (Flanegan and others 1977; Lee and others 1977). The genome ends with a 3' poly(A) tract, which is encoded rather than being added posttranscriptionally.

c. The 5' internal ribosomal entry sites of picornaviruses

The 5′-UTRs of picornaviral RNAs are unusually long (624 to 1,199 nucleotides) and highly structured (Fig. 1-3). This region of the genome contains sequences that control replication and translation, and the RNA is predicted to fold into complex secondary structures (Agol 1991). The 5'-UTR have numerous AUG triplets that do not appear to be used as translation initiation sites, despite the fact that some of them have a local sequence context that would be very favorable to initiation according to the rules of the scanning ribosome model. A more remarkable feature about these upstream AUG codons is that most of them are not conserved, even between different isolates of the same virus (Pöyry and others 1992). These characteristics together with the fact that the RNA is not capped imply that the RNAs cannot possibly be translated efficiently by the conventional scanning mechanism.

Initiation by direct internal ribosome entry was demonstrated by experiments in which insertion of a picornavirus 5'-UTR led to dramatic enhancement of expression of the downstream cistron in a transfected bicistronic plasmid (Jang and others 1988;

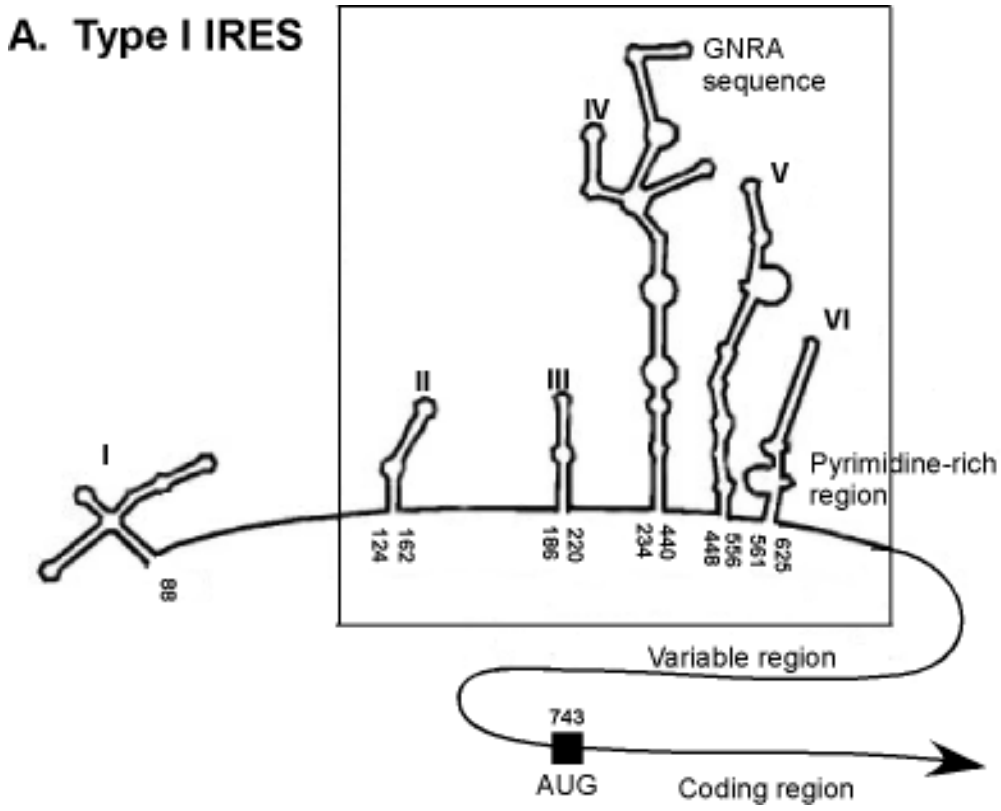
Pelletier and Sonenberg 1988). The minimal IRES element of several picornavirus has been mapped. In all cases it is about 450 nucleotides long, but the surprising result is that there is no single consensus IRES sequence common to all picornaviruses. Based on IRES sequence conservation and, more importantly, RNA secondary structure, two major classes (Fig. 1-3) and one minor class of IRES elements have been defined. The IRESes of enteroviruses and rhinoviruses belong to type I (Fig. 1-3, top panel), whereas the IRESes of cardioviruses and aphthoviruses belong to type II (Fig. 1-3, bottom panel). The IRES of hepatoviruses differs from type I and type II IRESes and constitutes a third class, type III (Stewart and Semler 1997).

d. The picornaviral polyprotein and its cleavage

The polyprotein encoded by the picornavirus genome is divided into three regions: P1, P2, and P3 (Fig. 1-4). Aphthoviruses and cardioviruses encode a leader (L) protein before the P1 region. The P1 region encodes the viral capsid proteins, whereas the P2 and P3 regions encode proteins involved in protein processing ($2A^{pro}$, $3C^{pro}$, $3CD^{pro}$) and genome replication (2B, 2C, 3AB, $3B^{VPg}$, $3CD^{pro}$, $3D^{pol}$).

e. 2A protease

2A protease of enteroviruses and rhinoviruses cleaves at the P1-P2 junction of the viral polyprotein. 2A protease also cleaves host cellular proteins, such as eIF4G, PABP, and dystrophin (Lamphear and others 1993; Zhao and others 2003; Kerekatte and others 1999; Joachims and others 1999; Badorff and others 2000). 2A protease encoded by enterovirus and rhinovirus RNA cleaves eIF4G-1 initially between Arg-682 and Gly-683 (Lamphear and others 1993) and, secondarily, between Leu-674 and Gly-675


A. Type I IRES
GNRA
sequence
IV
V
VI
II
III
I
Pyrimidine-rich
region
124
162
186
220
234
440
448
556
561
625
89
Variable region
743
AUG
Coding region

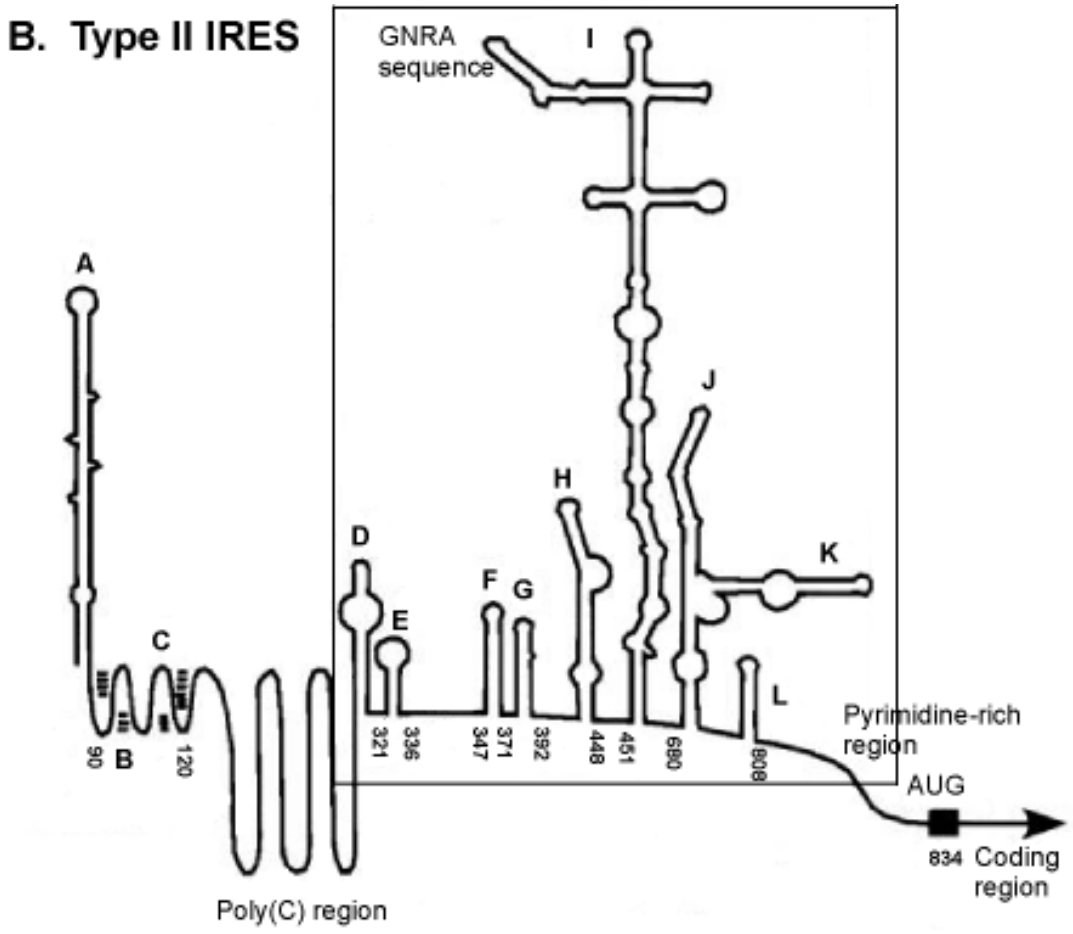

B. Type II IRES
GNRA
sequence
I
A
J
H
D
K
F
G
E
C
L
Pyrimidine-rich
region
AUG
90
B
120
321
336
347
371
392
448
451
680
808
834
Coding
region
Poly(C) region

Fig. 1-3. Two major classes of picornavirus internal ribosome entry sites. **(A).** The 5′-UTR of poliovirus (type I IRES). (**B).** The same region from EMCV (type II IRES). The IRES is indicated by a box. Predicted secondary and tertiary structures (RNA pseudoknots) are labeled. The GNRA loop and pyrimidine-rich region are features that are conserved in both the type I and type II IRESes. (Figure reproduced from Racaniello 2001)

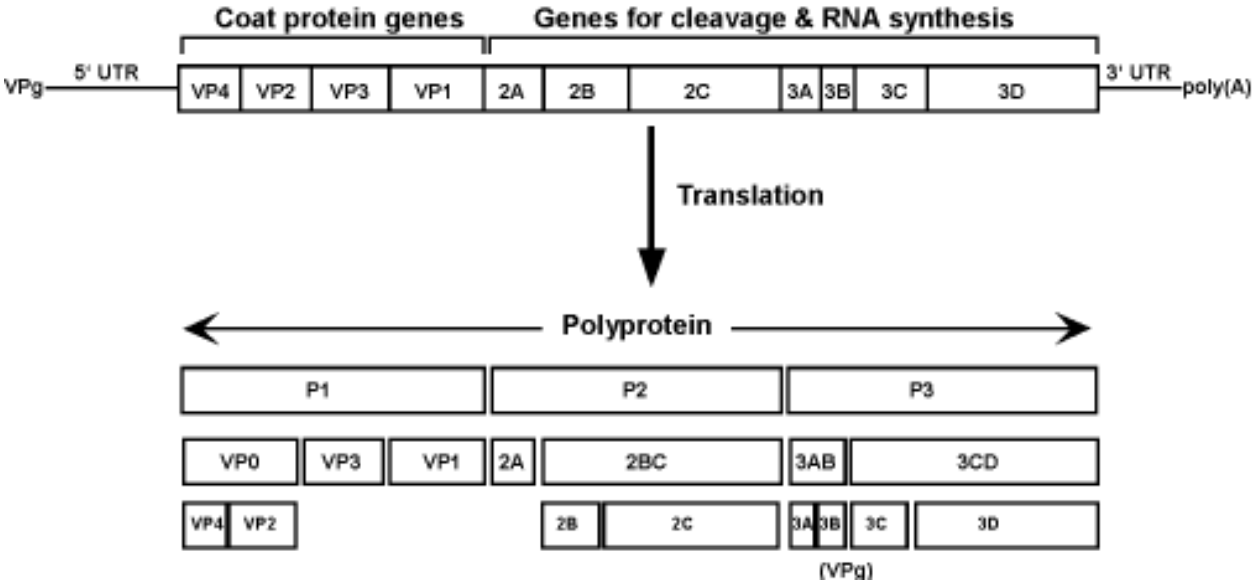

Coat protein genes
Genes for cleavage & RNA synthesis
VPg
5' UTR
VP4
VP2
VP3
VP1
2A
2B
2C
3A
3B
3C
3D
3' UTR
poly(A)
Translation
Polyprotein
P1
P2
P3
VP0
VP3
VP1
2A
2BC
3AB
3CD
VP4
VP2
2B
2C
3A
3B
3C
3D
(VPg)

Fig. 1-4. Organization of the picornaviral genome. Top: Diagram of the viral RNA genome, with the genome-linked protein (VPg) at the 5′-end, the 5′-UTR, the protein-coding region, the 3′-UTR, and the poly(A) tract. Coding regions for the viral proteins are indicated. **Bottom:** Processing pattern of the picornavirus polyprotein. The coding region has been divided into three regions, P1, P2, and P3, which are cleaved into functional proteins by two viral proteases, 2A and 3C. Intermediate and final cleavage products are indicated. In cells infected with enteroviruses and rhinoviruses, nascent P1 is cleaved from P2 by 2A. The remaining cleavages are carried out by 3C and its precursor, 3CD. (Figure reproduced from Racaniello 2001)

(Zhao and others 2003, and Chapter 2). L protease produced by aphthoviruses cleaves eIF4G-1 between Gly-675 and Arg-676 (Kirchweger and others 1994).

2. Picornaviral strategies to shut off host protein synthesis

The viral 2A protease of enterovirus and rhinovirus cleaves eIF4G-1 (Lamphear and others 1993; Zhao and others 2003), which results the separation of the N-terminus containing the eIF4E-binding site from the C-terminus containing the eIF3, eIF4A, and RNA binding sites (Lamphear and others 1995). The C-terminus is sufficient to support internal initiation. Picornaviral RNA, as discussed above, has an IRES element at its 5'-UTR and is able to bind the central domain of eIF4G directly (Pestova and others 1996a; Kolupaeva and others 1998). On the other hand, cap-dependent translation is shut off due to the failure of the C-terminal fragment to recruit mRNA through eIF4E and PABP. When cells are infected with these viruses, eIF4G is cleaved. The current model is that the cleavage of eIF4G causes a switch in the predominant mode from cap-dependent initiation to internal initiation. Capped mRNA translation is inhibited, whereas internal initiation and initiation of uncapped mRNAs are either unaffected or even stimulated (Liebig and others 1993, Ohlmann and others 1995, Borman and others 1997). This results in host protein synthesis shutoff and allows viral protein synthesis to continue.

D. Statement of the Problem

Cleavage of eIF4G-1 by 2A protease was initially thought to be the major mechanism utilized by entero-, rhino-, and aphothoviruses to shut down host protein synthesis and allow viral protein synthesis to continue (Ehrenfeld 1996). Subsequently, evidence was presented (reviewed in Chapter 2) that eIF4G-1 cleavage is not responsible,

or is only partially responsible, for the shutoff of host protein synthesis. Creating a 2A protease-resistant form eIF4G-1 could help us to study the mechanism of host protein synthesis shutoff and the relative importance of eIF4G integrity upon picornavirus infection.

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CHAPTER 2

CREATION OF EIF4G-1A^{DM} AND EIF4G-1E^{DM}: RESISTANCE TO CLEAVAGE BY 2A PROTEASE AND FUNCTION *IN VITRO* AND *IN VIVO*

A. Introduction

Cleavage of eIF4G-1 by 2A protease was initially thought to be the major mechanism utilized by entero- and rhinoviruses to shut down host protein synthesis and allow viral protein synthesis to continue by IRES-driven translation (Ehrenfeld 1996). Subsequently, evidence was presented that eIF4G-1 cleavage is not responsible, or is only partially responsible, for the shutoff of host protein synthesis. When cells are infected with poliovirus in the presence of a viral replication inhibitor, eIF4G-1 is completely degraded but host protein synthesis is only partially shut off (Bonneau and Sonenberg 1987; Perez and Carrasco 1992). Expression of poliovirus 2A protease in COS-1 cells has a much greater inhibitory effect on transcription by RNA polymerase II than on translation, suggesting that both processes may be involved in the host shutoff (Davies and others 1991). *In vivo* expression of 2A protease alone activates viral mRNA translation, independent of its role in the host shutoff (Hambidge and Sarnow 1992; Macadam and others 1994). Complete cleavage of eIF4G-1 in *Xenopus* oocytes (Keiper and Rhoads 1997) or HeLa cells (Novoa and Carrasco 1999) by introduction of exogenous 2A protease results in only a modest reduction of protein synthesis. Cleavage of eIF4G-2 appears to correlate better with host shutoff than that of eIF4G-1 (Gradi and others 1998). Finally, PABP is also cleaved during Coxsackievirus (Kerekatte and others 1999) and poliovirus (Joachims and others 1999) infection; partial cleavage coincides with the most rapid period of viral protein synthesis while complete cleavage coincides with the cessation of viral protein synthesis (Kerekatte and others 1999). Thus, it is possible that events accompanying picornavirus infection *other* than eIF4G-1 cleavage are responsible, wholly or partially, for the host shutoff.

Creating an eIF4G-1 variant that is resistant to 2A protease would provide a useful tool to study the mechanism of host protein synthesis shutoff upon viral infection. Previously, our laboratory determined the cleavage site in eIF4G-1 for human rhinovirus and CVB4 2A protease (Lamphear and others 1993) and developed a variant form in which the Gly on the C-terminal side of the scissile bond (position P1') was changed to Glu (Lamphear and Rhoads 1996). Even though the variant (eIF4G-1a^{SM}) was 100-fold more resistant to cleavage than eIF4G-1a^{WT}, it was still digested, at sufficiently high protease concentrations and extended time periods, into fragments of apparently the same size as those derived from eIF4G-1a^{WT}. This raised the question whether cleavage occurred at the same or a different site. In this chapter, we mapped the CVB4 2A protease-cleavage site in eIF4G-1^{SM} and found that it is different from the site in eIF4G-1^{WT}. This information was used to create a second variant in which both cleavage sites were altered, eIF4G-1^{DM}. This variant is 1,000- to 10,000-fold more resistant than eIF4G-1^{WT} to CVB4 2A protease *in vitro* and highly resistant to cleavage upon Coxsackievirus B serotype 3 (CVB3) infection. The functional activity of eIF4G-1^{DM} was verified *in vitro*.

B. Experimental Procedures

1. Materials

A C4 column for reverse phase purification of cp_C was obtained from Vydac (Hesperia, CA). HPLC-grade acetonitrile and trifluoroacetic acid were obtained from Fisher Scientific (Fairlawn, NJ) and Pierce Chemical Co. (Rockford, IL), respectively. Rabbit reticulocytes lysate (RRL) was prepared from New Zealand white rabbits (Chu and Rhoads 1980). Globin mRNA was prepared from high-salt washed ribosomal pellets from rabbit reticulocytes. The vectors pET29A and pCITE2a as well as S-Protein Agarose were from Novagen (Madison, WI). Benzamidine, Pepstatin, Pefabloc and Expand^{TM} High Fidelity PCR System polymerase were purchased from Boehringer Mannheim (Indianapolis, IN). T7 RNA polymerase, T4 polynucleotide kinase, all restriction enzymes unless otherwise specified, RQ DNase, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitro blue tetrazolium chloride (NBT) were from Promega (Madison, WI). [^{35}S]Met was obtained from ICN Biochemicals (Irvine, CA). Oligonucleotides used for site-directed mutagenesis and sequencing were acquired from Gibco BRL (Gaithersburg, MD). [5'-^{32}P]pCp was obtained from ICN Biochemicals (Irvine, CA). The T7-1 primer (5'-AATACGACTCACTATAG-3') was provided by the DNA sequencing facility at Iowa State University. Anti-T7-Tag antibody was purchased from Novagen (Madison, WI). The production of an antibody against eIF4G-1 was described previously (Yan and others 1992). Anti-PABP antibodies were a gift from Dan Schoenberg (The Ohio State University, Columbus, OH). An antibody against the N-terminus of eIF4G-1e, termed anti-NT, was prepared against the peptide CRAQPPSSAASR and generously donated by Chris Bradley, LSUHSC-S. This peptide,

consisting of an N-terminal Cys residue and aa 56-66 of eIF4G-1, was synthesized by BioSynthesis, Inc. (Lewisville, TX). The peptide was coupled to keyhole limpet hemocyanin (Sambrook and others 1989) and used to raise antibodies in rabbits (500 μg/immunization, bimonthly for eight months). Antibodies were purified by Chris Bradley on columns of Affi-Gel 501 mercury Agarose (Bio-Rad, Hercules, CA) to which the synthetic peptide was linked via the Cys residue. For western analysis of recombinant eIF4G-1e^{DM} and endogenous rabbit eIF4G-1, affinity-purified anti-NT antibodies were used at 1:50 for 12-16 h at 4°C followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution, Vector Laboratories, Burlingame, CA) for 1 h at 23°C.

2. Construction of recombinant plasmids

The plasmid pSΔN1 and pSΔN1E were constructed by inserting the *Sma*I/*Xho*I fragments from pAD4G^{WT} and pAD4G^{G486E} (Lamphear and Rhoads 1996), respectively, into pET29A between the *Eco*RV and *Xho*I sites. This resulted in plasmids coding for a portion (aa 590-1601) of eIF4G-1 containing either the wild type Gly [S-eIF4G-1(590-1601)^{WT}] or the variant Glu [S-eIF4G-1(590-1601)^{SM}] at aa 683, each protein also containing an N-terminal tag consisting of the S-peptide of RNase A.

Construction of the plasmid pCITE4G-1a^{DM}, which encodes eIF4G-1a with a second aa substitution (G675A), was achieved by site-directed mutagenesis of pCITE4G^{G486E} (Lamphear and Rhoads 1996). A mutated PCR product from nt 1221 to 2033 was synthesized from pCITE4G^{G486E} using the sense primer 5'-AGCCCCCTCGCCACCAG-3' (nt 1221-1237) and the antisense primer 5'-GTTGTGCGCGCAAGGTTGGCAAAGGATGGAG-3' (nt 2003-2033). A second

mutated PCR product from nt 2016 to 3052 was produced from pCITE4G^{G486E} using the sense primer 5'-CAACCTTGCGCGCACAACCCTTAGCACCCG-3' (nt 2016-2045) and the antisense primer 5'-CACGTCCTGCAGCATAAAGCGG-3' (nt 2931-2952). PCR reaction mixtures (100 µl) were composed of 100 ng of template DNA, 1 µM primers, 200 µM dNTP, 1 µl Expand^{TM} High Fidelity PCR system polymerase, and 10 µl of 10X buffer + Mg^{2+} supplied by Boehringer Mannheim. DNA was initially denatured at 95°C (5 min), annealed at 61°C (30 s), and extended at 72°C (30 s). The reactions were then cycled an additional 20 times at 95°C (1 min), 61°C (30 s), and 72°C (30 s), with a final extension at 72°C (5 min). Two DNA fragments, one derived from the PCR product of nt 1221-2033 by cutting with *Bam*HI and *Bss*HII, and the other from the PCR product of 2016-3052 by cutting with *Bss*HII and *Nsi*I, were ligated into pCITE4G^{G486E} that had been digested with *Bam*HI and *Nsi*I. *E. coli* was transformed with the ligation product. The mutagenesis not only changed aa 675 from Gly to Ala but also created a restriction site for *Bss*HII at nt 2023-2028. Therefore, plasmids from colonies were initially screened by restriction digestion with *Bss*HII, and the presence of the mutation was confirmed by DNA sequencing. A plasmid was chosen in which no other mutations occurred.

The plasmid pAD4G-1a^{DM} was constructed by cutting pCITE4G-1a^{DM} with *Eco*RV and *Not*I and ligating the 4.7-kbp fragment into pAD4G^{WT} (Lamphear and Rhoads 1996) digested with the same enzymes.

A plasmid expressing eIF4G-1e^{DM}, termed pCITE4G-1e^{DM}, was constructed by inserting the DNA sequence that encodes the 156-aa N-terminal extension into pCITE4G-1a^{DM}. This was accomplished by digesting a plasmid containing this sequence

in pCR2.1 (Johannes and Sarnow 1998; kindly donated by Peter Sarnow) with *Xma*I and *Eco*RV, treating the reaction mixture with the Klenow fragment of DNA polymerase I (Promega), and then purifying the 501-bp fragment by gel electrophoresis. The fragment was ligated into pCITE4G-1a^{DM} that had been digested with *Eco*RV. Plasmids from colonies were first screened by restriction digestion and then confirmed by DNA sequencing across the ligated regions.

3. Expression and purification of the cp_C fragment

One-liter cultures of the *E. coli* expression strain BL21(DE3)pLysS bearing the plasmids pSΔN1 and pSΔN1E were grown in LB medium (Sambrook and others 1989) containing 25 μg/ml kanamycin and 34 μg/ml chloramphenicol to an OD_{595} of 0.3. Expression of S-eIF4G-1(590-1601)^{WT} or S-eIF4G-1(590-1601)^{SM} was induced with 1 mM isopropyl-1-thio-β-D-galactoside for 3 h at 30°C. Cells were cooled in an ice water bath, pelleted by centrifugation, and stored at -80°C. Cells were thawed in the presence of 30 ml Buffer A (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM dithiothreitol, 2 mM EDTA) containing 1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 1 mg/ml Leupeptin, 1 mg/ml Pepstatin, and 1 μg/ml Pefabloc. Lysis was completed by sonication (3-6 bursts of 10 sec each), and insoluble debris was removed by centrifugation at 30,000*g* for 30 min. The supernatant was combined with 2 ml of S-Protein Agarose equilibrated in Buffer A and incubated with rotation at 4°C for 2 h. The resin was washed with 80 volumes of Buffer A containing 0.1% Triton X-100. The protein, still bound to S-Protein Agarose, was subjected to digestion in 4 ml of Buffer A containing 50 μg/ml CVB4 2A protease and 0.1% Triton X-100 at 30°C for 2 h. This treatment released the cp_C fragments from the resin. The resin was allowed to settle, and

2 ml of the supernatant were removed and further fractionated by reverse-phase HPLC on a 4.5 × 15 cm C4 column equilibrated in Buffer B (0.1% aqueous trifluoroacetic acid) using a Waters Model 625 LC System. The column was developed with 5 ml of Buffer B followed by a 45-ml linear gradient of Buffer B to 80% Buffer C (0.1% trifluoroacetic acid in 95% acetonitrile). Peak fractions containing cp_C were subjected to automated Edman degradation using an Applied Biosystems Model 470A sequenator at the University of Kentucky Macromolecular Structure Analysis Facility.

4. Preparation of 2A protease

2A protease was prepared and generously donated by Aili Cai, LSUHSC-S. Plasmid pET8c/CVB4 2A, a gift from Dr. Tim Skern, University of Vienna, encodes a fusion protein consisting of 13 aa of the T7-tag, 34 aa of CVB4 VP1, and 150 aa of CVB4 2A protease. This plasmid was used to produce recombinant 2A protease in *E. coli* (Liebig, et al. 1993). The enzyme was purified and stored at 4°C at a concentration of 5 mg/ml in Buffer C (50 mM Tris-HCl, pH8.0, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 50% glycerol.

5. *In vitro* transcription

Various pCITE4G-1 plasmids were purified and linearized with *Xho*I and transcribed *in vitro* at 37°C for 120 min using T7 polymerase, rNTPs, 5X transcription buffer and RNasin ribonuclease inhibitor (Promega). The transcripts were purified by phenol/chloroform extraction and precipitated with ethanol.

6. *In vitro* translation

Rabbit reticulocyte lysate was depleted of endogenous mRNA by treatment with micrococcal nuclease (Pelham and Jackson 1976) and is referred to in this work as MDL. In some cases, the MDL was also made dependent on added eIF4G for cap-mediated translation by pretreating with recombinant CVB4 2A protease at 50 μg/ml for 30 min at 30°C.

A two-phase cell-free translation protocol was used to test the function of eIF4G-1^{DM}. During Phase I, *in vitro* translation was performed to synthesize the eIF4G-1 isoforms in the absence of radioactivity in CVB4 2A protease-pretreated MDL by internal initiation of translation. (The mRNA transcribed from pCITE vectors contains an EMCV IRES and is efficiently translated in 2A protease-treated MDL.) During Phase II, *in vitro* translation of globin mRNA was used to test whether the eIF4G-1 isoforms synthesized in Phase I were functional.

Reaction mixtures of 25 μl in Phase I contained 10 μl of either mock-treated or 2A protease-pretreated MDL and the following components: 150 mM potassium acetate, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 μM of each aa with the exception of Met, 2 μM Met, 50 mM HEPES (pH 8.0), and 20 μg/ml eIF4G-1 mRNA. The latter was transcribed from pCITE4G-1a^{DM} and pCITE4G-1e^{DM} plasmids linearized by *Xho*I as described previously (Joshi and others 1994). Reactions were carried out at 30°C for 1 h.

Phase II reaction mixtures (25 μl) contained 12.5 μl of Phase I reaction mixtures and 12.5 μl of fresh reaction mixtures consisting of 5 μl of either mock-treated or 2A protease-pretreated MDL, 160 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP,

15 mM creatine phosphate, 40 μM of each aa with the exception of Met, 1 mCi/ml [^{35}S]Met, 50 mM HEPES (pH 8.0), and 40 μg/ml globin mRNA. The Phase II reaction mixtures were incubated at 30°C for 30 min. Aliquots were then analyzed by SDS-PAGE followed by immunoblotting and autoradiography. eIF4G-1 synthesis was measured by immunoblotting, and globin synthesis, by incorporation of [^{35}S]Met into protein.

7. Electrophoresis

SDS-PAGE was performed for 90-120 min at 100 V using a Hoefer minigel electrophoresis system (Laemmli 1970). Proteins were visualized by western blotting or autoradiography if labeled with [^{35}S]Met and quantitated by densitometry. For western blotting analysis, unstained proteins were transferred to Immobilon-P Transfer membrane (PVDF) (Millipore Corporation, Bedford, MA) using a Bio-Rad Mini Trans-Blot cell.

C. Results

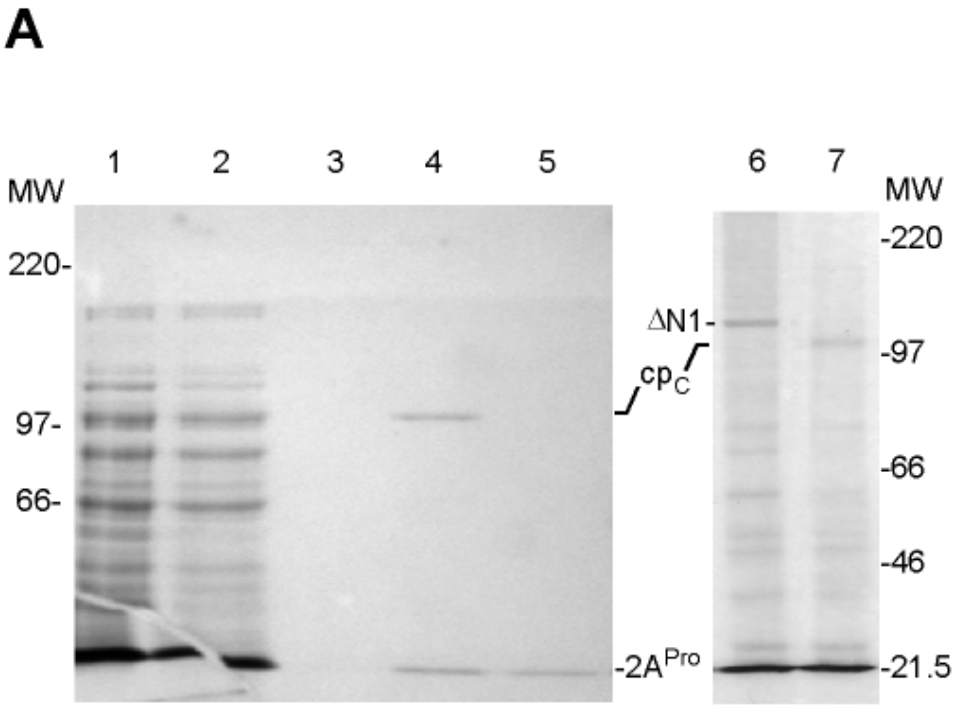
1. The cleavage site for CVB4 2A protease in eIF4G-1a^{SM} is different from the site previously identified in eIF4G-1a^{WT}

The fact that the eIF4G-1a^{SM} variant is cleaved at sufficiently high 2A protease concentrations (Lamphear and Rhoads 1996) suggests one of two possibilities: either the single aa substitution is insufficient to completely prevent cleavage at the original location, or the protease cleaves eIF4G-1 at an alternate site close to the primary site. To distinguish between these possibilities, we determined the cleavage site in eIF4G-1^{SM}, *i.e.*, the site that is cleaved when the primary site in eIF4G-1 is altered. We expressed in *E. coli* truncated versions of eIF4G-1^{WT} and eIF4G-1^{SM} containing the S-peptide of

RNase A fused at the N-terminus, designated S-eIF4G-1(590-1601)^{WT} and S-eIF4G-1(590-1601)^{SM}, respectively. This allowed immobilization of the products on S-Protein Agarose. Digestion of the bound proteins with CVB4 2A protease released soluble cp_C (Fig. 2-1, panel A, lane 4). The eluates were subjected to reverse phase HPLC to separate cp_C from CVB4 2A protease and other contaminating proteins (Fig. 2-1, panel B). cp_C from both S-eIF4G-1(590-1601)^{WT} (data not shown) and S-eIF4G-1(590-1601)^{SM} (panel B) eluted at 40 min, similar to the elution position of natural rabbit cp_C (Lamphear and others 1993). N-terminal sequencing of cp_C from S-eIF4G-1(590-1601)^{WT} confirmed the primary cleavage site between Arg-682 and Gly-683 (Lamphear and others 1993; Table 2-1). N-terminal analysis of cp_C for S-eIF4G-1(590-1601)^{SM} demonstrated that cleavage of S-eIF4G-1(590-1601)^{SM} occurred at a site eight aa residues upstream of the original site (Table 2-1). Cleavage at this alternate site was not due to the fact that the substrate was made in bacteria, since S-eIF4G-1(590-1601)^{WT}, was cleaved at the site previously determined in natural rabbit eIF4G-1 (Lamphear and others 1993). These results indicate that alteration of the primary 2A protease cleavage site in eIF4G-1 results in cleavage at a secondary site (labeled 2° in Fig. 1-2).

2. Treatment of eIF4G-1^{SM} and eIF4G-1^{DM} with CVB4 2A protease *in vitro*

This finding suggested that alteration of both primary and secondary cleavage sites might confer even greater resistance to CVB4 2A protease. We therefore performed site-directed mutagenesis to further modify eIF4G-1^{SM} (single mutant) and create a new variant, eIF4G-1^{DM} (double mutant), in which both of the P1' Gly residues are substituted (G675A and G683E). To test the effect of this on CVB4 2A protease susceptibility, we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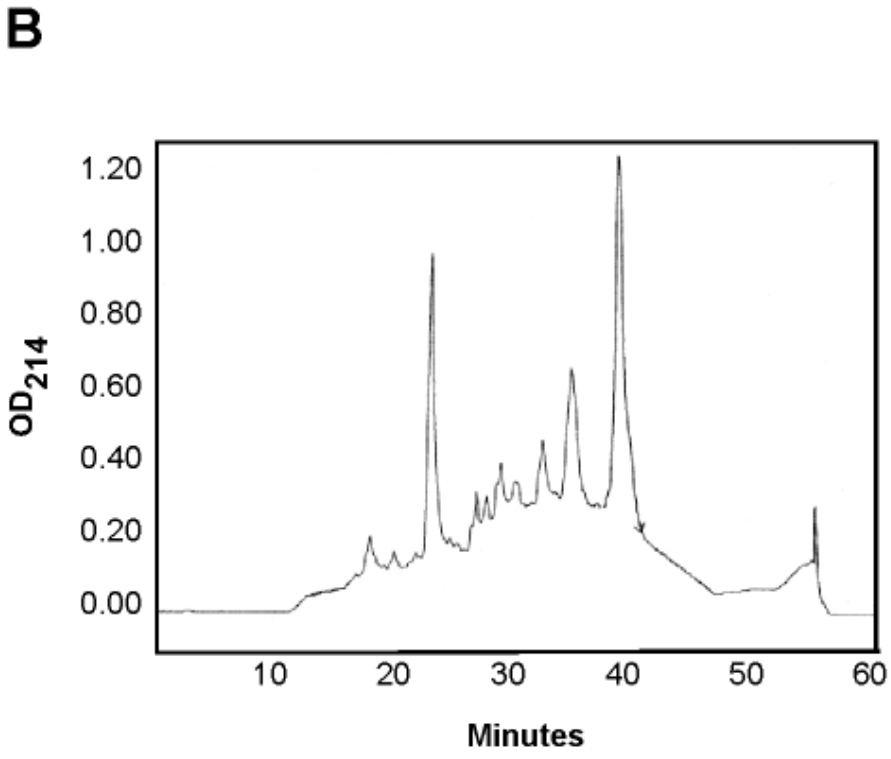
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Fig. 2-1. Purification of cp_C from eIF4G-1^{SM}. (A) The truncated, S-peptide-tagged eIF4G form S-eIF4G-1(590-1601)^{SM} was produced in *E. coli* and bound to S-Protein-agarose. The cp_C fragment was cleaved from the resin by CVB4 2A protease as described in Experimental Procedures, and fractions were analyzed by SDS-PAGE on a 7.5% gel (lanes 1 through 5). Lane 1, bacterial extract; lane 2, fraction unbound to S-Protein agarose; lane 3, last wash fraction; lane 4, eluate after CVB4 2A protease treatment; lane 5, CVB4 2A protease alone. In a separate experiment, S-Protein-agarose with bound S-eIF4G-1(590-1601)^{SM} was eluted with SDS-PAGE loading buffer and analyzed on an 8% gel before (lane 6) and after (lane 7) incubation with CVB4 2A protease. ΔN1 refers to uncleaved S-eIF4G-1(590-1601)^{SM}. (B) The eluate from panel A, lane 4, containing cp_C, CVB4 2A protease, and contaminating proteins, was fractionated by reverse phase HPLC on a C4 column. Proteins were monitored by absorbance at 214 nm. The peaks at 24 and 40 min were identified by SDS-PAGE as 2A protease and cp_C, respectively (data not shown).

Table 2-1. *In vitro* cleavage sites for CVB4 2A protease in human S-eIF4G-1(590-1601)^{WT} and S-eIF4G-1(590-1601)^{SM}

Source of sequence	Sequence[c]
Protein sequence predicted from eIF4G-1^{WT} cDNA[a]	^{675}GRTTLSTR↓GPPRGGPGGEL^{693}
Edman degradation of cp_C derived from S-eIF4G-1(590-1601)^{WT} by CVB4 2A protease digestion	XPXRGGPGXEL
Protein sequence predicted from eIF4G-1^{SM} cDNA[b]	^{671}FANL↓GRTTLSTR EPPRGGPGGEL^{693}
Edman degradation of cp_C derived from S-eIF4G-1(590-1601)^{SM} by CVB4 2A protease digestion	XRTTLSTR EPPRG

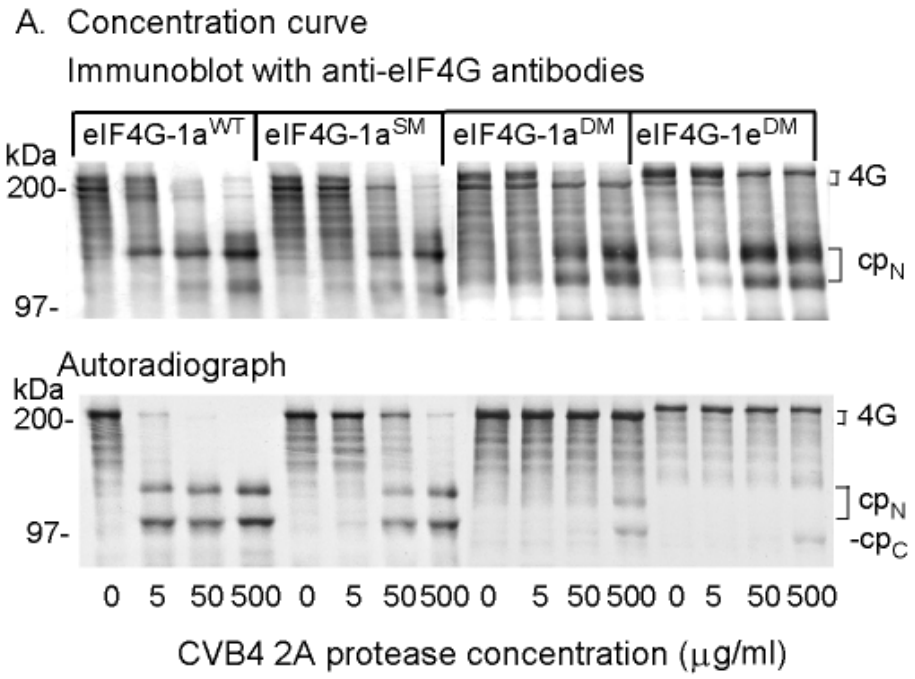
[a]Ref. Yan and others 1992.

[b]Ref. Lamphear and Rhoads 1996.

[c]X indicates that an unambiguous assignment could not be made. The gaps in some sequences are for alignment purposes only. Downward arrows indicate cleavage sites in the aa sequence that would explain the results of Edman sequencing.

synthesized various isoforms of eIF4G-1 in an MDL system programmed with mRNAs encoding eIF4G-1a^{WT}, eIF4G-1a^{SM}, eIF4G-1a^{DM}, and eIF4G-1e^{DM}, the latter of which has an extended open reading frame encoding 156 aa at the N-terminus and has a PABP-binding site (Fig. 1-2). The reaction mixtures were then incubated with increasing concentrations of CVB4 2A protease. Immunoblotting with anti-eIF4G-1 antibodies detected both endogenous rabbit and exogenous (*in vitro*-synthesized) human eIF4G-1 (Fig. 2-2, panel A, top portion), while autoradiography detected only the exogenous eIF4G-1 (panel A, lower portion). Newly synthesized eIF4G-1^{WT} was completely cleaved at 5 μg/ml of CVB4 2A protease, whereas endogenous eIF4G-1^{WT} was cleaved at 50 μg/ml. Most of the eIF4G-1^{SM} was cleaved at 500 μg/ml, as observed previously (Lamphear and Rhoads 1996). However, newly synthesized eIF4G-1a^{DM} and eIF4G-1e^{DM} were only slightly cleaved at 500 μg/ml. The degree of cleavage of eIF4G-1^{DM} isoforms at 500 μg/ml was intermediate between that of eIF4G-1a^{SM} at 5 μg/ml and 50 μg/ml. Thus, eIF4G-1^{DM} was 10- to 100-fold more resistant than eIF4G-1^{SM}.

We also tested the resistance of eIF4G-1a^{WT}, eIF4G-1a^{SM}, eIF4G-1a^{DM}, and eIF4G-1e^{DM} to 100 μg/ml CVB4 2A protease cleavage as a function of time (Fig. 2-2, panel B). Newly synthesized eIF4G-1^{WT} (lower portion) was completely cleaved by 5 min, while the endogenous eIF4G-1^{WT} (top portion) was cleaved somewhat more slowly. Most of the eIF4G-1^{SM} was converted to cp$_C$ by 60 min, but the eIF4G-1^{DM} variants were only slightly converted. The cleavage of eIF4G-1a^{DM} and eIF4G-1e^{DM} at 60 min was intermediate between that of eIF4G-1a^{SM} at 0 min and 5 min. By this criterion, eIF4G-1^{DM} was at least 12-fold more resistant than eIF4G-1^{SM}.


A. Concentration curve
Immunoblot with anti-eIF4G antibodies
eIF4G-1a^WT
eIF4G-1a^SM
eIF4G-1a^DM
eIF4G-1e^DM
kDa
200-
97-
4G
cp_N
Autoradiograph
kDa
200-
97-
4G
cp_N
-cp_C
0 5 50 500 0 5 50 500 0 5 50 500 0 5 50 500
CVB4 2A protease concentration (μg/ml)

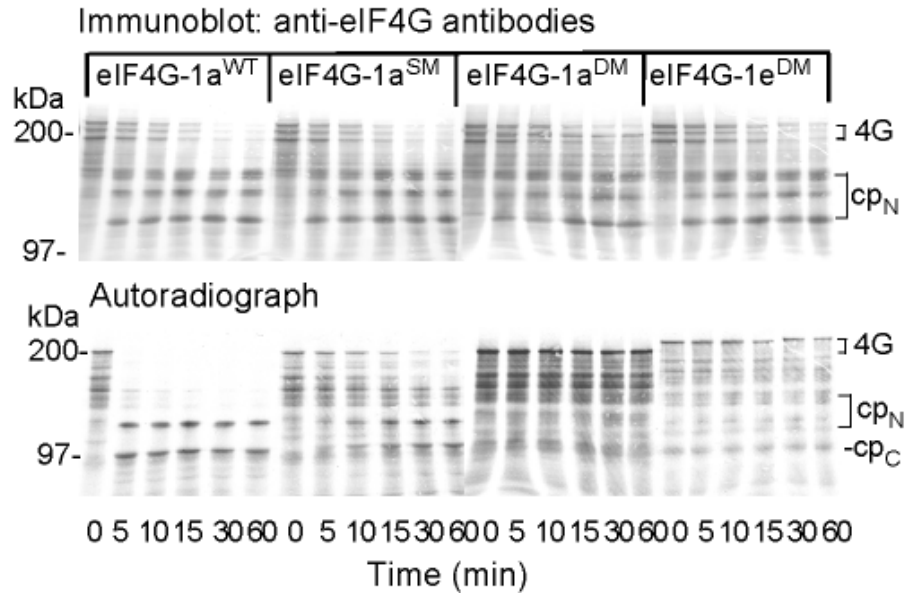

B. Time course
Immunoblot: anti-eIF4G antibodies
eIF4G-1a^WT
eIF4G-1a^SM
eIF4G-1a^DM
eIF4G-1e^DM
kDa
200-
97-
4G
cp_N
Autoradiograph
kDa
200-
97-
4G
cp_N
-cp_C
0 5 10 15 30 60 0 5 10 15 30 60 0 5 10 15 30 60 0 5 10 15 30 60
Time (min)

Fig. 2-2. Resistance of eIF4G-1^{SM} and eIF4G-1^{DM} to cleavage by CVB4 2A protease *in vitro*. (A) mRNAs encoding eIF4G-1a^{WT}, eIF4G-1a^{SM}, eIF4G-1a^{DM} and eIF4G-1e^{DM} were transcribed from pCITE4G^{WT} (Lamphear and Rhoads 1996), pCITE4G^{G486E} (Lamphear and Rhoads 1996), pCITE4G-1a^{DM} (Experimental Procedures), and pCITE4G-1e^{DM} (Experimental Procedures), respectively, and translated in a MDL system. Translation reactions then were incubated with the indicated concentrations of CVB4 2A protease at 30°C for 20 min, and 2-μl aliquots were subjected to SDS-PAGE on 6% gels. Proteins were transferred to PVDF membranes and immunoblotted with anti-eIF4G-1 antibodies (top panel). The blots were then exposed to x-ray film (lower panel). The top panel shows both endogenous rabbit eIF4G-1 and newly synthesized human eIF4G-1, whereas the lower panel shows only the latter. (B) Translation reactions programmed with the same mRNAs were incubated with CVB4 2A protease at 30°C at a final concentration of 100 μg/ml. Aliquots (10 μl) were taken at the indicated times and analyzed as in panel A.

3. Resistance of eIF4G-1^{DM} to 2A protease *in vivo*

CVB3 infection of 293 cells results in the expression of 2A protease and complete cleavage of eIF4G-1 within the first three hours (Kerekatte and others 1999). To test whether the second amino acid substitution in eIF4G-1 produced a protein that was more stable *in vivo* upon CVB3 infection, we first transiently transfected 293 cells with mammalian expression plasmids encoding eIF4G-1a^{WT}, eIF4G-1a^{SM}, or eIF4G-1a^{DM} and then infected them with CVB3, variant H3. This work was performed in collaboration with the laboratory of Kirk Knowlton, University of California, San Diego. Cleavage is more reliably monitored by the appearance of cp_N (Fig. 2-3, lower arrow), since cp_N is transferred more efficiently to the PVDF membrane than the higher molecular weight intact eIF4G-1. The cp_N of eIF4G-1a^{WT} and eIF4G-1a^{SM} initially increased for 3 h after infection and then remained constant for up to 8 h. The cp_N of eIF4G-1a^{DM}, however, increased only slightly up to 8 h. There was a general decrease in the eIF4G-1 signal with time (Fig. 2-3, lane 14-19), perhaps due to nonspecific proteolysis. However, the ratio of uncleaved eIF4G-1a^{DM} to cp_N after 8 h (lane 19) was similar to that of eIF4G-1a^{WT}, eIF4G-1a^{SM}, and eIF4G-1a^{DM} at 0 h (lanes 2, 8 and 14). These results demonstrate that cleavage of eIF4G-1a^{SM} in CVB3-infected cells was delayed compared with eIF4G-1a^{WT}, and cleavage of eIF4G-1a^{DM} was delayed even more. The *in vivo* data thus show that the differences in sensitivities of eIF4G-1a^{WT}, eIF4G-1a^{SM}, and eIF4G-1a^{DM} observed in Fig. 2-2 were not an artifact of the *in vitro* assay.

4. Relationship of eIF4G-1a and eIF4G-1e to endogenous eIF4G-1 isoforms

Mammalian eIF4G-1 proteins are heterogeneous; multiple bands can normally be

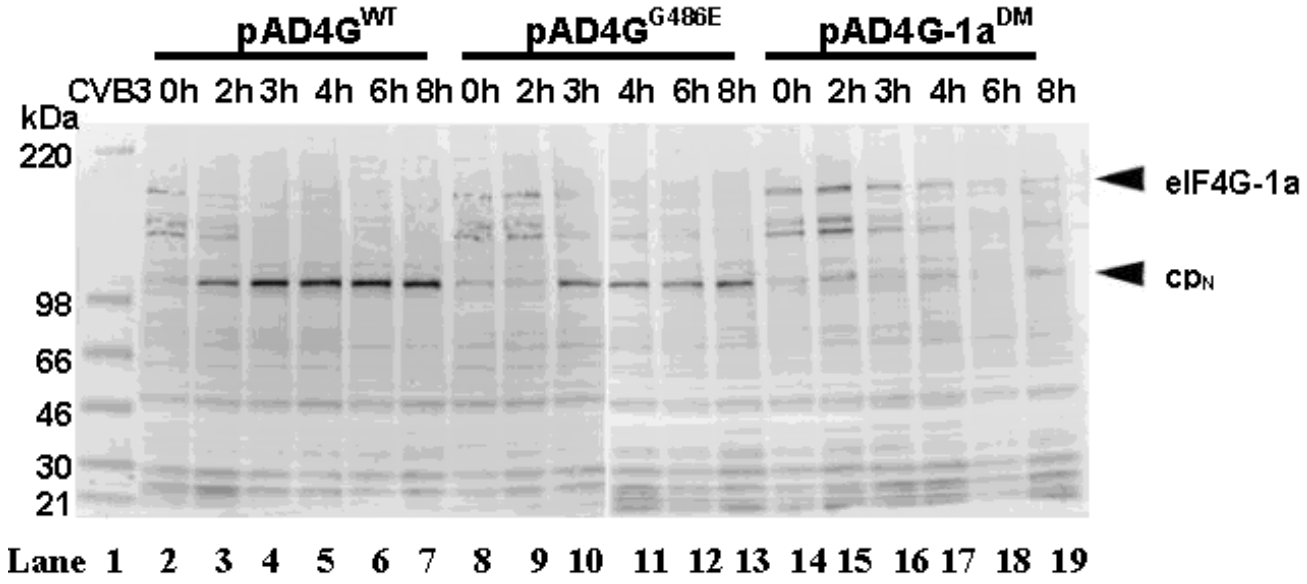

pAD4G^WT
pAD4G^G486E
pAD4G-1a^DM
CVB3 0h 2h 3h 4h 6h 8h 0h 2h 3h 4h 6h 8h 0h 2h 3h 4h 6h 8h
kDa
220
98
66
46
30
21
eIF4G-1a
cpN
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig. 2-3. Delayed cleavage of eIF4G-1a^{SM} and marked resistance of eIF4G-1a^{DM} in 293 cells following infection with CVB3. T7 epitope-tagged forms of eIF4G-1a^{WT}, eIF4G-1a^{SM} and eIF4G-1a^{DM} were expressed in 293 cells by transient transfection with pAD4G^{WT} (Lamphear and Rhoads 1996), pAD4G^{G486E} (Lamphear and Rhoads 1996), or pAD4G-1a^{DM} (Experimental Procedures). After 40 h, the cells were infected with wild type CVB3 and harvested 0, 2, 3, 4, 6, and 8 h after infection. Cell extracts were separated by SDS-PAGE on 4-12% gradient gels and immunoblotted with anti-T7-Tag antibodies. Molecular weight markers are shown on the left. The mobilities of intact eIF4G-1a and cp$_N$ are shown by the upper and lower arrows, respectively.

seen on SDS-PAGE with mobilities corresponding to 200-220 kDa (Etchison and others 1982; Duncan and others 1987; Lamphear and Panniers 1990; Yan and others 1992). To gain more insight into this, plasmids encoding both eIF4G-1a^{DM} and eIF4G-1e^{DM} were transcribed *in vitro* and the resultant mRNAs translated in the MDL translation system. Anti-eIF4G-1 antibodies detected endogenous rabbit eIF4G-1 as well as exogenous human eIF4G-1a and eIF4G-1e (Fig. 2-4, panel A), whereas an autoradiograph of the same blot detected only the exogenous forms (panel B). eIF4G-1a^{DM}, a protein calculated to be 154 kDa, migrated at 205 kDa, which was slightly faster than the fastest band (206 kDa) of endogenous rabbit eIF4G-1. This is in agreement with results obtained previously (Joshi and others 1994). Human eIF4G-1e, a protein calculated to be 172 kDa, migrated at 218 kDa, exactly corresponding with the second slowest band of endogenous rabbit eIF4G-1 (panel B). To obtain better separation of the two slowest bands, we used a larger gel with higher polyacrylamide concentration and ran the electrophoresis overnight (panel C and D). The eIF4G-1e^{DM} product (panel D) was found to co-migrate with the middle of the three immunoreactive eIF4G-1 bands (panel C). The slowest migrating band is most likely to be eIF4G-1f (Bradley and others 2002).

To better distinguish between eIF4G-1 isoforms, we developed an antibody (α-NT) against aa 56-66, which are present in eIF4G-1e but not eIF4G-1a. Only the two slowest bands reacted with this antibody (panel E, lanes 1 and 2). Translation of eIF4G-1a mRNA yielded a protein that was detected by autoradiography (panel F, lane 1) but not by immunoblotting with the anti-NT antibody (panel E, lane 1). This is consistent with the absence of an N-terminal extension in eIF4G-1a. The product of eIF4G-1e mRNA translation, on the other hand, was detectable by both methods and co-migrated

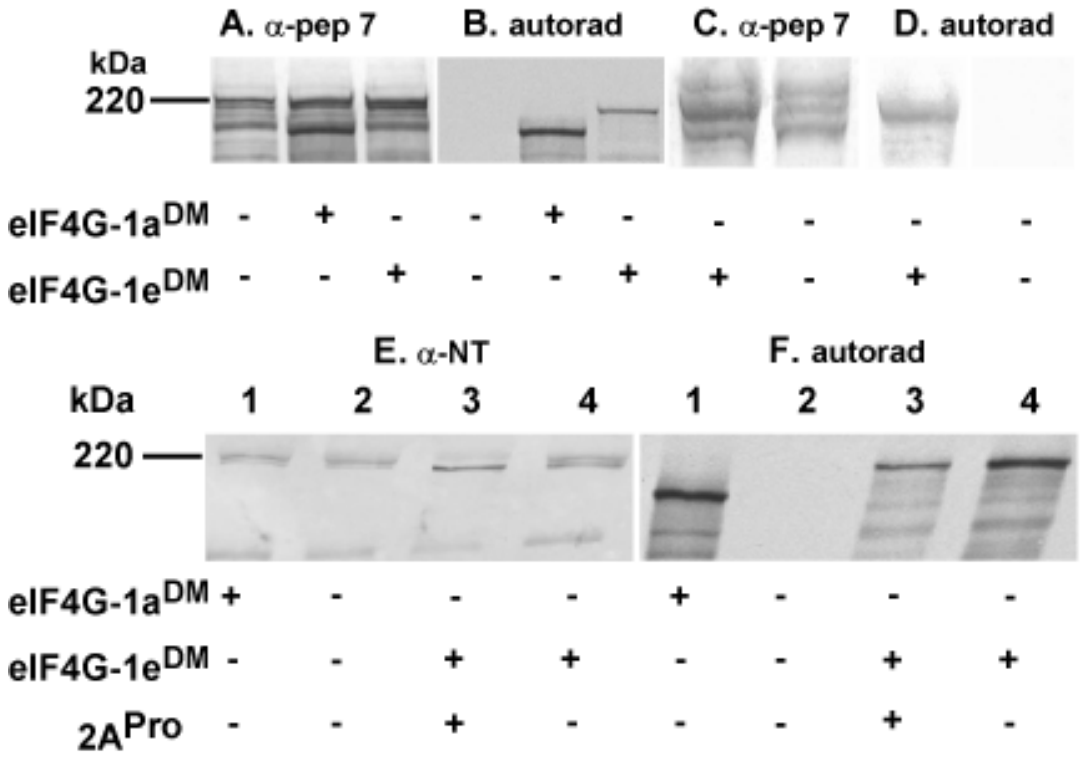

A. α-pep 7
B. autorad
C. α-pep 7
D. autorad
kDa
220
eIF4G-1a^DM - + - - + - - - - -
eIF4G-1e^DM - - + - - + + - + -
E. α-NT
F. autorad
kDa 1 2 3 4 1 2 3 4
220
eIF4G-1a^DM + - - - + - - -
eIF4G-1e^DM - - + + - - + +
2A^Pro - - + - - - + -

Fig. 2-4. Relationship of recombinant human eIF4G-1a^{DM} and eIF4G-1e^{DM} to endogenous rabbit eIF4G-1. The mRNAs encoding eIF4G-1a^{DM} and eIF4G-1e^{DM} were synthesized *in vitro* as described in Experimental Procedures. Recombinant eIF4G-1a^{DM} and eIF4G-1e^{DM} were synthesized in a MDL system at 30°C for 1 h. The translation reactions were subjected to SDS-PAGE on Hoefer 8 × 10 cm minigels of 6% polyacrylamide (panels A, B, E and F) or a Bio-Rad Protean Iixi 20 × 20 cm gel of 8.7% polyacrylamide (panels C and D). Proteins were transferred to PVDF membranes and immunoblotted with either anti-peptide 7 antibodies (Yan and others 1992), which react with both eIF4G-1a and eIF4G-1e, or anti-NT antibodies, which are specific for eIF4G-1e (see Fig. 1-2). The membranes were then exposed to x-ray film. (A) Immunoblot of translation reactions programmed with eIF4G-1a^{DM}, eIF4G-1e^{DM} or no mRNA, probed with anti-peptide 7 antibodies. (B) Autoradiograph of the PVDF membrane shown in panel A. (C) Immunoblot of translation reactions programmed with eIF4G-1e^{DM} mRNA or no mRNA, probed with anti-peptide 7 antibodies. (D) Autoradiograph of the PVDF membrane shown in panel C. (E) Immunoblot probed with anti-NT antibodies of translation reactions programmed with either eIF4G-1a^{DM} (lane 1), no exogenous mRNA (lane 2), or eIF4G-1e^{DM} mRNA (lanes 3 and 4). After synthesis of the proteins, the reaction in lane 3 was treated with 50 μg/ml of CVB4 2A protease for 30 min at 30°C. (F) Autoradiograph of the PVDF membrane shown in panel E. (Gels C-F were run by Chris Bradley).

with the faster member of the anti-NT-reactive doublet (panel E and F, lanes 4). Treatment with CVB4 2A protease eliminated the upper band (endogenous rabbit eIF4G-1) but not the lower band (exogenous eIF4G-1e^{DM}; panel E and F, lane 3 *versus* lane 4). These results indicate that the theoretical human proteins represented by eIF4G-1a and eIF4G-1e are similar in electrophoretic mobility and immunoreactivity to natural rabbit isoforms of eIF4G-1. Furthermore, they show that all but the two slowest rabbit isoforms of eIF4G-1 lack the N-terminal epitope derived from aa 56-66. Subsequent to these studies, the relationship of these polypeptides to the various cDNAs that have been cloned was determined by mass spectrometry (Bradley and others 2002). At least one mechanism responsible for multiple bands appears to be the use of alternate translation initiation codons (Byrd and others 2002).

5. Restoration of eIF4G-1 function by eIF4G-1e^{DM} to 2A protease-pretreated MDL

We sought to determine whether the two aa substitutions made in eIF4G-1^{DM} interfered with the intrinsic activity of this initiation factor. We therefore conducted a two-phase *in vitro* translation assay to test if eIF4G-1^{DM} could restore activity to MDL that had been pretreated with CVB4 2A protease. In Phase I, exogenous eIF4G-1e^{DM} was synthesized in 2A protease-pretreated MDL by IRES-driven translation. In Phase II, the newly synthesized eIF4G-1e^{DM} was tested for its ability to restore translation of globin mRNA (see Experimental Procedures). Endogenous eIF4G-1 was cleaved in the 2A protease-pretreated MDL (Fig. 2-5, panel A, lanes 3 through 7) but not in mock-treated MDL (lanes 1 and 2). eIF4G-1a^{DM} (lane 6) and eIF4G-1e^{DM} (lane 7) were shown by immunoblotting (panel A) and autoradiography (panel B) to be resistant to CVB4 2A

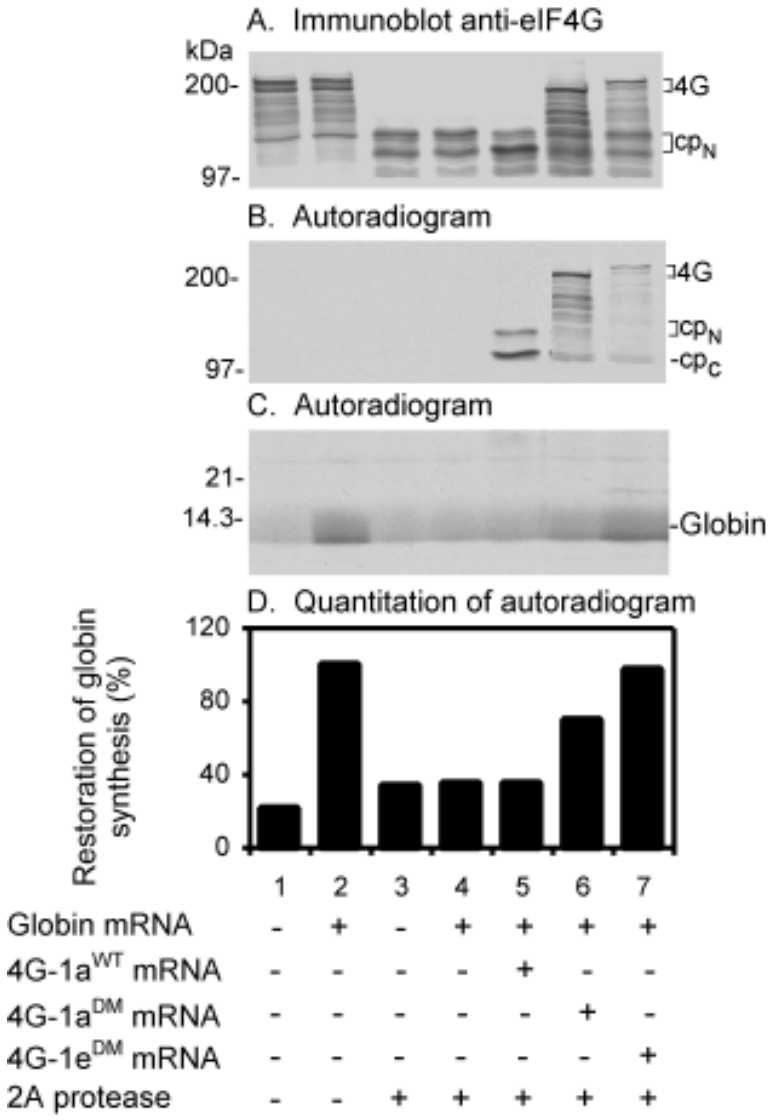

A. Immunoblot anti-eIF4G
kDa
200-
97-
4G
cp_N
B. Autoradiogram
200-
97-
4G
cp_N
-cp_C
C. Autoradiogram
21-
14.3-
-Globin
D. Quantitation of autoradiogram
Restoration of globin synthesis (%)
120
80
40
0
1 2 3 4 5 6 7
Globin mRNA - + - + + + +
4G-1a^WT mRNA - - - - + - -
4G-1a^DM mRNA - - - - - + -
4G-1e^DM mRNA - - - - - - +
2A protease - - + + + + +

Fig. 2-5. Restoration of cap-dependent translation to CVB4 2A protease-pretreated MDL by eIF4G-1^{DM}. Two-phase *in vitro* translation reactions were performed as described in Experimental Procedures. During Phase I, either eIF4G-1a^{WT}, eIF4G-1a^{DM}, eIF4G-1e^{DM} or no eIF4G-1 was synthesized in the absence of radioactivity in 2A protease-pretreated MDL using an EMCV IRES. Phase II was initiated by the addition of fresh 2A-treated MDL, globin mRNA and [^{35}S]Met. Aliquots were removed after 30 min and subjected to SDS-PAGE on 6% and 15% gels. (A) The 6% gel was immunoblotted with anti-eIF4G-1 antibodies. The positions of intact eIF4G-1 and the N-terminal cleavage products (cp_N) are indicated. (B) The 6% gel was subjected to autoradiography. (C) The 15% gel was subjected to autoradiography. (D) Globin synthesis was quantitated from the autoradiograph in C with ImageQuant software (Molecular Dynamics).

protease. Globin was efficiently synthesized in mock-treated MDL (panels C and D, lane 2) but not in 2A-pretreated MDL in the absence of exogenous eIF4G (lane 4) or in the presence of eIF4G-1e^{WT} (lane 5). Globin synthesis was partially restored by eIF4G-1a^{DM} (lane 6), and completely restored by eIF4G-1e^{DM} (lane 7). These results show that both eIF4G-1a^{DM} and eIF4G-1e^{DM} are functional but that eIF4G-1e^{DM} has higher translational activity than eIF4G-1a^{DM}, especially since more eIF4G-1a^{DM} was produced in the two-phase translation system than eIF4G-1e^{DM} (Fig. 2-5, panels A and B). Apparently synthesis of eIF4G-1e^{DM} is less efficient than eIF4G-1a^{DM}, since the Phase I reactions contained the same amount of mRNA. Thus, the two aa substitutions in eIF4G-1^{DM} do not interfere with its activity as a translation initiation factor.

D. Discussion

Comparison of the cleavage sites for 2A proteases in the viral polyproteins of 22 entero- and rhinoviruses yields the consensus sequence Leu-Thr-Thr-X↓Gly-Pro… (P4-P3-P2-P1↓P1'-P2'…; Blom and others 1996). The most conserved positions are P1', which is Gly in all cases, and P2, which is Thr in 16 out of 22 cases. The 2A proteases of HRV2 and CVB4 cleave eIF4G-1 initially at ^{679}Leu-Ser-Thr-Arg↓Gly-Pro^{684}, a sequence that contains both of these consensus aa residues (Lamphear and others 1993). Our previous observation that the G683E variant eIF4G-1a^{SM} was still cleaved (Lamphear and Rhoads 1996), albeit 100-fold more slowly than eIF4G-1a^{WT}, meant either that CVB4 2A protease had a preference but not an absolute requirement for Gly at P1', or that it cleaved at another site. Finding a secondary cleavage site between Leu-674 and Gly-675, eight aa residues upstream from the primary site, supports the latter

mechanism. While the P1' position in the secondary cleavage site is the highly conserved Gly, the P2 position is the less conserved Asn, which occurs in only two of the 22 viral polyprotein sequences (Blom and others 1996). This may account for the slower rate of cleavage at the secondary site. It is possible that both sites are cleaved in eIF4G-1^{WT}, but that our method of analyzing cp_C by Edman sequencing detected only the downstream site (Lamphear and others 1993). Even if this occurs, the results of Fig. 2-2 indicate that the secondary cleavage is ~100-fold slower. Interestingly, the Lb protease of foot-and-mouth virus, which is a papain-like protease, cleaves eIF4G-1 between Gly-675 and Arg-676 (Kirchweger and others 1994), only one aa residue from the secondary cleavage site of CVB4 2A protease. Thus, this region of eIF4G-1 may be particularly accessible to proteases. Because of this proximity of sites, the eIF4G-1^{DM} variant may have some resistance to Lb protease as well.

Careful examination of the immunoblots in Fig. 2-2 reveals that the newly synthesized eIF4G-1^{WT} is cleaved more readily than endogenous eIF4G-1 (Fig. 2-2, panel A and 3B, top *versus* lower portions). This may indicate that newly synthesized eIF4G-1 associates more readily with eIF4E, since eIF4G-1 alone is a poorer substrate for rhinovirus 2A protease than the eIF4G-1●eIF4E complex (Haghighat and others 1996). Alternatively, human eIF4G-1 may be a better substrate for CVB4 2A protease than rabbit eIF4G-1; notably, the human sequence matches the consensus CVB4 2A protease recognition sequence better than does rabbit (Lamphear and others 1993).

We tested the ability of recombinant human eIF4G-1a and eIF4G-1e to replace endogenous rabbit eIF4G-1 in a MDL translation system. This assay is reasonable since the aa sequence of human eIF4G-1a (Yan and others 1992; GenBank accession number

D12686; with corrections published in Lamphear and Rhoads 1996 and Imataka and Sonenberg 1997; accession number 2660712) is 87.2% identical and 93.9% similar to endogenous rabbit eIF4G-1 (accession number P41110). We found that human eIF4G-1a migrates on SDS-PAGE slightly faster than the fastest major band of rabbit eIF4G-1, in agreement with previous results (Joshi and others 1994), and that human eIF4G-1e exactly co-migrates with the second slowest major band of rabbit eIF4G-1 (Fig. 2-4). The slowest major band reacted with anti-NT antibodies (Fig. 2-4, panel E) and is most likely to be eIF4G-1f. Thus, it is likely that both eIF4G-1a and eIF4G-1e are similar to endogenous forms.

The ability of eIF4G-1e^{DM} to restore translation to 2A protease pretreated MDL was significantly higher than that of eIF4G-1a^{DM} when the system contained natural globin mRNA. This is in agreement with the finding of Imataka and others (1998) that eIF4G-1e but not eIF4G-1a contains a PABP-binding site. The higher translation efficiency is likely due to the PABP-binding site at the N-terminus of eIF4G-1e^{DM}, which makes the 48S complex formed by this eIF4G-1e^{DM} able to connect the 5'-end of the mRNA with 3'-end to facilitate translation reinitiation (see Chapter 1). By contrast, eIF4G-1a^{DM}, which lacks the PABP-binding site, is not able to form this mRNA loop structure in the 48S complex, and each round of translation initiation would occur *de novo*. Another possible reason for the higher activity of eIF4G-1e^{DM} might be that the interaction between eIF4G-1e^{DM} and PABP causes a conformational change in eIF4G, increasing its affinity for eIF4E. Alternatively, this interaction may change the conformation of the eIF4G•eIF4E complex and increase its affinity for the cap structure.

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CHAPTER 3

THE TRANSLATIONAL ACTIVITY OF TWO DIFFERENT EIF4G-1 ISOFORMS: EIF4G-1A^{DM} AND EIF4G-1E^{DM}

A. Introduction

The existence of a poly(A) tract at the 3' end of mRNA was discovered about thirty years ago (Lim and Canellakis 1970; Darnell and others 1971; Edmonds and others 1971). Following this discovery, its formation and function have been extensively studied. The poly(A) tract is not encoded by the gene (Philipson and others 1971; Birnboim and others 1973; Jacobson and others 1974) but is added by posttranscriptional modification (Wickens 1990; Proudfoot 1991; Wahle and Keller 1992). Poly(A)-tract length is relatively homogeneous in the nucleus, about 200-250 adenylate residues in mammals and 60-80 adenylate residues in yeast (Brawerman and Diez 1975; Sheiness and others 1975; Palatnik and others 1979), but it is much more heterogeneous after transport from the nucleus to the cytoplasm (Palatnik and others 1981; Palatnik and others 1984). The poly(A) tract is dynamic and undergoes shortening in the cytoplasm (Sheiness and Darnell 1973; Sheiness and others 1975; Palatnik and others 1979; Palatnik and others 1980). The rates of poly(A) shortening of different mRNAs are quite different, that of stable mRNAs being slower than that of unstable mRNAs (Herrick and others 1990; Decker and Parker 1993; Mercer and Wake 1985; Shyu and others 1991). Polyadenylation and deadenylation are regulated in response to developmental progress and physiological stimuli (McGrew and others 1989; Vassalli and others 1989).

The poly(A) tract is involved in mRNA transport from nucleus to cytoplasm, mRNA stability, and translational efficiency. Early studies of polyadenylated and deadenylated forms of various mRNAs failed to demonstrate that the poly(A) tract stimulates translation *in vitro* (Sippel and others 1974; Soreq and others 1974; Williamson and others 1974; Spector and Baltimore 1975). However, subsequent studies

showed that these conclusions were erroneous, due to the low initiation activity of the translational systems used. In more active cell-free translation systems such as the rabbit reticulocyte lysate, polyadenylated mRNAs are translated more efficiently than deadenylated mRNAs (Doel and Carey 1976, Hruby and Roberts 1977; Iizuka and others 1994).

Exogenous poly(A) is a potent and specific inhibitor of the translation of polyadenylated but not deadenylated mRNAs in rabbit reticulocyte, wheat-germ, L-cell, pea seed, and *Xenopus* oocyte systems (Jackson and Hunt 1983b; Bablanian and Banerjee 1986; Lemay and Millward 1986; Grossi de Sa and others 1988; Munroe and Jacobson 1990a; Drummond and others 1985). Poly(A) inhibition can be rescued by adding back purified PABP to reticulocyte lysates (Grossi de Sa and others 1988). The PABP of germinated pea embryo axes stimulates translation from poly(A) mRNA *in vitro* (Sieliwanowicz 1987). The abundance and stability of PABP correlates with the rate of translational initiation in developing or heat shocked *Dictyostelium* amoebae (Manrow and Jacobson 1986, Manrow and Jacobson 1987). These experiments do not, however, exclude the possibility that exogenous poly(A) has a high affinity for other components of the translation apparatus than PABP, and that the addition of purified PABP simply competes for this interaction. Poly(A) inhibition of translation can also be reversed by simultaneous addition of translation initiation factors eIF4F, eIF4B, and eIF4A (Gallie and Tanguay 1994). Uncapped mRNAs are much more sensitive to poly(A) inhibition than capped mRNAs, regardless of their polyadenylation status (Munroe and Jacobson 1990a; Gallie and Tanguay 1994).

How does the poly(A) tract enhance translation initiation efficiency? More than ten year ago, a closed-loop model was proposed (Jackson and Hunt 1983a; Palatnik and others 1984) and was further developed subsequently (Munroe and Jacobson 1989; Munroe and Jacobson 1990b, Munroe and Jacobson 1990a; Sachs and Davis 1989; Muhlrad and Parker 1994). Poly(A) binds to PABP, which in turn binds to the N-terminal region of eIF4G. This closed loop model was first demonstrated directly in yeast (Tarun and Sachs 1996; Tarun and others 1997; Wells and others 1998). In plants, the binding of PABP to eIF4G or eIF(iso)4G increases the RNA-binding activity of PABP (Le and others 1997). In HeLa cells, PAIP-1 binds PABP and eIF4A, enhancing translation reinitiation *in vivo* (Craig and others 1998). eIF4G-2 (Imataka and others 1997), a closely related functional homologue of eIF4G-1 (Yan and others 1992), binds PABP at the N-terminal region. eIF4G-1e, the N-terminal extended form of eIF4G-1a, also binds PABP (Imataka and others 1998).

In Chapter 2, we found that eIF4G-1e has much higher translational activity than eIF4G-1a. We hypothesize that the increased activity is due to the PABP-binding site at the N-terminus of eIF4G-1e but not eIF4G-1a. In order to test this hypothesis and to elucidate the different functions of these two isoforms of eIF4G-1, we used eIF4G-1a^{DM} and eIF4G-1e^{DM} as translation initiation factors to compare the translation of deadenylated and polyadenylated globin mRNA.

B. Experimental Procedures

1. Materials

MDL was prepared from New Zealand white rabbits (Chu and Rhoads 1980). Globin mRNA was prepared from high-salt washed ribosomal pellets from rabbit reticulocytes. T7 RNA polymerase, T4 polynucleotide kinase, RNase H, RQ DNase, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium chloride were from Promega (Madison, WI). [^{35}S]Met, [5'-^{32}P]pCp, and [γ-^{32}P]ATP were obtained from ICN Biochemicals (Irvine, CA). T4 RNA ligase was purchased from New England Biolabs (Beverly, MA). The production of an antibody against eIF4G-1 was described previously (Yan and others 1992). Anti-PABP antibodies were a gift from Dan Schoenberg.

2. 3'-End labeling of globin mRNA

Rabbit globin mRNA was labeled at 4°C overnight in a 100-µl total reaction mixture containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 50 µg/ml bovine serum albumin, 1 U/µl RNasin, 10% demethysulfoxide, 500 µCi/ml [5'-^{32}P]pCp, 200 µg/ml globin mRNA, and 100 U/ml T4 RNA ligase. Separation of globin mRNA from nucleotides was carried out by size exclusion chromatography on Sephadex G-50. The RNA was precipitated with ethanol and dissolved in 10 µl H_2O.

3. Deadenylation of globin mRNA

Rabbit globin mRNA (400 µg/ml) containing a trace amount of ^{32}P-labeled globin mRNA was hybridized in 50 µl to oligo$(dT)_{15}$ (20 µg/ml) by heating in 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 30 µg/ml bovine serum albumin at 65°C for 5 min and slowly cooling down to 37°C. RNase H (5 U) was added and the reaction mixture incubated at 37°C for 20 min. DNase (1 U) was added and the reaction

continued at 37°C for 15 min. The RNA was isolated by phenol extraction and ethanol precipitation.

4. 5'-End labeling of oligonucleotides

A 5'-dephosphorylated antisense oligonucleotide against β-globin mRNA (nt 464-445) (5'-ACCAGCCACCACCUUCUGAU-3') was phosphorylated with T4 polynucleotide kinase by incubating 30 pmol of oligonucleotide in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 4 mCi/ml [γ-^{32}P]ATP, and 20 U T4 polynucleotide kinase in a total volume of 50 μl for 30 min at 37°C. The reaction mixture was heated at 65°C for 15 min to inactivate T4 polynucleotide kinase.

5. Northern blotting

In vitro translation of natural or deadenylated globin mRNA was performed at 30°C for 30 min. Aliquots of 15 μl were removed at 0 and 30 min and added to 400 μl of RNA extraction buffer containing 100 mM Tris-HCl (pH 7.5), 250 mM NaCl, 10 mM EDTA, 2% SDS, and 100 μg/ml yeast RNA. The RNA was purified by phenol/chloroform extraction and ethanol precipitation, and then dissolved in 10 μl H_2O. An antisense oligonucleotide corresponding to β-globin mRNA (nt 464-445) (5'-ACCAGCCACCACCUUCUGAU-3'), labeled at the 5'-end, was used as probe to detect β-globin mRNA. A 4.5-μl aliquot was separated by 1% formaldehyde agarose gel electrophoresis at 50 V for 2.5 h. The gel was washed in diethyl pyrocarbonate-treated H_2O twice for 30 min and then in 0.5X TBE buffer for another 30 min. RNA was transferred to a nitrocellulose membrane using a Trans-Blot SD apparatus (BIO-RAD) at 400 mA for 40 min. The membrane was prehybridized at room temperature for 4 h in a solution containing 5X SSC, 5X Denhardt's reagent, 50% formamide, 1% SDS, and

100 μg/ml denatured salmon sperm DNA, and was then hybridized with the antisense probe overnight. The membrane was washed with 2X SSC and 0.1% SDS three times, 0.2X SSC and 0.1% SDS twice, 0.1X SSC and 0.1% SDS once, 0.05X SSC once, and 0.1% SDS once, each time for 5 min. All of the washes were carried out at room temperature. Radioactivity was detected by exposing the membrane to x-ray film.

C. Results

1. eIF4G-1a^{DM} and eIF4G-1e^{DM} differ in their ability to restore translation of polyadenylated mRNA *in vitro*

eIF4G-1a^{DM} and eIF4G-1e^{DM} differ by 156 amino acid at the N terminus (Fig. 1-2). The extended N-terminal sequence contains a PABP-binding site (Imataka and others 1998). We hypothesize that a PABP-binding site at the N-terminus of eIF4G-1e but not eIF4G-1a accounts for the difference in translation activity between eIF4G-1a^{DM} and eIF4G-1e^{DM}. Our hypothesis is based on the fact that PABP participates in the translation reaction. From our and others' previous work, we know PABP is also cleaved by 2A protease but at a much slower rate, about one tenth that of eIF4G-1 (Kerekatte and others 1999; Joachims and others 1999). We therefore checked PABP status by western blotting. Two thirds of the PABP was full-length in 2A protease-pretreated MDL (Fig. 3-1, lane 2). There was some full-length PABP left even at the end of the translation reaction (lane 3).

To test our hypothesis, we deadenylated rabbit globin mRNA with oligo(dT) and RNase H. Ethidium bromide staining showed that untreated globin mRNA was

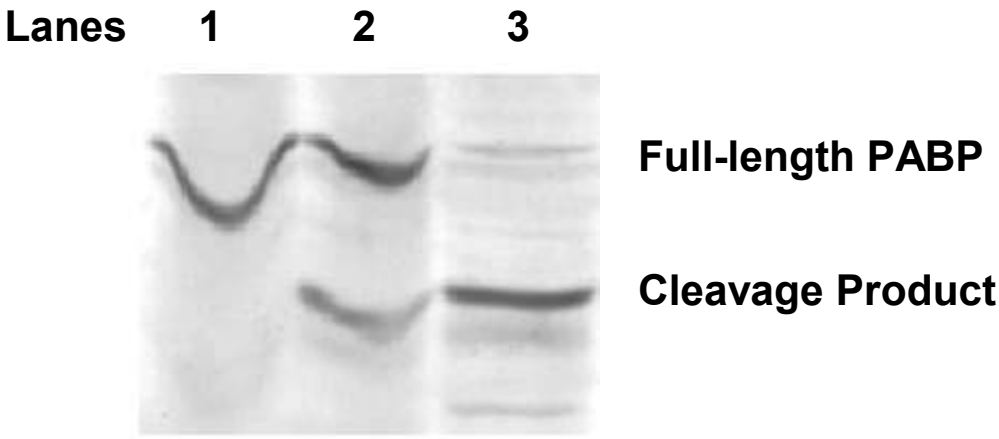

Lanes 1 2 3
Full-length PABP
Cleavage Product

Fig. 3-1. Cleavage of PABP by 2A protease in an *in vitro* translation system. MDL were loaded on SDS-PAGE gels, transferred to PVDF a membrane, and probed with anti-PABP antibody. PABP in MDL (lane 1), PABP in 2A-pretreated MDL before the translation reaction was incubated (lane 2), PABP in 2A-pretreated MDL after the translation reaction was incubated (lane 3).

heterogenous but resolved into distinct bands respresenting α- and β-globin mRNA after RNase H treatment (Fig. 3-2, panel B). Concomitantly, the radioactivity in3'-end-labeled globin mRNA was lost (panel A). Both results indicate that the poly(A) tract of globin mRNA was removed from the 3' end.

The untreated and deadenylated globin mRNA were then used to test the activity of eIF4G-1^{DM} isoforms. eIF4G-1a^{DM} and eIF4G-1e^{DM} were synthesized in a Phase I translation reaction by cap-independent translation (Fig. 3-3, panel A). In Phase II, the newly synthesized eIF4G-1a^{DM} and eIF4G-1e^{DM} were used to facilitate translation of untreated or deadenylated globin mRNA (panel B).

Deadenylated globin mRNA was translated at the same translation efficiency in the presence of either eIF4G-1a^{DM} or eIF4G-1e^{DM} (panel B, lane 9 *versus* 10, $p = 0.36$, n=3). The polyadenylated globin mRNA, however, was translated at considerably higher effeciency when eIF4G-1e^{DM} was used as translation initiation factor than eIF4G-1a^{DM} (lane 7 *versus* 8, $p < 0.008$, $n = 3$). Northern blotting showed that the amount of untreated and deadenylated mRNA was the same at the beginning and end of the reaction (Fig 3-4), indicating that the difference in translational activity was not due to a difference in mRNA stability.

2. The translational activity of eIF4G-1e^{DM} but not eIF4G-1a^{DM} is inhibited by poly(A)

In order to test how the poly(A) tract of mRNA affects the translation activity of eIF4G-1a^{DM} and eIF4G-1e^{DM}, we performed competition experiments. The same two-phase *in vitro* translation system was used. In Phase I, exogenous eIF4G-1^{DM} was synthesized; in Phase II, globin synthesis from natural globin mRNA was used to test

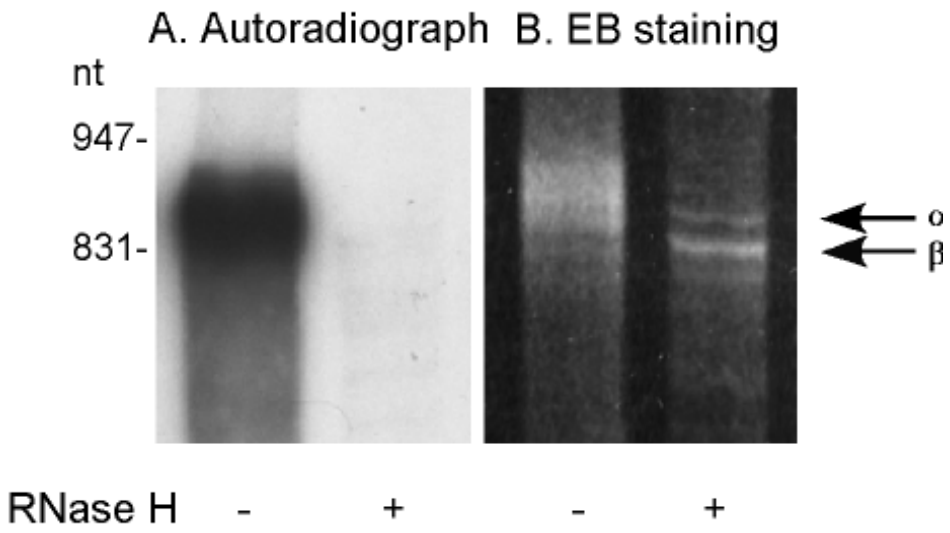

A. Autoradiograph
B. EB staining
nt
947-
831-
α
β
RNase H - + - +

Fig. 3-2. Deadenylation of globin mRNA. Globin mRNA labeled at the 3' end with [5'-^{32}P]pCp was mixed with unlabeled globin mRNA, hybridized to oligo$(dT)_{15}$, and treated with RNase H. The RNA was separated on a 6% denaturing polyacrylamide gel and analyzed by autoradiography (A) and ethidium bromide staining (B). α and β designate the mRNAs for α-globin and β-globin, respectively.

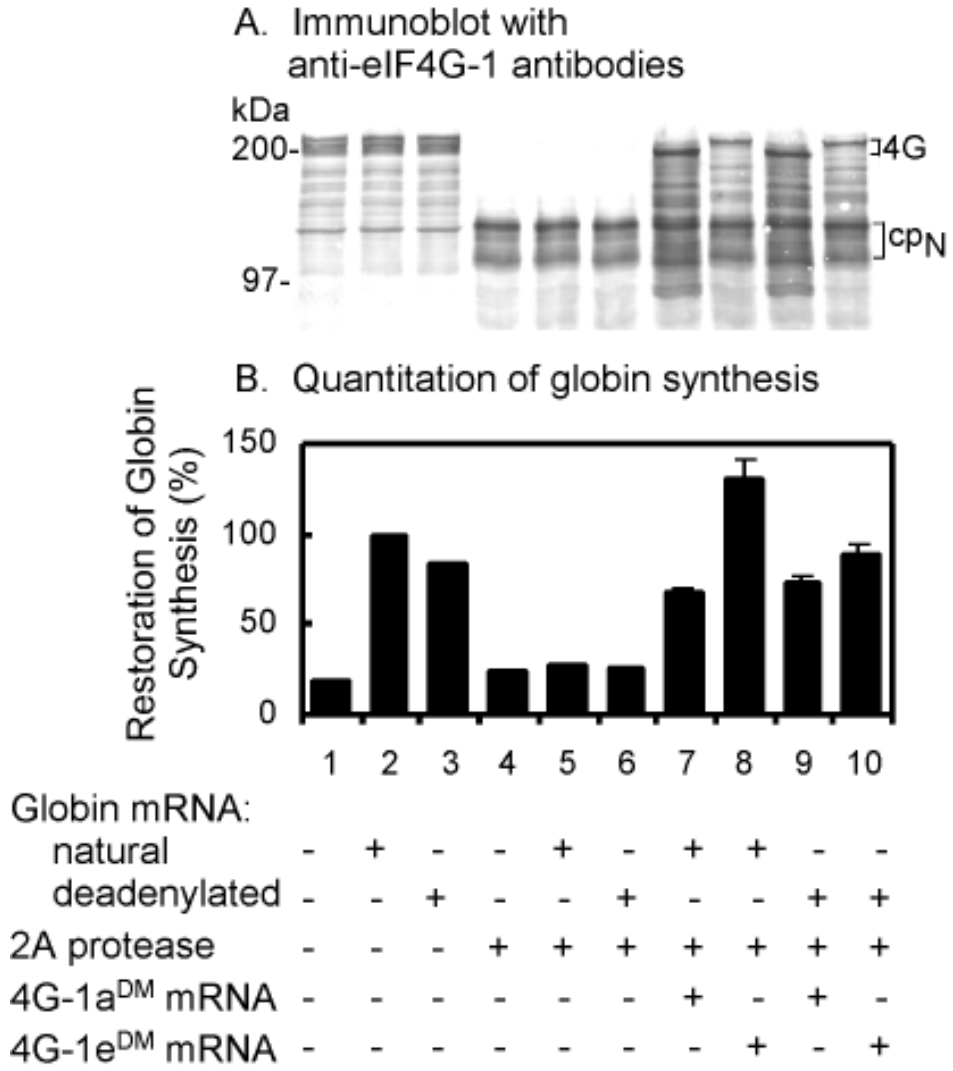

A. Immunoblot with
anti-eIF4G-1 antibodies
kDa
200-
97-
4G
cpN
B. Quantitation of globin synthesis
Restoration of Globin Synthesis (%)
150
100
50
0
1 2 3 4 5 6 7 8 9 10
Globin mRNA:
natural - + - - + - + + - -
deadenylated - - + - - + - - + +
2A protease - - - + + + + + + +
4G-1a^DM mRNA - - - - - - + - + -
4G-1e^DM mRNA - - - - - - - + - +

Fig. 3-3. Translational activity of eIF4G-1a^{DM} and eIF4G-1e^{DM} on polyadenylated and deadenylated rabbit globin mRNA. The two-phase *in vitro* translation assay described in Experimental Procedures in Chapter 2 was utilized. During Phase I, eIF4G-1a^{DM} or eIF4G-1e^{DM} was synthesized in the absence of radioactivity in 2A protease-pretreated MDL. During Phase II, globin was synthesized by the addition of fresh 2A protease-pretreated MDL, polyadenylated or deadenylated rabbit globin mRNA, and [^{35}S]Met. Aliquots were removed after 30 min of reaction and subjected to 6% and 15% SDS-PAGE gradient gels. (A) The 6% gel was immunoblotted with anti-eIF4G-1 antibodies. The positions of intact endogenous eIF4G-1 and the N-terminal cleavage products (cp_N) are indicated. (B) Globin synthesis was quantitated from the autoradiograph of the 15% gel with ImageQuant software.

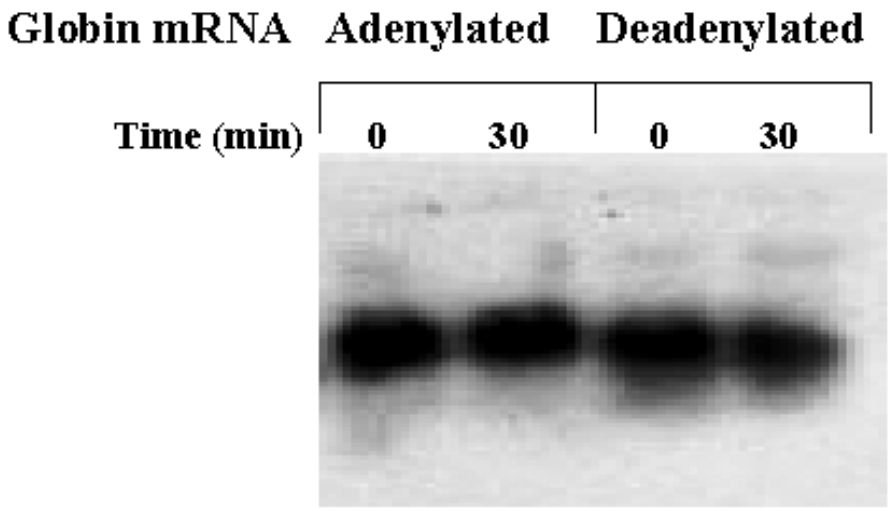

Globin mRNA
Adenylated
Deadenylated
Time (min)
0
30
0
30

Fig. 3-4. Northern blot analysis of polyadenylated and deadenylated globin mRNA from translation reaction mixtures. Aliquots of *in vitro* translation mixtures were removed at the indicated time points. RNAs were purified by phenol/chloroform and precipitated by ethanol. RNAs were separated on 1% agarose gels containing formaldehyde, transferred to nitrocellulose membranes, and probed with a 5'-end-labeled antisense oligonucleotide specific to β-globin mRNA.

the function of eIF4G-1^{DM} synthesized in Phase I. Poly(A) was added to the translation reactions at the beginning of Phase II. The total amount of eIF4G-1^{DM} present (endogenous plus exogenous) is shown by western blotting (Fig. 3-5, panel A). The amount of eIF4G-1^{DM} synthesized in Phase II is shown by autoradiography of the western blot (panel B). Globin synthesis is represented by the autoradiogram in panel C. Globin synthesis was not affected by increasing the concentration of poly(A) when eIF4G-1a^{DM} functioned as the translation initiation factor (lanes 2 through 5). In contrast, globin synthesis decreased with increasing concentrations of poly(A) when eIF4G-1e^{DM} was present (lanes 6 through 9). The intensities of eIF4G-1^{DM} and globin synthesis were quantitated by ImageQuant software and the ratio of globin synthesis to eIF4G-1^{DM} was calculated (panel D). Poly(A) had no effect on the ratio of globin synthesis to eIF4G-1a^{DM} (lanes 2 through 5), whereas it decreased the ratio of globin synthesis to eIF4G-1e^{DM} (lanes 6 through 9). These results suggest that the translational activity of eIF4G-1e^{DM} but not eIF4G-1a^{DM} is inhibited by poly(A).

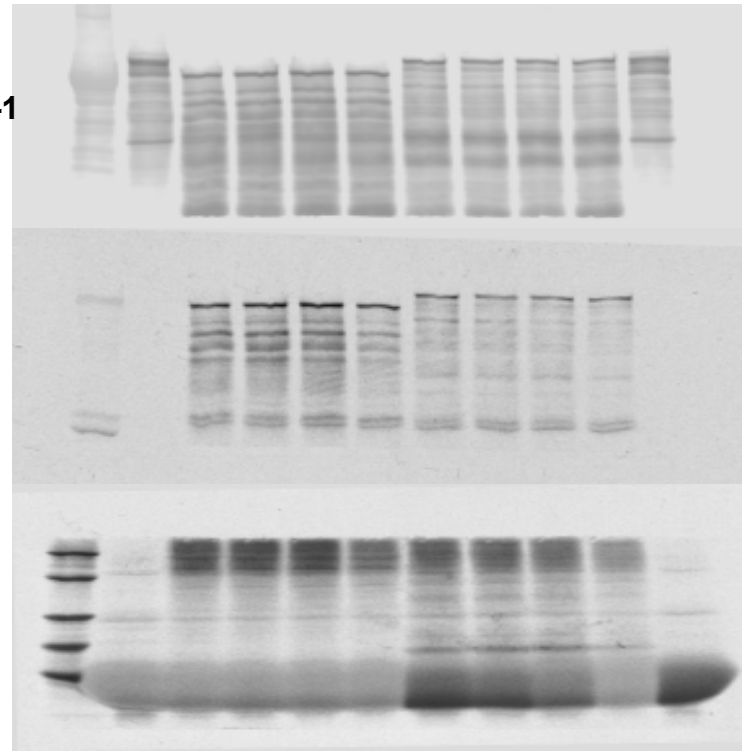
3. The translational activity of eIF4G-1e^{DM} but not eIF4G-1a^{DM} is inhibited by oligo(dT)$_{15}$

We further used oligo(dT)$_{15}$ to block the poly(A) tract of globin mRNA. The same experimental procedure was used as in the poly(A) competition experiment except that oligo(dT)$_{15}$ was used instead of poly(A). Oligo(dT)$_{15}$ had no effect on the translational activity of eIF4G-1a^{DM} or the ratio of globin synthesis to eIF4G-1a^{DM} (Fig. 3-6, panel D, lanes 2 through 5), whereas it decreased the translational activity of eIF4G-1e^{DM} and the ratio of globin synthesis to eIF4G-1e^{DM} (lanes 6 through 9).

A. Immunoblot of 6% gel with anti-eIF4G-1 antibodies

B. Autoradiogram of immunoblot

C. Autoradiogram of 15% gel



Globin mRNA	–	+	+	+	+	+	+	+	+	+
2A protease	–	+	+	+	+	+	+	+	+	–
eIF4G-1a^{DM} mRNA	–	+	+	+	+	–	–	–	–	–
eIF4G-1e^{DM} mRNA	–	–	–	–	–	+	+	+	+	–
polyadenylic acid	–	▲	▲	▲	▲	▲	▲	▲	▲	–

D.

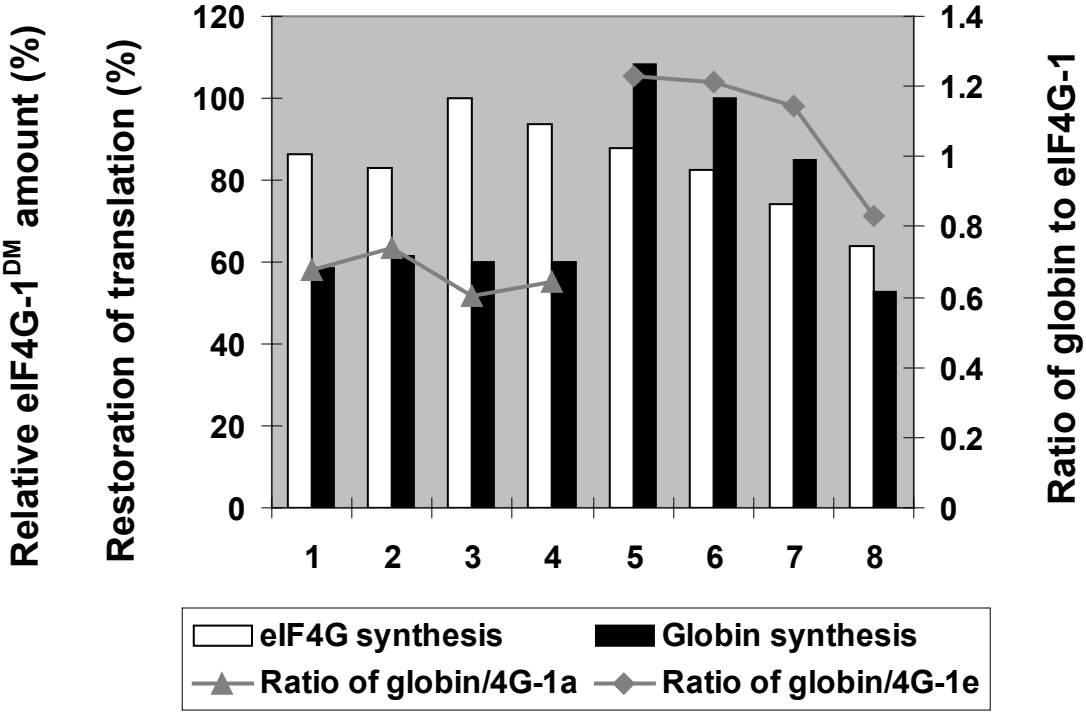


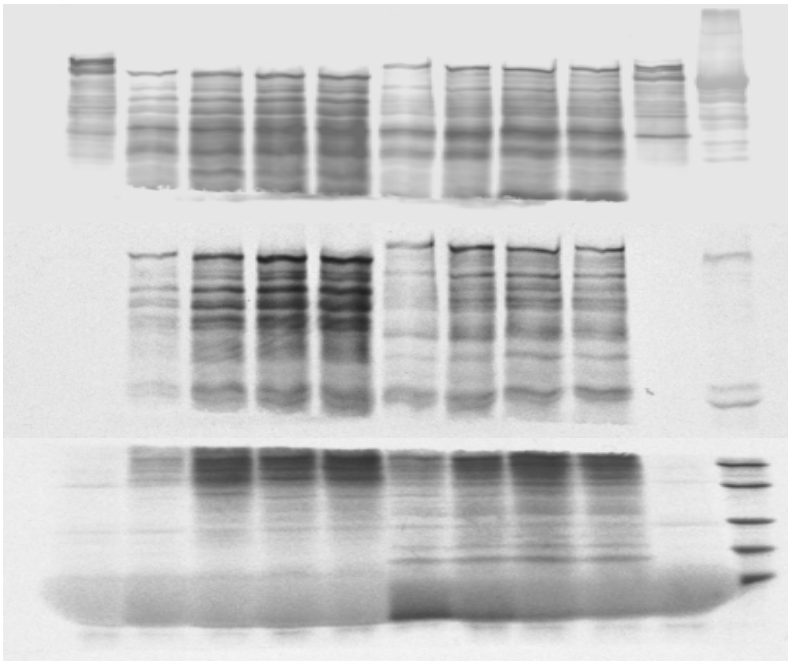
Fig. 3-5. The translational activity of eIF4G-1e^{DM} but not eIF4G-1a^{DM} is inhibited by poly(A). A two-phase *in vitro* translation assay was utilized as described in Experimental Procedures in Chapter 2. Aliquots were removed from translation reaction mixtures and subjected to SDS-PAGE on 6% and 15% gradient gels. (A) The 6% gel was immunoblotted with anti-eIF4G-1 antibodies. (B) Newly synthesized eIF4G was detected on the same membrane by autoradiography. (C) The 15% gel was dried and subjected to autoradiography. (D) The intensities of eIF4G-1 and globin were quantitated from the immunoblot (A) and autoradiogram (C), respectively, with ImageQuant software. For eIF4G-1, 100% corresponds to the maximum amount present (lane 3). For restoration of globin synthesis, 100% is defined as synthesis in a reaction untreated with 2A protease (last lane of panel C). The ratios of globlin synthesis to eIF4G-1a^{DM} (triangles) and globin synthesis to eIF4G-1e^{DM} (diamonds) are shown.

.

A. Immunoblot of 6% gel with anti-eIF4G-1 antiobodies

B. Autoradiogram of immunoblot

C. Autoradiogram of 15% gel



Globin mRNA	–	+	+	+	+	+	+	+	+	+
2A protease	–	+	+	+	+	+	+	+	+	–
eIF4G-1a^{DM} mRNA	–	+	+	+	+	–	–	–	–	–
eIF4G-1e^{DM} mRNA	–	–	–	–	–	+	+	+	+	–
Oligo(dT)$_{15}$	–	▲	▲	▲	▲	▲	▲	▲	▲	–

D.

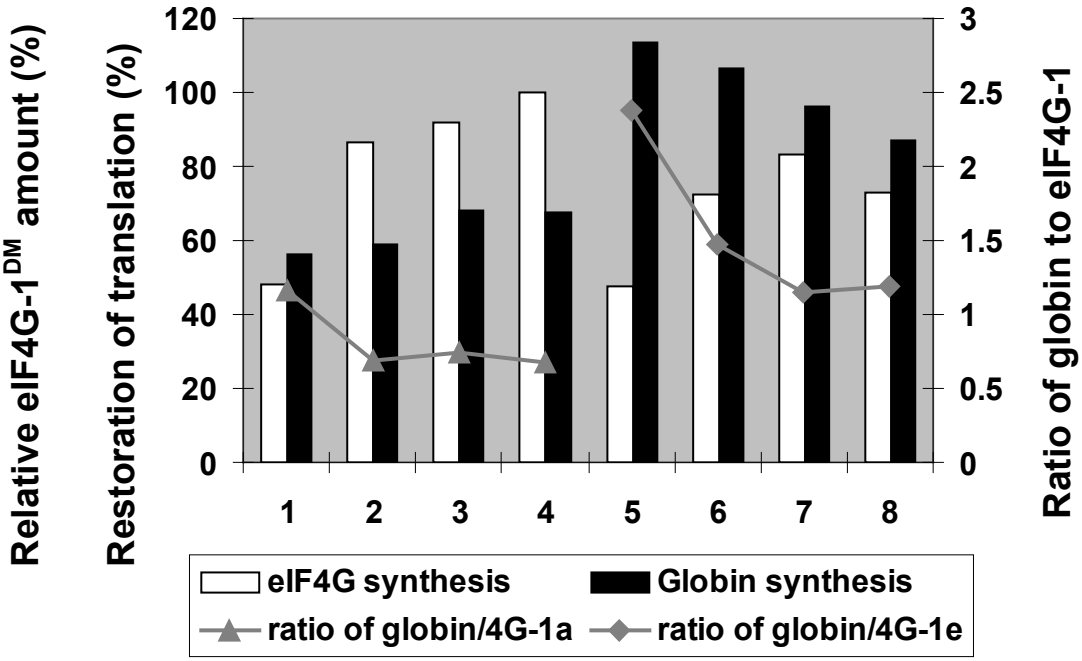


Fig. 3-6. The translational activity of eIF4G-1e^{DM} but not eIF4G-1a^{DM} is inhibited by oligo(dT)$_{15}$. A two-phase *in vitro* translation assay was utilized as described in Experimental Procedures in chapter 2. Aliquots were removed from translation reaction mixtures and subjected to SDS-PAGE on 6% and 15% gradient gels. (A) The 6% gel was immunoblotted with anti-eIF4G-1 antibodies. (B) Newly synthesized eIF4G was detected on the same membrane by autoradiography. (C) The 15% gel was dried and subjected to autoradiography. (D) The intensity of eIF4G-1 and globin was quantitated from the immunoblot (A) and autoradiogram (C), respectively, with ImageQuant software. The ratios of globlin synthesis to eIF4G-1a^{DM} (triangles) and globin synthesis to eIF4G-1e^{DM} (diamonds) are shown.

D. Discussion

We found that the ability of eIF4G-1e^{DM} to restore translation to 2A protease-pretreated MDL was significantly higher than that of eIF4G-1a^{DM} when the system contained natural globin mRNA. By contrast, the activity of eIF4G-1a^{DM} and eIF4G-1e^{DM} were similar with deadenylated globin mRNA. This is consistent with the finding of Imataka and others (1998) that eIF4G-1e but not eIF4G-1a contains a PABP-binding site. It might be argued that 2A protease-pretreated MDL lacks both functional eIF4G-1 and PABP (Kerekatte and others 1999, Joachims and others 1999). However, our laboratory has previously shown that PABP cleavage occurs at only approximately one-tenth the rate of eIF4G-1 cleavage (Kerekatte and others 1999). Under the conditions actually used in the present study, we demonstrated, by immunoblotting with specific anti-PABP antibodies, that pretreatment of MDL with CVB4 2A protease resulted in only one-third cleavage of PABP but complete cleavage of eIF4G-1 (Fig. 3-1). The fact that eIF4G-1e^{DM} is able to fully restore translation of globin mRNA indicates that 1) any hypothetical changes to the translational system caused by 2A protease pretreatment other than eIF4G cleavage do not affect the overall rate of translation, and 2) the two aa substitutions in eIF4G-1e^{DM} do not affect its activity in translation of capped and polyadenylated mRNA.

Identification of longer isoforms of eIF4G-1 with PABP binding sites (Johannes and Sarnow 1998; Imataka and others 1998; Bradley and others 2002; Byrd and others 2002) suggests that the ability of eIF4G-1 to bind eIF4E and PABP simultaneously results in concomitant circularization of the mRNA. A circularized mRNA may allow ribosomes to rapidly reload following termination to reinitiate the next round of

translation. By contrast, eIF4G-1a^{DM}, lacking the PABP binding site, cannot form a loop structure, and each round of translation initiation occurs *de novo*. The fact that eIF4G-1e^{DM} has much higher translational activity on polyadenylated than deadenylated globin mRNA whereas eIF4G-1a^{DM} has the same translational activity on both (Fig. 3-3) suggests that the poly(A)•PABP•eIF4G-1 interaction is responsible for the higher translational activity of eIF4G-1e^{DM}. The inhibition of eIF4G-1e^{DM} but not eIF4G-1a^{DM} by poly(A) and oligo(dT)$_{15}$ further supports this hypothesis (Fig. 3-5 and 3-6). Another possible mechanism for the higher translational activity of eIF4G-1e^{DM} over eIF4G-1a^{DM} is that eIF4G-1e binding to PABP increases its affinity for mRNA. As noted above, the interaction of wheat germ PABP with eIF4F increases the affinity of eIF4F for a cap analogue (Wei and others 1998) and the affinity of PABP for poly(A) (Le and others 1997). These two changes are not exclusive but may function mutually to enhance the translational activity of eIF4G-1e^{DM}.

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CHAPTER 4

FUNCTIONAL STUDIES OF EIF4G-1A^{DM} AND EIF4G-1E^{DM} *IN VIVO* USING INDUCIBLE, STABLE CELL LINES

B. Introduction

N4G3, a cell line that overexpresses eIF4G-1 about 80- to 100-fold of normal NIH3T3 cells, was made by stable transfection of NIH3T3 cells with human eIF4G-1a cDNA (Fukuschi-Shimogori and others 1997). This cell line formed transformed foci on monolayers of cells, showed anchorage-independent growth, and formed tumors in nude mice. In our laboratory, several years ago, the HeLa cell line was transfected with a plasmid expressing eIF4G-1a and selected by drug resistance. In the resulting cell line, eIF4G-1 was overexpressed 2- to 5-fold. This stable HeLa cell line was also found to form foci, show anchorage-independent growth, and lose contact inhibition. Attempts to infect these cells with rhinovirus, in collaboration with the laboratory of Tim Skern, University of Vienna, were unsuccessful, perhaps because of the grossly altered phenotype. More recently, we performed transient transfections with plasmids expressing eIF4G-1a WT, SM, and DM variants to determine whether these modified eIF4G-1 forms could protect cells against the development of cytopathology upon infection with Coxsackievirus (in collaboration with Kirk Knowlton, University of California, San Diego). By contrast to the studies with stable cell lines, these experiments were inconclusive because the transfection efficiency was too low. To solve these overexpression and transfection efficiency problems, we initiated experiments to establish cell lines that stably express exogenous eIF4G-1 (hence, transfection efficiency is not an issue since every cell is capable of expressing eIF4G-1) and in which the expression level can be regulated (hence, avoiding the problem of aberrent cell growth due to constitutively elevated eIF4G-1 levels).

A system that would allow stringent control of the expression of the eIF4G-1 gene, and not just mediate "on/off" gene activity, would be ideal for our study. Inducible eukaryotic promoters responsive to, for example, heavy metal ions (Brinster and others 1982; Searle and others 1985), heat shock, or hormones (Israel and Kaufman 1989) have generally suffered from leakiness of the inactive state (the metallothionein promoter) or from pleiotropic effects caused by the nature of the induction method itself (elevated temperature or glucocorticoid hormone action; Lee and others 1988). Regulatory systems that do not rely on endogenous control elements, such as *lac* repressor/operator (*lac*R/O)-based system, have been extensively studied. However, the utility of *lac*R/O-based systems in mammalian cells is limited since the inducer IPTG, despite its rapid uptake and intracellular stability (Wyborski and Short 1991), acts rather slowly and inefficiently, resulting in only moderate induction.

Tetracyline–responsive promoters allow regulation of expression of an individual gene over up to five orders of magnitude under tight control (Gossen and Bujard 1992). This system is based on regulatory elements of the Tn10-specified tetracycline-resistance operon of *E. coli*, in which transcription of resistance-mediating genes is negatively regulated by the tetracycline repressor (TetR). In the presence of the antibiotic tetracycline, TetR does not bind to its operator, located within the promoter region of the operon, permitting transcription to proceed. A hybrid transactivator (rTA) has been generated by combining TetR with the C-terminal domain of VP16 from herpes simplex virus (HSV), known to be essential for the transcription of immediate early viral genes (Triezenberg and others 1988). rTA stimulates minimal promoters that have been fused to tetracycline operator (*tet*O) sequences. These promoters are virtually silent in the

presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to *tet*O sequences.

Since the first construction of a tetracycline-responsive promoter, numerous studies have improved and utilized this system to study gene function in complex genetic environments, such as mammalian cells and transgenic mice (Resnitzky and others 1994; Gossen and others 1995; Tremblay and others 1998; Mansuy and Bujard 2000). A tetracycline-regulated gene expression system, the Tet Gene Expression System, has been commercially introduced by Clontech. It is based on the tetracycline-resistance operon of the *E. coli* Tn10 transposon, *tet*O, and the protein that binds to it, TetR. The Tet Gene Expression System has been developed into Tet-Off and Tet-On systems according to how the gene of interest is regulated. In the Tet-Off system, the gene of interest under the control of *tet*O is repressed in the presence of tetracycline (Tc) or doxycycline (Dox); removing Tc or Dox from the cell culture medium induces the activation of gene expression. The regulatory protein of the Tet-On system is based on the "reverse" TetR (rTetR), which differs from the wild-type TetR by four amino acids. When fused to the VP16 AD, rTetR becomes a "reverse" transactivator (rtTA) that activates transcription in the presence of Dox. Thus, in the Tet-On system, the gene of interest is activated in the presence of Dox.

We chose the Tet-On system to develop HeLa cell lines that stably and inducibly express eIF4G-1^{DM} isoforms.

B. Experimental Procedures

1. Materials

The vectors pIRES2-EGFP, pEGFP-C1, and pTRE2pur were obtained from Clontech (Palo Alto, CA). A plasmid expressing CVB3 VP-1-2A protease domain from an EMCV IRES was described previously and is here referred to as pVP12A (Badorff and others 2000).

2. Construction of recombinant plasmids

pTRE2pur4G-1a^{DM} and pTRE2pur4G-1e^{DM} were constructed by digesting pFastBac4G-1a^{DM} and pFastBac4G-1e^{DM} first with *Ssp*I and then partially with *Nhe*I. The inserts, consisting of the entire eIF4G-1a^{DM} or eIF4G-1e^{DM} coding regions, were purified by electrophoresis and ligated into pTRE2pur previously cut with *Pvu*II and *Nhe*I. Plasmids were screened by restriction enzyme digestion first and confirmed by DNA sequencing.

3. Establishment of stable cell lines expressing tetracycline-regulated eIF4G-1a^{DM} and eIF4G-1e^{DM}

The Tet-On HeLa cell line was transfected by electroporation with pTRE2pur4G-1a^{DM} or pTRE2pur4G-1e^{DM} linearized with *Xho*I. After 24 h, the cells were selected in medium containing 2 μg/ml puromycin. The medium was changed every 3-4 days. Colonies were transferred to 6-well plates and culture continued. Expression of eIF4G-1^{DM} was detected by adding Dox to the culture medium in the amounts and times described in the figure legends. Cell lysates were treated with 2A protease for 2 h on ice. eIF4G-1 detected by immunoblotting with anti-eIF4G-1 antibodies but resistant to 2A protease digestion was scored as eIF4G-1^{DM}. Colonies

were selected that had the lowest expression of eIF4G-1^{DM} in the absence of Dox but highest expression in its presence.

4. Cotransfection of cell lines with GFP and 2A protease-expressing plasmids

Stably transfected Tet-On HeLa cell lines expressing eIF4G-1a^{DM} or eIF4G-1e^{DM} were cultured in 6-well plates. Cells were either induced by Dox to express exogeneous eIF4G-1^{DM} for 24 h or mock induced. Cells were then cotransfected with pVP12A and either pEGFP-C2 or pIRES2-EGFP using Polyfectin (Qiagen). Culture was continued for 24 h, and then cells were observed with a Nikon Eclipase TE 300 fluorescence microscope (Melville, NY). In each well, five random fields were selected and green fluorescence protein (GFP)-expressing cells were analyzed by IPLab Scientific Imaging Software from Scanalytics, Inc. (Fairfax, VA). The averages and standard deviations from the five fields were computed.

5. Quantitation of GFP mRNA by real-time PCR

RNA was isolated from the cells in each well and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). mRNA was transcribed into cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real-time PCR was performed to detect GFP mRNA levels using an ABI PRISM 7700 Sequence Detection System. The antisense primer for GFP was 5'-GTACAGCTCGTCCATGCCGA-3', sense primer, 5'- CAACGAGAAGCGCGATCACAT-3', and probe, 6-FAM-CTGCTGGAGTTCGTGACCGCCGC-TAMRA. GFP mRNA levels were normalized by taking the ratio to endogenous glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA using reagents from Applied Biosystems.

C. Results

1. Establishment of inducible, stable cell lines expressing eIF4G-1a^{DM} and eIF4G-1e^{DM}

To establish a regulatable stable cell line that expresses endogenous protein level of eIF4G-1a^{DM} and eIF4G-1e^{DM}, we used the Tet–On Gene Expression System of Clontech (Fig. 4-1). The gene to be expressed is cloned into the pTRE2 "response" plasmid, a 5.3-kb pTRE2pur vector expressing the gene of interest under the control of the TRE and containing the puromycin-resistance gene as a selectable marker. We used the Tet-On^{TM} (Clontech) HeLa cell line, which already stably expresses rtTA under the maintenance of a G418 resistance marker, to establish the eIF4G-1a^{DM} and eIF4G-1e^{DM} integrated cell lines. Thus, cells transfected with pTRE-eIF4G-1 plasmids were selected in the presence of both puromycin and G418. We wished to distinguish exogenous from endogenous eIF4G-1, so we incorporated a His-tag sequence at the N-terminus of eIF4G-1.

2. eIF4G-1^{DM} restores cap-dependent translation *in vivo*

Two stable cell lines that had the highest expression of exogenous eIF4G-1 in the presence of Dox and lowest expression of exogenous eIF4G-1 in the absence of Dox were selected. To distinguish endogenous eIF4G-1 from eIF4G-1^{DM}, we treated cell extracts with CVB4 2A protease before immunoblotting with anti-eIF4G-1 antibodies.

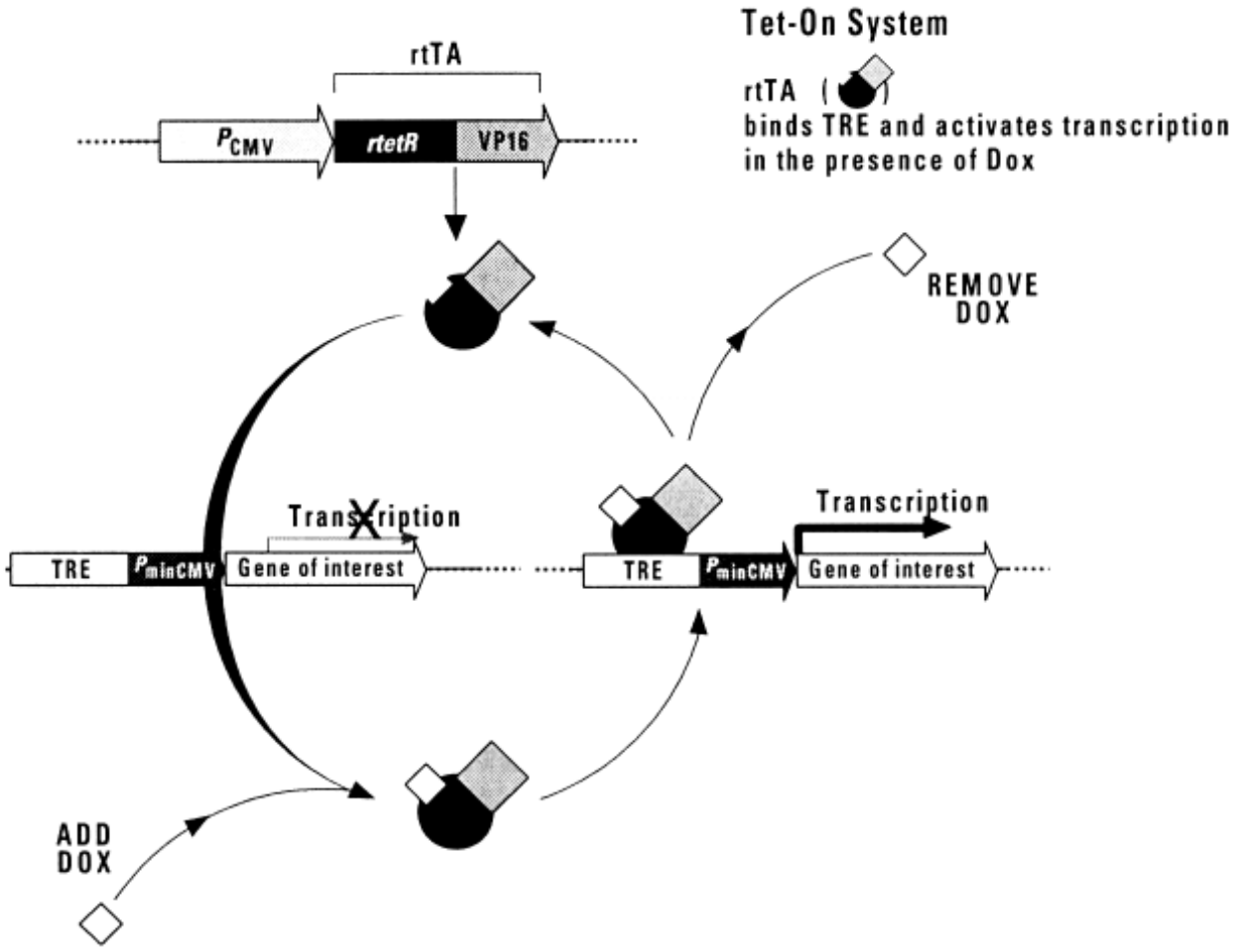

Tet-On System
rtTA
rtTA
binds TRE and activates transcription
in the presence of Dox
rtTA
P_{CMV}
rtetR
VP16
REMOVE
DOX
Transcription
Transcription
TRE
P_{minCMV}
Gene of interest
TRE
P_{minCMV}
Gene of interest
ADD
DOX

Fig. 4-1. Gene regulation in the Tet-On System. The TRE (tetracycline-response element) is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}) of the response plasmid, which is silent in the absence of activation. rtTA, expressed from the regulatory plasmid, binds Dox to activate the expression of the gene of interest from the response plasmid. (This figure is reproduced from the Tet Systems User Manual, Clontech Laboratories, Inc.)

The level of exogenous eIF4G-1a^{DM} (Fig. 4-2, panel A) and eIF4G-1e^{DM} (panel B) increased with increasing Dox concentration. Unfortunately, the expression level of exogenous eIF4G-1^{DM} was only a small fraction of endogenous eIF4G-1, despite the fact that we surveyed numerous clonal lines.

We tested the translational activity of eIF4G-1a^{DM} and eIF4G-1e^{DM} by co-transfecting the cell lines with vectors expressing GFP and CVB4 2A protease. To distinguish between cap-dependent and internal initiation, we used vectors expressing GFP mRNA with either a normal 5'-untranslated region (pEGFP-C1) or an EMCV IRES (pIRES2-EGFP). The plasmid producing CVB3 2A protease also contained an EMCV IRES, so the protease was produced regardless of the cleavage status of eIF4G. eIF4G-1^{DM} isoforms were induced with Dox for 24 h. Cells were then transiently co-transfected with the GFP- and 2A protease-expressing vectors.

It was necessary to control for differences in GFP mRNA levels under the various conditions tested, since eIF4G strongly enhances the rate of initiation and since the stability of an mRNA is affected by its state of translation. We therefore obtained quantitative estimations of GFP mRNA levels by real-time PCR, normalizing for RNA yield and reverse transcription efficiency using GAPDH mRNA as endogenous control (Fig. 4-3). The threshold cycle (C_T) for all GAPDH cDNA samples fell between 18 and 20 (Fig. 4-3, panel A), whereas those for GFP cDNA fell between 15 and 18 (Fig. 4-3, panel B). Table 4-1 shows the average number of fluorescent cells in five randomly selected sites for each cell culture well. The levels of GFP mRNA for each cell type and condition were measured in duplicate. Rates of GFP synthesis were calculated by

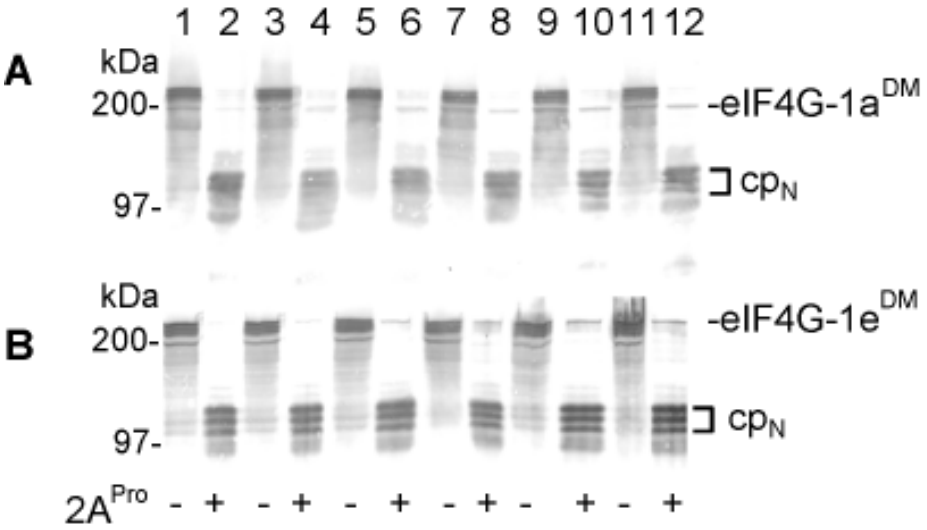
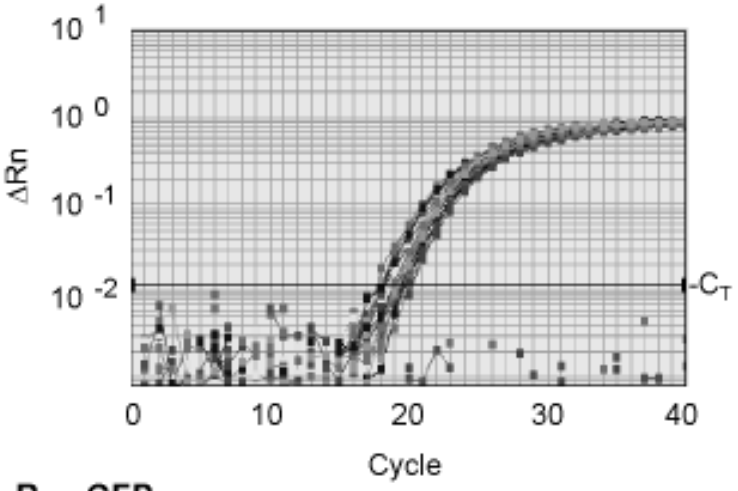

1 2 3 4 5 6 7 8 9 10 11 12
A
kDa
200-
97-
-eIF4G-1a^DM
cp_N
B
kDa
200-
97-
-eIF4G-1e^DM
cp_N
2A^Pro - + - + - + - + - + - +

Fig. 4-2. Synthesis of eIF4G-1a^{DM} and eIF4G-1e^{DM} in stably transfected HeLa cells as a function of increasing Dox. eIF4G-1a^{DM} (A) and eIF4G-1e^{DM}(lanes 1 and 2), or at Dox at 100 (lanes 3 and 4), 200 (lanes 5 and 6), 300 (lanes 7 and 8), 500 (lanes 9 and 10), 1000 (lanes 11 and 12) ng/ml.ell lysates were either treated (+) or mock-treated (-) with CVB4 2A protease on ice for 2 h. Samples were subjected to SDS-PAGE on 6% gels, transferred to a PVDF membrane, and immunoblotted with anti-eIF4G-1 antibodies.

A. GAPDH


10 1
10 0
10 -1
10 -2
ΔRn
-C_T
0
10
20
30
40
Cycle

B. GFP

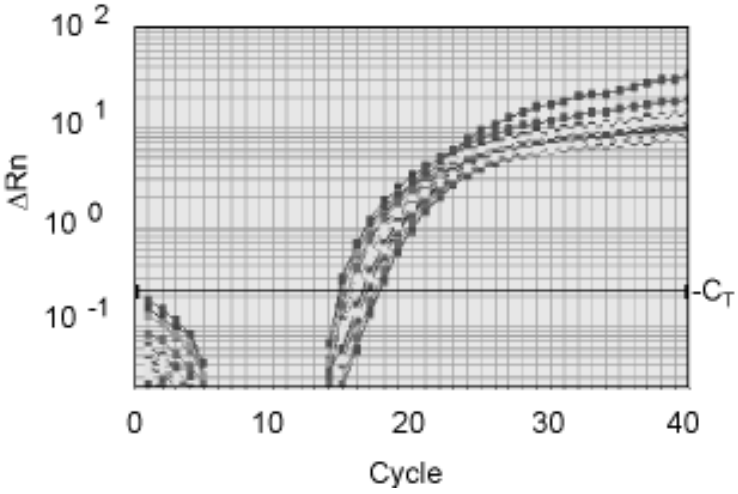

10 2
10 1
10 0
10 -1
ΔRn
-C_T
0
10
20
30
40
Cycle

Fig. 4-3. Measurement of GFP mRNA levels by real-time PCR. RNA was isolated from cells transiently transfected with plasmids expressing GFP or co-transfected with plasmids expressing GFP and 2A protease. The RNA was subjected to reverse transcription using oligo$(dT)_{15}$ as primer. Real-time PCR was used to amplify the endogenous control GAPDH cDNA (A) and GFP cDNA (B) as described under Experimental Procedures. ΔRn and C_T are defined in the ABI PRISM® 7700 Users Manual and correspond to the difference of fluorescence intensity between reactions with or without template and the cycle in which there occurs a significant increase of fluorescence intensity, respectively.

Table 4-1. Cap-dependent and IRES-driven synthesis of GFP in cells transfected with plasmids producing CVB4 2A protease as a function of eIF4G-1^{DM} induction

GFP synthesis[a]	Cells	Doxy-cycline[b]	CVB4 2A[c]	GFP Fluorescence[d]	GFP mRNA[e]	GFP Synthesis[f]
Cap-dependent	eIF4G-1a^{DM}	-	-	907±51	10.7±2.0	85±21
		+	-	881±37	8.9±0.8	99.6±13
		-	+	162±12	5.3±0.2	30±4
		+	+	533±42	7.3±1.5	73±21
	eIF4G-1e^{DM}	-	-	718±29	14.3±3.0	50.4±13
		+	-	745±19	17.3±2.3	43±7
		-	+	62±6.8	7.1±0.5	8.8±1.9
		+	+	228±7	7.4±0.7	31±3.7
IRES-dependent	eIF4G-1a^{DM}	-	-	226±44	9.8±1.5	23±9.5
		+	-	281±57	7.9±0.4	36±14
		-	+	360±32	3.7±0.01	98±17
		+	+	317±51	3.7±0.5	86±30
	eIF4G-1e^{DM}	-	-	349±44	7.5±0.4	47±11
		+	-	153±33	6.4±0.8	24±11
		-	+	392±27	3.2±0.1	122±16
		+	+	336±41	9.1±0.3	37±8.6

[a]Cells were transfected with either vector pEGFP-C1, for which GFP synthesis is cap-dependent, or vector pIRES2-EGFP, for which GFP synthesis is cap-independent due to the presence of the EMCV IRES in the mRNA.

Table 4-1 (continued)

[b]Dox was added to the indicated cells 48 h before fluorescence measurements.

[c]The indicated cells were transfected with the pVP12A vector expressing CVB4 2A protease 24 h before fluorescence measurements.

[d]The number of fluorescent cells was measured in the cell culture well using 16 adjacent microscopic field for each location. The averages ± SEM are given for five randomly selected locations.

[e]GFP mRNA levels were measured by real-time PCR and normalized to endogenous GAPDH mRNA levels.

[f]GFP synthesis rate over 24 h was determined by dividing the number of fluorescent cells by the GFP mRNA level. The SEM for the quotient was calculated from the formula

$$SEM_{\frac{F}{M}} = \frac{F}{M}\sqrt{\left(\frac{SD_F^2}{F^2}+\frac{SD_M^2}{M^2}\right)\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}$$

where F and M refer to number of fluorescent cells and GFP mRNA level, respectively.

dividing the number of fluorescent cells by the GFP mRNA level. The results are presented graphically in Fig. 4-4.

When mediated by cap-dependent translation, GFP synthesis was not significantly different in the presence or absence of Dox in eIF4G-1a^{DM} cells not expressing 2A protease (Fig. 4-4, panel A, GFP, open *versus* filled bars; $0.5 < p < 0.8$; eight degrees of freedom). This was also true for eIF4G-1e^{DM} cells (panel B, GFP; $0.5 < p < 0.8$). Expression of 2A protease dramatically decreased GFP synthesis in eIF4G-1a^{DM} cells uninduced with Dox (panel A, open bars, GFP + 2A *versus* GFP; $p < 0.05$). This was also true for eIF4G-1e^{DM} cells (panel B, open bars, GFP + 2A *versus* GFP; $p < 0.02$). However, induction of eIF4G-1a^{DM} almost completely rescued GFP expression (panel A, GFP + 2A, $0.05 < p < 0.1$). Similarly, induction of eIF4G- 1e^{DM} partially rescued GFP expression (panel B, GFP + 2A; $p < 0.01$).

When GFP was expressed by IRES-dependent translation, the results with 2A protease were quite different. In eIF4G-1a^{DM} cells not expressing 2A protease, GFP expression was not affected by Dox (panel C, IGFP, $0.2 < p < 0.5$). This was also true in eIF4G-1e^{DM} cells not expressing 2A protease (panel D, IGFP, $0.1 < p < 0.2$). Unlike cap-dependent GFP synthesis, IRES-dependent GFP synthesis increased dramatically with 2A protease expression in uninduced eIF4G-1a^{DM} cells (panel C, open bars, IGFP + 2A *versus* IGFP; $p < 0.01$). This was also true for uninduced eIF4G-1e^{DM} cells (panel D, open bars, IGFP + 2A *versus* IGFP; $p < 0.01$). This increase in GFP expression was suppressed by the induction of eIF4G-1e^{DM} by Dox (panel D, IGFP + 2A, $p < 0.01$) but not eIF4G-1a^{DM} (panel C, IGFP + 2A; $0.5 < p < 0.8$).

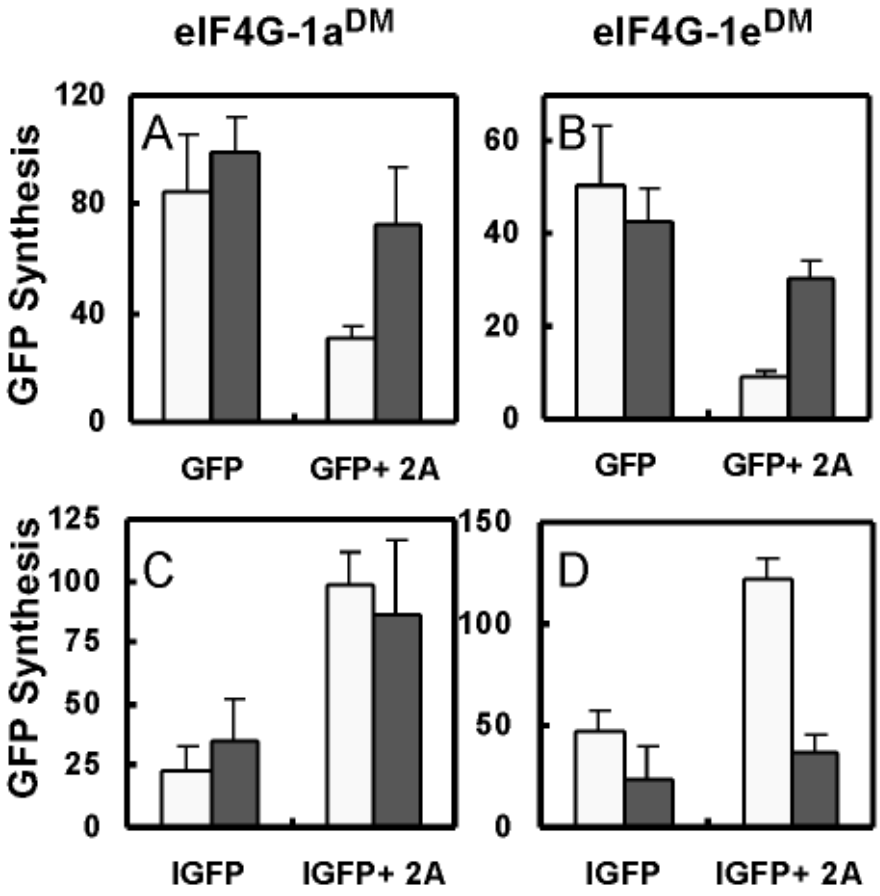

eIF4G-1a^DM
eIF4G-1e^DM
GFP Synthesis
A
B
C
D
120
80
40
0
60
20
125
100
75
50
25
150
GFP
GFP+ 2A
IGFP
IGFP+ 2A

Fig. 4-4. GFP synthesis in stable cell lines expressing eIF4G-1^{DM} isoforms.

GFP synthesis, as calculated in Table 4-1, was measured in cells stably expressing either eIF4G-1a^{DM} (A and C) or eIF4G-1e^{DM} (B and D). Expression of eIF4G-1^{DM} was either uninduced (*open bars*) or induced with Dox for 24 h (*filled bars*). Cells were then co-transfected with vectors transiently expressing 2A protease (pVP12A) and GFP by either cap-dependent (pEGFP-C1; A and B) or IRES-dependent (pIRES2-EGFP; C and D) translation, and GFP synthesis was measured after an additional 24 h.

D. Discussion

We chose to create tetracycline-regulated eIF4G-1^{DM}-expressing cell lines for two reasons. First, it was necessary to restrict the overexpression of eIF4G-1 to specific, short periods during which measurements would be taken, since long-term overexpression of eIF4G-1 tends to cause the transformation of cells. Second, we needed to solve the problem that the transfection does not occur in 100% of the cells. Unfortunately, despite our surveying of many clones, the maximal expression level of eIF4G-1^{DM} was only 5 to 10% of the endogenous level.

Co-transfection of the cell lines with plasmids that express GFP and 2A protease indicated that eIF4G-1^{DM} isoforms substantially protect cap-dependent protein synthesis from inhibition by 2A protease *in vivo*. The response of GFP synthesis to 2A protease was opposite depending on how translation of GFP mRNA was initiated. Cap-dependent GFP synthesis was dramatically inhibited by 2A protease expression whereas IRES-dependent GFP synthesis was greatly increased. This latter result agrees with the observation that initiation of both IRES-containing and uncapped mRNAs is either unaffected or even stimulated upon eIF4G cleavage (Liebig and others 1993; Ohlmann and others 1995; Borman and others 1997). This may be due to two factors: i) cp$_C$ is more active than intact eIF4G-1 for initiation of IRES-containing mRNAs, and ii) the inhibition of cap-dependent translation of exogenous mRNAs by 2A protease makes limiting components of the translational machinery available for IRES-driven synthesis.

Dox-induced expression of eIF4G-1e^{DM} substantially reversed the effects of 2A protease in both cases: inhibition of cap-dependent translation by 2A protease was relieved and stimulation of IRES-dependent translation by 2A protease was suppressed.

For reasons we do not understand, eIF4G-1a^{DM} was able to restore cap-dependent translation but not suppress the IRES-dependent stimulation.

E. References

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CHAPTER 5

DELAY OF PICORNAVIRAL REPLICATION IN HELA CELLS EXPRESSING A 2A PROTEASE-RESISTANT FORM OF EIF4G-1

A. Introduction

A variety of viral strategies are utilized to suppress host and promote viral protein synthesis, many of which act through mechanisms involving the initiation of translation (Gale and others 2000; Schneider and Mohr 2003). Several components of the 48S translational initiation complex can become viral targets to usurp the host translational machinery or antagonize antivirus responses. Phosphorylation of eIF2α restricts translation of viral RNAs, but a wide variety of viruses express either RNAs or proteins that antagonize this host defense mechanism (Pe'ery and Mathews 2000). eIF4E, which is required for host but not picornaviral protein synthesis, is sequestered by poliovirus- and EMCV-activated dephosphorylation of 4E-BP1 (Gingras and others 1996). eIF4E phosphorylation is inhibited by adenoviral disruption of the interaction between eIF4G and Mnk1 (Cuesta and others 2000). Poly(A)-dependent host mRNA translation is antagonized by replacement of PABP with the rotaviral protein NSP3, which is specific for rotaviral RNAs (Piron and others 1999).

One of the most studied mechanisms by which host protein synthesis is shutoff in favor of viral protein synthesis is the cleavage of eIF4G by picornviral 2A or L proteases (Ehrenfeld 1996), as discussed in Chapter 1 and Chapter 2. The prior studies, however, have dealt with correlations rather than cause-and-effect relationships between eIF4G levels and the virus-induced changes in translational specificity. To address this directly, we created eIF4G-1 variants, containing either a single amino acid substitution (eIF4G-1^{SM}) or two substitutions (eIF4G-1^{DM}) that are highly resistant to 2A protease *in vitro* (Lamphear and Rhoads 1996; Zhao and others 2003) in Chapter 2. Full functional activity of eIF4G-1^{DM} was demonstrated *in vitro* by its ability to restore cap-dependent

translation to a 2A protease-pretreated rabbit reticulocyte system (Chapter 2). An isoform containing the binding site for PABP, eIF4G-1e^{DM}, was more active in this assay than an isoform lacking it, eIF4G-1a^{DM}, but only with polyadenylated mRNA (Chapter 3). Functional activity was also demonstrated *in vivo* with stably transfected HeLa cells expressing eIF4G-1^{DM} from a tetracycline-regulated promoter (Chapter 4). In transient cotransfection experiments, cap-dependent protein synthesis was inhibited by 2A protease and partially rescued by either eIF4G-1a^{DM} or eIF4G-1e^{DM} induction. IRES-dependent protein synthesis, on the other hand, was stimulated by 2A protease and suppressed by induction of eIF4G-1e^{DM} (but not eIF4G-1a^{DM}).

These *in vitro* and *in vivo* studies with 2A protease-resistant eIF4G-1 have not, however, tested whether eIF4G cleavage affects the change in translational specificity during an actual virus infection. In the present chapter, we infected the HeLa cell line inducibly expressing eIF4G-1e^{DM} with various strains of poliovirus and found a reduction in infectivity as well as extracellular virus production compared to control cells. A more detailed investigation with one strain, PV-1 Mahoney MMd, showed that eIF4G-1e^{DM} induction prolonged host protein synthesis, delayed viral protein synthesis, and reduced virus production.

B. Experimental Procedures

1. Cell Culture

Dulbecco-modified Eagle medium (DMEM), cell culture supplements, geneticin, and bovine calf serum were obtained from Gibco BRL (Grand Island, NY). Chang's human conjunctival cells (HCC) were obtained from the ATCC (Rockville, MD) and

were maintained as previously described in DMEM (Langford and others 1995). The stable HeLa cell line that expresses eIF4G-1e^{DM} from a tetracycline-inducible promoter described in Chapter 4 (Zhao and others 2003) is termed HeLa-G in this chapter. The cell line from which HeLa-G cells were derived is termed HeLa-O in this chapter (HeLa Tet-On, Clonetch, Palo Alto, CA). HeLa-O cells were cultured in DMEM supplemented with 100 μg/ml geneticin, 40 μg/ml gentamycin (Sigma Chemical Co., St Louis, MO), and 10% bovine calf serum in a humidified 5% CO_2 atmosphere at 37°C. HeLa-G cells were maintained in the same medium except containing 1 μg/ml puromycin. Expression of eIF4G-1e^{DM} was induced by adding 1 μg/ml Dox for 24 h.

2. Viruses

Poliovirus type 3 (PV-3) vaccine strains Leon and Saukett, the Indiana strain of vesicular stomatis virus (VSV), and adenovirus type 7 (Ad 7) were obtained from the ATCC. The PV-1 Mahoney antibody escape mutant (MMd), which is neutralized and immunoprecipitated by PV-1 Sabin-specific monoclonal antibodies (Blondel and others 1986), was obtained from Radu Crainic (Institut Pasteur, Paris, France). PV-1 strain Sabin was obtained from Marie Chow (University of Arkansas School of Medical Sciences). All viruses were passaged twice in HCC, aliquoted, and stored at -100°C.

3. Plaque assays

Virus titers were determined in both virus stocks and in cell culture supernatants from HeLa-O and HeLa-G cells by infection of at least triplicate cultures of HCC. The number of infectious virons/ml was determined after 48 h of incubation (Langford and others 1991). Each virus stock contained $\geq 10^7$ HCC plaque-forming units (pfu)/ml.

4. Viral replication efficiency in HeLa cells

The infectivity of various viral strains was determined in HeLa-O and HeLa-G cells induced with Dox for 24 h (subsequently termed OD and GD, respectively) or mock induced (O and G, respectively) by standard methods (Reid 1968). Briefly, a 10-fold dilution series of virus stocks was prepared in DMEM with or without 1 μg/ml Dox, and 50 μl of each dilution were added to quadruplicate cultures of either G, GD, O, or OD cells in 96-well tissue culture plates (Sarstedt, Inc., Newton, NC). After 24 h of incubation, the culture medium was removed and stored at -100°C for plaque assay. Concomitantly, the cell cultures were stained and fixed with 0.1% crystal violet and 20% methanol. Excess dye was removed from the cultures by rinsing three times in tap water. The optical density (OD_{500}) of the cell-associated dye was determined spectrophotometrically using a Microtiter Plate Autoreader (Cayman Chemical, Ann Arbor, MI). The 50% tissue culture cytolytic dose ($TCCD_{50}$)/ml was determined for each viral strain in each cell type (Reed and Muench 1938). Viral replication efficiency is expressed as moi/$TCCD_{50}$, where moi (multiplicity of infection) is derived from plaque assays of the original virus stocks on HCC cultures, and $TCCD_{50}$ is derived from infection of the HeLa cell cultures.

5. Metabolic labeling of proteins with [^{35}S]Met

The incorporation of [^{35}S]Met into proteins by MMd-infected HeLa-O and HeLa-G cells was compared by pulse-labeling experiments over an 8-h single replication cycle. Cells were induced or mock induced with Dox for 24 h in 12-well plates (Sarstedt, Inc., Newton, NC; 3 to 5×10^5 cells/well) prior to infection with MMd at moi of 10 pfu/cell. After 1 h, the cultures were washed with three 0.5-ml aliquots of PBS to

remove unadsorbed virus. The cells were labeled for 30 min in 1 ml of Met-deficient medium (Gibco BRL) containing 30 μCi/ml [^{35}S]Met (ICN) at various times postinfection (pi), beginning immediately after removal of unadsorbed virus. Cells were collected, centrifuged, washed with PBS, and lysed with 50 μl lysis buffer consisting of 50 mM Tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethylsulfonylfluoride, 1% Nonidet P-40, and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates (10 μg of protein) were resolved by SDS-PAGE on 8% gels and stained with Coomassie blue. The radioactivity was analyzed using a Storm 260 Phosphoimager (Molecular Dynamics). The intensity of selected bands was quantitated using ImageQuant software (Molecular Dynamics).

6. Trichloroacetic acid precipitation of [^{35}S]Met-labeled cell lysates

One microliter of cell lysate was spotted on an S&S filter (Schleicher & Schuell Inc., Keene, NH). The filter was place into cold 10% trichloroacetic acid (TCA), washed with 10% TCA three times and 5% TCA twice, heated in 5% TCA at 90°C for 10 minutes, washed with 5% TCA once and acetone once, and dried at room temperature. Dried filter papers were placed in vials, and 4 ml of scintillation solution containing 5.5 g Permablend™ (Packard Instrument Co. Inc, Downers Grove, Ill) in 1 l toluene were added to each vial. The radioactivity was measured in a LS 6500 scintillation counter (Beckman) and normalized to protein concentration.

7. One-cycle poliovirus growth curves

HeLa-O and HeLa-G cells were infected with MMd (10 pfu/cell) as described above. At various times pi, the cells and a 200-μl aliquot of medium were collected. The cells were washed three times with PBS, suspended in 100 μl of H_2O containing protease

inhibitor cocktail, and submitted to five freeze-thaw cycles. The number of infectious virions in the medium samples (extracellular viral titer) and cell lysates (intracellular viral titer) was determined by plaque assay.

8. Protein analysis

Protein concentration was measured by the Bradford protein assay (Bradford 1976). Lysates obtained from cells used to develop PV-1 growth curve containing 10 μg total protein were subjected to SDS-PAGE on 6% gels, and the proteins were transferred to Immobilon-P Transfer membrane (Millipore Corporation, Bedford, MA). Western blot analysis was performed with antibody against peptide 7 of eIF4G-1 described previously (Yan and others 1992**).**

C. Results

1. Viral replication efficiency and production are reduced in cells expressing eIF4G-1e^{DM}

Our previous characterization of HeLa-G cells indicated that the maximal levels of eIF4G-1e^{DM} upon induction with Dox were only ~5% of endogenous eIF4G-1 levels (Chapter 4). Despite this low level, we were encouraged to test this cell line for viral infection because induction of eIF4G-1e^{DM} caused substantial rescue of cap-dependent translation upon cotransfection of vectors expressing Coxsakievirus 2A protease and a reporter protein.

We tested viral replication efficiency and virus production for various poliovirus strains in Dox-induced HeLa-G (GD) and HeLa-O (OD) cells or control HeLa-G (G) and HeLa-O (O) cells (Fig. 5-1). Viral replication efficiency was judged by the moi required

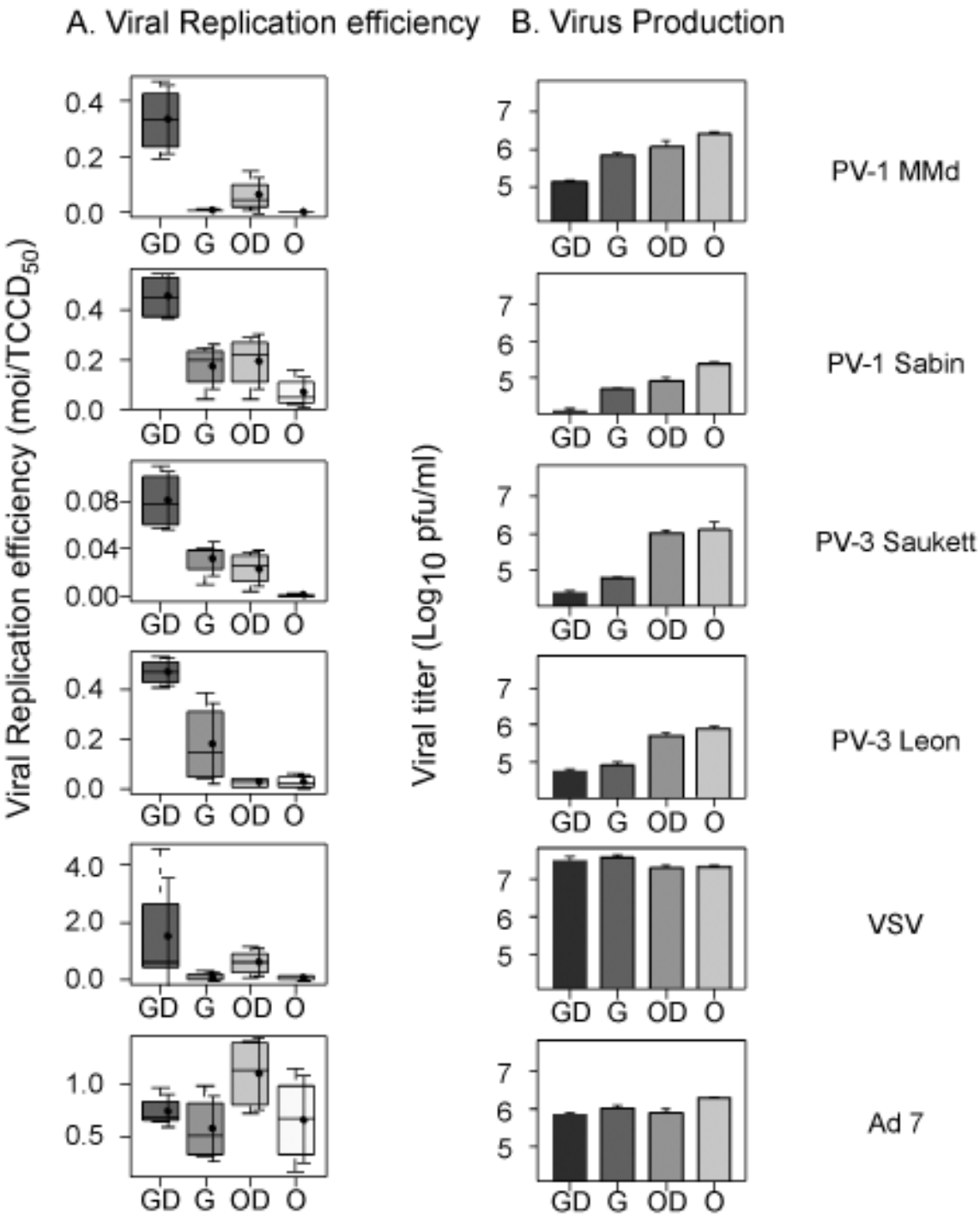

A. Viral Replication efficiency
B. Virus Production
Viral Replication efficiency (moi/TCCD50)
Viral titer (Log10 pfu/ml)
GD G OD O
PV-1 MMd
PV-1 Sabin
PV-3 Saukett
PV-3 Leon
VSV
Ad 7

Fig. 5-1. Polioviral replication efficiency and production in HeLa cells is reduced by expression of eIF4G-1e^{DM}. HeLa-G and HeLa-O cells were incubated in 96-well plates for 24 h in DMEM either with Dox (GD and OD, respectively) or without Dox (G and O, respectively). Quadruplicate cultures were infected with the indicated viral strains at seven moi differing by factors of 10, and incubation was continued for 24 h. (A) Viral replication efficiency, represented as the moi required to reach $TCCD_{50}$, is plotted by in box plots. The horizontal line inside the box represents the median, the box represents values falling between the lower and upper quartiles, and the whiskers extend to the minimum and maximum values. For each measurement, the mean ± standard deviation is also shown by the solid circle connected to error bars. (B) The medium was removed at 24 h pi and used for determination of extracellular virus production by plaque assay on confluent HCC cultures. The titer for the well closest to the $TCCD_{50}$ is shown.

to obtain $TCCD_{50}$ in the cell cultures (moi/$TCCD_{50}$; panel A). For the most important comparison, GD *versus* G (*i.e.*, the same cell line with or without Dox induction for 24 h), the moi required for $TCCD_{50}$ was significantly elevated for infection with the laboratory strain MMd (0.33 ± 0.12 *versus* 0.007 ± 0.004; $p < 0.013$). Similarly, there was a significant increase in moi/$TCCD_{50}$ for GD cells infected with the vaccine strains Sabin (0.45 ± 0.09 *versus* 0.17 ± 0.09; $p < 0.005$), Saukett (0.081 ± 0.025 *versus* 0.031 ± 0.015; $p < 0.018$), and Leon (0.47 ± 0.05 *versus* 0.18 ± 0.16; $p < 0.034$) strains. By contrast, no significant differences between GD and G were observed for the control viruses VSV ($p = 0.249$) and Ad 7 ($p = 0.388$). These results indicate that expression of eIF4G-1e^{DM} reduces infectivity of the strains tested.

To determine whether the expression of eIF4G-1e^{DM} also inhibited virus production, we measured viral titers in the culture medium by plaque assay (panel B). All of the polioviral strains had significant reduction in viral titer for GD *versus* G cells and also for G *versus* OD cells. However, for the control viruses VSV and Ad 7, there were no significant differences among the four HeLa lines and growth conditions.

2. Host protein synthesis is prolonged and viral protein synthesis is delayed by expression of eIF4G-1e^{DM}

The level and integrity of eIF4G-1 were monitored by western blotting at various times postinfection (Fig. 5-2). eIF4G-1 was completely cleaved at 2 h postinfection in both OD and O cells (panels C and D), but it persisted until 5 h in GD cells (panel A). The figure also shows that expression of eIF4G-1 is slightly leaky in G cells (panel B), although there is a significant difference in expression between G and GD cells.

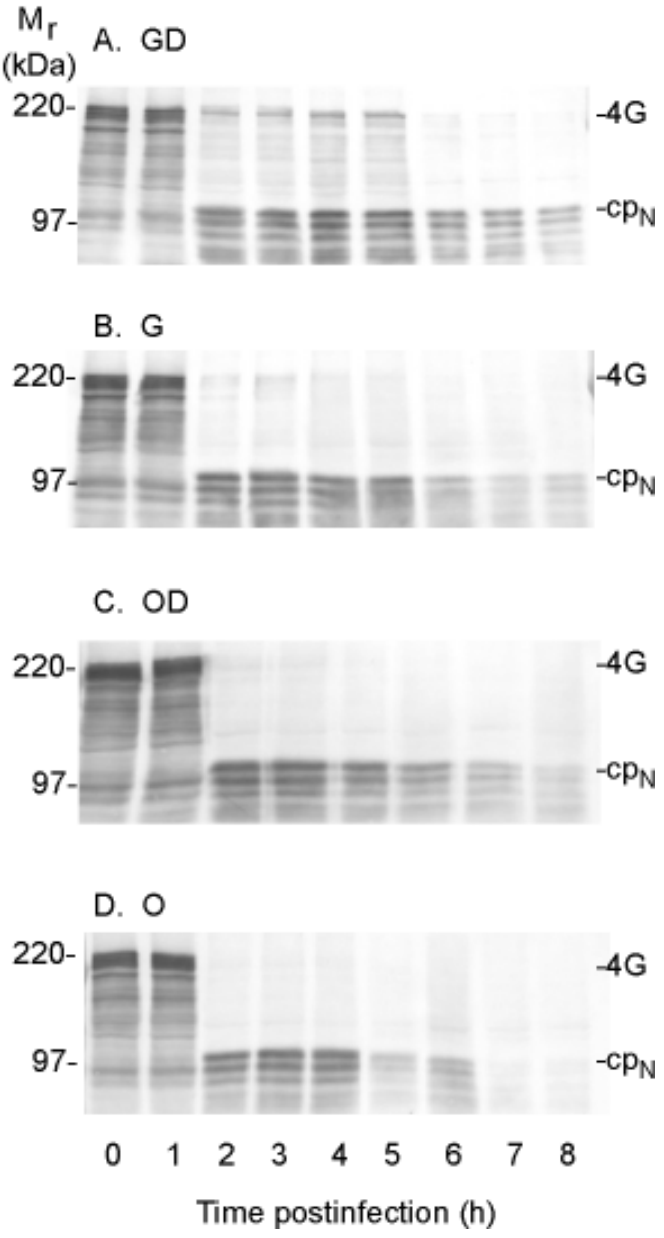

Mr
(kDa)
A. GD
220-
97-
-4G
-cpN
B. G
220-
97-
-4G
-cpN
C. OD
220-
97-
-4G
-cpN
D. O
220-
97-
-4G
-cpN
0 1 2 3 4 5 6 7 8
Time postinfection (h)

Fig. 5-2. Integrity of eIF4G-1 in HeLa-G and HeLa-O cells infected with MMd virus. HeLa-G and HeLa-O cells were cultured in 6 well plates and induced or mock-induced with Dox for 24 h before MMd infection at 10 moi. Cells were collected at the indicated time points postinfection. Cell lysates were subjected to SDS-PAGE on 6% gels and immunoblotted with anti-eIF4G-1 antibodies. 4G and cp_N refer to intact and N-terminal cleavage products, respectively.

We further investigated the effect of eIF4G-1e^{DM} expression on cellular and viral protein synthesis in GD, G, OD, and O cells infected with MMd over one generation cycle. A pulse-labeling experiment with [^{35}S]Met was performed to monitor rates of protein synthesis and/or processing. The total protein synthesis rate was assayed by TCA precipitation (Fig. 5-3). Protein synthesis was slightly higher in GD and G cells between 0-2 h pi than in OD and O cells. Subsequently, synthesis was higher in OD and O cells between 3-6 h and it dropped after 6 h pi. The protein synthesis rate of GD and G cells remained higher until 8 h pi.

The proteins labeled each hour were separated by SDS-PAGE (Fig. 5-4, panel A-D). Synthesis of most host proteins was shut down by 4 h pi, at which time viral proteins were first detected. Although the patterns of host and viral proteins were similar for the four conditions, subtle differences could be detected. For instance, in O and OD cells, viral protein synthesis was strongest at 4 h pi, but in G and GD cells, the peak of synthesis did not occur until 5 h pi.

To visualize changes in protein synthesis patterns more clearly, we chose one host protein band (indicated by H1 in Fig. 5-4, panel D) and three viral protein bands (V1-V3 in panel B) and quantitated their intensities by PhosphoImager and ImageQuant analysis as a function of time pi (panel E-H). Based on the mobilities of these viral proteins, Viral Protein 1 (100 kDa), Viral Protein 2 (70 kDa), and Viral Protein 3 (30 kDa) are likely to be P1, 3CD, and VP3, respectively (Bell and others 1999). Although Host Protein 1 synthesis no longer occurred beyond 3 h pi in OD and O cells, it persisted to 4 h pi in G cells and to 5 h pi in GD cells (panel E). The three viral protein bands behaved just the opposite; their appearance was delayed by expression of eIF4G-1e^{DM}. The maximal

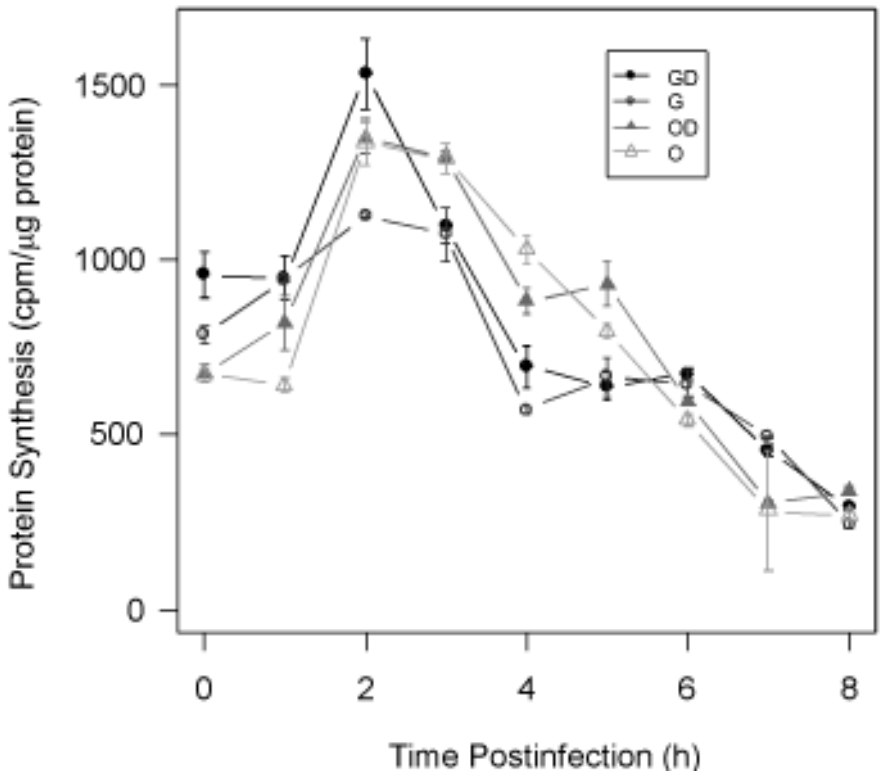
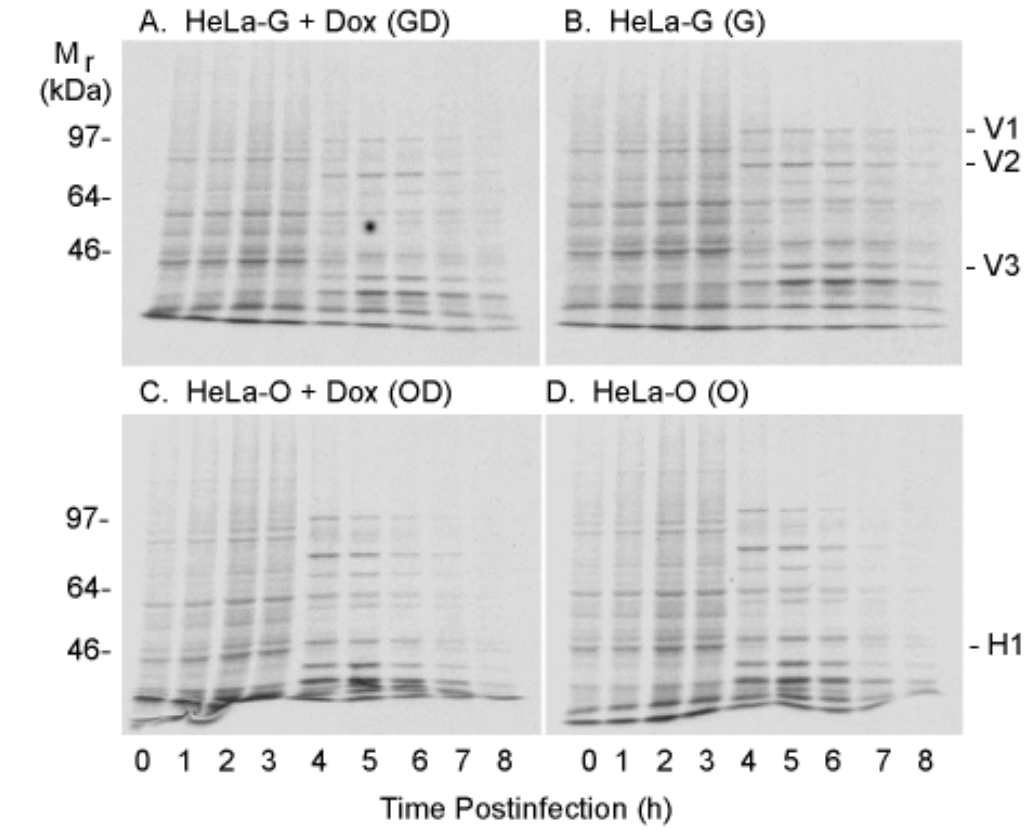

GD
G
OD
O
Protein Synthesis (cpm/μg protein)
1500
1000
500
0
0
2
4
6
8
Time Postinfection (h)

Fig. 5-3. Total protein synthesis in MMd virus-infected HeLa-G and HeLa-O cells. HeLa-G and HeLa-O control cells were pulse labeled for 30 min with [^{35}S]Met while infected with MMd virus as described in Experimental Procedures. Cells were collected at the indicated time points. Radioactivity incorporated into protein was assayed by TCA precipitation as described in Experimental Procedures.


A. HeLa-G + Dox (GD)
B. HeLa-G (G)
C. HeLa-O + Dox (OD)
D. HeLa-O (O)
Mr (kDa)
97-
64-
46-
- V1
- V2
- V3
- H1
0 1 2 3 4 5 6 7 8
Time Postinfection (h)

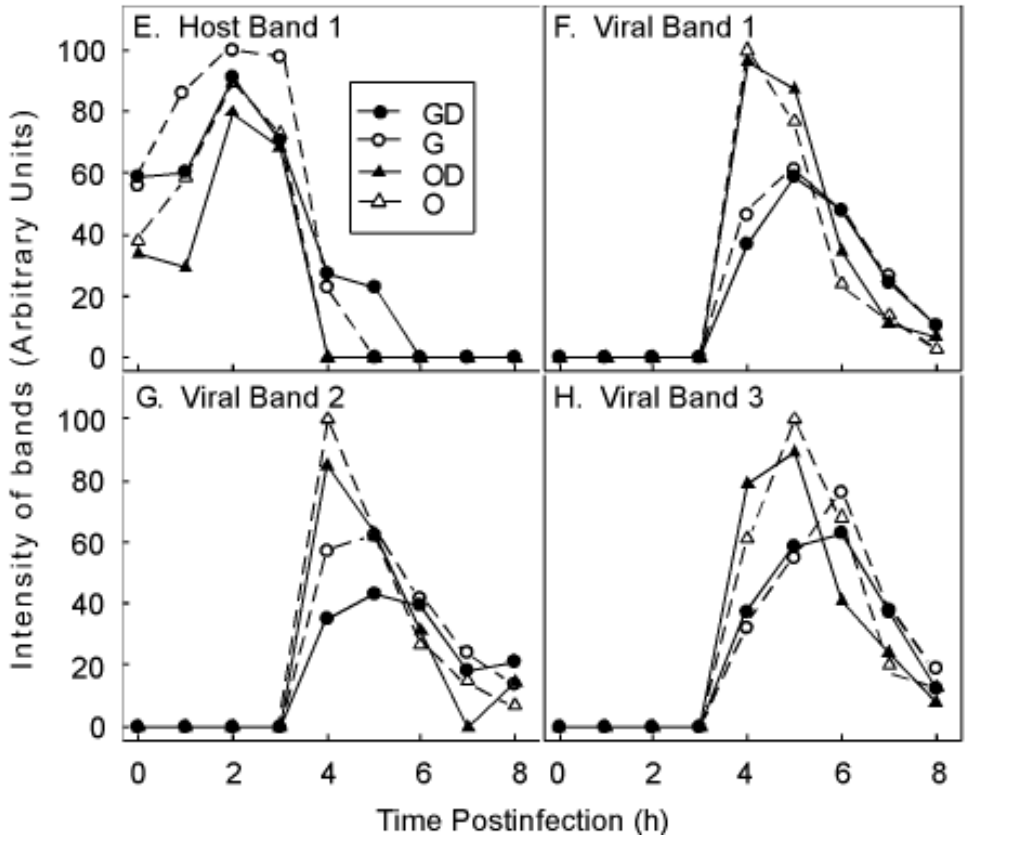

E. Host Band 1
F. Viral Band 1
G. Viral Band 2
H. Viral Band 3
GD
G
OD
O
Intensity of bands (Arbitrary Units)
0 20 40 60 80 100
0 2 4 6 8
Time Postinfection (h)

Fig. 5-4. The shutoff of host protein synthesis and onset of viral protein synthesis are delayed in cells expressing eIF4G-1e^{DM}. HeLa-G and HeLa-O cells, each either pretreated with Dox or mock induced as in Fig. 5-1, were pulse-labeled with [^{35}S]Met for 30 min at the indicated time points following infection with PV-1 Maloney MMd. Aliquots of 10 μg of protein were subjected to SDS-PAGE on 8% gels. The images produced by exposing the gels to a PhosphoImager screen for 2 d are shown in panels A-D. The intensities of selected host (H1) and viral (V1-V3) bands were quantitated by ImageQuant software (Molecular Dynamics) in panels E-H.

labeling of Viral Proteins 1 and 2 occurred at 4 h pi in OD and O cells but at 5 h pi in GD and G cells (panels F and G). For Viral Protein 1, there was little difference between GD and G cells (panel F), but for Viral Protein 2, the amount was diminished in GD *versus* G cells (panel G). Viral Protein 3 appeared later than Viral Proteins 1 and 2 in all cells (panel H). However, it was further delayed in GD and G cells, peaking at 6 h pi, compared with OD and O cells, where the maximum was at 5 h pi.

3. Expression of eIF4G-1e^{DM} reduces extracellular viral production more than intracellular viral production

Finally, we wished to examine in more detail the observation (Fig. 5-1, panel B) that virus accumulation in the culture medium is dramatically decreased in cells expressing eIF4G-1e^{DM}. Cells were infected with MMd, and infectious viral particles from the culture medium and from cell lysates were measured by plaque assay every hour pi. Intracellular viral titer was significantly lower at 5 h pi in G and GD cells compared with O and OD cells (Fig. 5-5, panel A). Extracellular viral titer was highest by 8 h pi in cells not expressing eIF4G-1e^{DM} (panel B, OD and O), less in cells having leaky eIF4G-1e^{DM} expression (G), and lowest in cells with demonstrable eIF4G-1e^{DM} expression (GD).

D. Discussion

As noted in Chapter 1 and the Introduction of Chapter 2, a wide variety of experimental findings have brought into question the importance of eIF4G-1 cleavage in mediating the host protein synthesis shutoff upon picornaviral infection. To test this

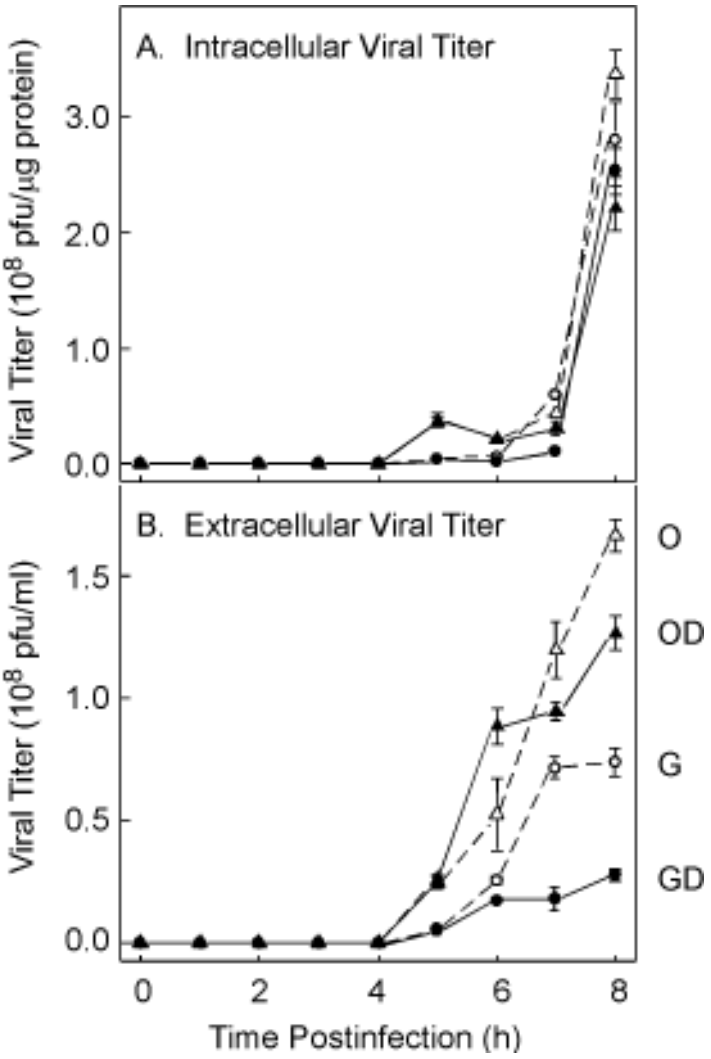

A. Intracellular Viral Titer
Viral Titer (10^8 pfu/μg protein)
3.0
2.0
1.0
0.0
B. Extracellular Viral Titer
Viral Titer (10^8 pfu/ml)
1.5
1.0
0.5
0.0
O
OD
G
GD
0
2
4
6
8
Time Postinfection (h)

Fig. 5-5. Virus production is reduced in HeLa cells expressing eIF4G-1e^{DM}.

HeLa-G and HeLa-O cells were cultured and infected with MMd at 10 pfu/cell as described in Experimental Procedures. Both cells and culture media were collected at the indicated time points. The viral titers from cells and media were quantified by plaque assay on confluent HCC cultures.

directly, we have altered the amino acid residues targeted by 2A protease, the major picornaviral protease responsible for eIF4G-1 cleavage, to produce a form of eIF4G-1 that is 1000- to 10,000-fold resistant to 2A protease i*n vitro* (Chapter 2; Lamphear and Rhoads 1996; Zhao and others 2003). Unfortunately, maximal induction of eIF4G-1e^{DM} in HeLa-G cells is only ~5% that of endogenous eIF4G-1. Another limitation of this system is that expression of eIF4G-1eDM is apparently leaky in the absence of Dox (Fig. 5-2, panel B). Thus, the translational properties of HeLa-G cells upon 2A protease challenge are intermediate between those of HeLa-O and Dox-induced HeLa-G cells. Despite this limitation, our results show that expression of intact eIF4G-1 inhibits the production and infectivity of several poliovirus strains, prolongs host protein synthesis, and delays viral protein synthesis. Previous studies have shown that eIF4G cleavage inhibits cap-dependent translation (Liebig and others 1993; Ohlmann and others 1995; Borman and others 1997). It is therefore logical that retention of a small amount of intact eIF4G could allow continued translation of some highly cap-dependent mRNAs, *e.g.*, Host Protein 1 in Fig. 5-4.

It is more difficult to rationalize the delay of viral protein synthesis by low amounts of eIF4G-1e^{DM}. The interaction between eIF4G and the IRES plays an important role in recruitment of picornavirus RNA to the 48S translation initiation complex. A single point mutation in secondary structure domain V of the IRES impairs binding to eIF4G and reduces translation of polioviral RNA (Ochs and others 2003). We and others have shown that the C-terminal cleavage product of eIF4G-1, cp$_C$, has a positive effect on entero- and rhinovirus IRES mediated translation (Ohlmann and others 1995; Ziegler and others 1995; Borman and others 1997). This is probably because

picornaviral IRESes bind more readily to the central portion of eIF4G than to intact eIF4G (Pestova and others 1996; Kolupaeva and others 1998; Lomakin and others 2000). However, since eIF4G-1e^{DM} is expressed at very low amounts, and furthermore is highly resistant to cleavage, the intracellular concentration of cp$_C$ should be the same in virus-infected HeLa-G, -GD, -O, and -OD cells. This has, in fact, been demonstrated for these cells transiently transfected with a vector expressing Coxsackievirus protease 2A (Chapter 4 and Zhao and others 2003). Thus, a difference in cp$_C$ levels cannot be responsible for the slower rate of viral protein synthesis in HeLa G cells (Fig. 5-4, panel F-H). We offer four possibilities for the delay in viral protein synthesis by eIF4G-1e^{DM}: 1) the small amount of host translation still occurring in Dox-induced HeLa-G cells prevents the full utilization of the protein synthesis machinery by viral mRNAs; 2) the existence of intact eIF4G-1e^{DM} competes with cp$_C$ for IRES-driven translation; 3) the IRES of attenuated poliovirus strains binds aberrantly to eIF4G-1e^{DM}; and 4) host proteins made in Dox-induced HeLa-G cells somehow antagonize viral mRNA translation and restrict viral replication. Regardless of the mechanism, the data in Fig. 5-4 indicate that the presence of intact eIF4G-1 acts to suppress the changeover from host to viral protein synthesis.

Although the effects on protein synthesis of eIF4G-1e^{DM} expression are modest, the consequences for virus production are striking. The 5-fold difference in extracellular virus between HeLa-G induced with Dox and HeLa-O after 8 h (Fig. 5-5, panel B) contrasts sharply with the synthesis rates of viral proteins, which are similar (Fig. 5-4, panel A *versus* D). Furthermore, even though there are differences between GD, G, OD, and O cells with respects to intracellular viral titer (Fig. 5-5, panel A), these are small

compared with the differences in extracellular viral titer (Fig. 5-5, panel B). This suggests that intact eIF4G-1 (or host proteins synthesized only in its presence) may interfere with late stages of virus production. This protective effect could play a defensive role during natural picornaviral infection to inhibit cell death and may be a factor in determining tissue tropism and virulence.

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CHAPTER 6

EXPRESSION OF EIF4G-1A^{DM} AND EIF4G-1E^{DM} IN A BACULOVIRUS SYSTEM

A. Introduction

Because of the difficulty of expressing full-length eIF4G-1 in *E. coli* we used a two-phase *in vitro* translation system to study the function of eIF4G-1a^{DM} and eIF4G-1e^{DM} (Chapter 2). The two-phase system is effective but has some limitations. First, the expression level of full-length eIF4G-1a^{DM} and eIF4G-1e^{DM} is hard to control. Second, there is always degraded eIF4G-1 present, as observed by western blotting. Third, the signal of globin synthesis is weak because nonradioactive Met is added during synthesis of eIF4G-1 in Phase I, and it dilutes the Antecol and others Met added in Phase II. Fourth, the synthesis of globin cannot be measured by scintillation spectrometry because eIF4G-1a^{DM} or eIF4G-1e^{DM} is still synthesized in Phase II. Fifth, the translational activity of 2A protease-pretreated MDL is compromised after the long incubation period of Phase I. We therefore sought another means of producing eIF4G-1^{DM} isoforms.

The baculovirus expression system is ideal for expression of full-length eIF4G-1 because it is a eukaryotic expression system, and therefore, the recombinant proteins are processed, modified, and targeted to their appropriate intracellular locations (O'Reilly and others 1992; King and Possee 1992; Luckow 1993). High levels of heterologous gene expression can be obtained compared to other eukaryotic expression systems. The recombinant proteins are soluble and easily recovered from infected cells late in infection when host protein synthesis is diminished. Baculoviruses have a restricted host range, limited to specific invertebrate species, which makes them safe to work with. Prolific cell lines that grow well in suspension cultures are available, permitting the production of recombinant proteins in large-scale bioreactors.

Baculovirus genomes are circular, double-stranded DNA, ranging from 100 to 200 kb, with multiple recognition sites for many restriction endonucleases. When AcMNPV was developed as an expression vector, there were no known restriction endonucleases that lacked recognition sites in this genome, so allelic replacement was adopted to insert foreign genes into the genome (Smith and others 1983b, Pennock and others 1984, Maeda and others 1985). As a result, recombinant baculoviruses are traditionally constructed in two steps. First, the gene to be expressed is cloned into a transfer vector downstream from a baculovirus promoter that is flanked by baculovirus DNA derived from a nonessential locus, usually the polyhedrin gene. This vector, with circular wild-type genomic viral DNA, is then transfected into insect cells. Typically, 0.1% to 1% of the resulting progeny are recombinant, with the heterologous gene inserted into the genome of the parent virus by homologous recombination *in vivo*. Higher percentages of recombinantion (about 30%) can be achieved by linearizing the baculovirus genome at one or more unique restriction sites near the target for insertion of the foreign gene (Kitts and others 1990; Hartig and Cardon 1992). Even higher recombinantion frequencies can be obtained by linearizing viral DNA that is missing an essential portion of the baculovirus genome downstream from the polyhedrin gene which is complemented by the transfer vector (Kitts and Possee 1993).

A rapid and efficient method to generate recombinant baculoviruses was developed by using site-specific transposition instead of homologous recombination (Luckow and others 1993) (Fig. 6-1). The gene of interest is cloned into a pFastBac transfer vector, and the transfer vector is used to transform competent DH10Bac cells that contain the bacmid with a mini-*att*Tn7 target site and a helper plasmid. The mini-Tn7

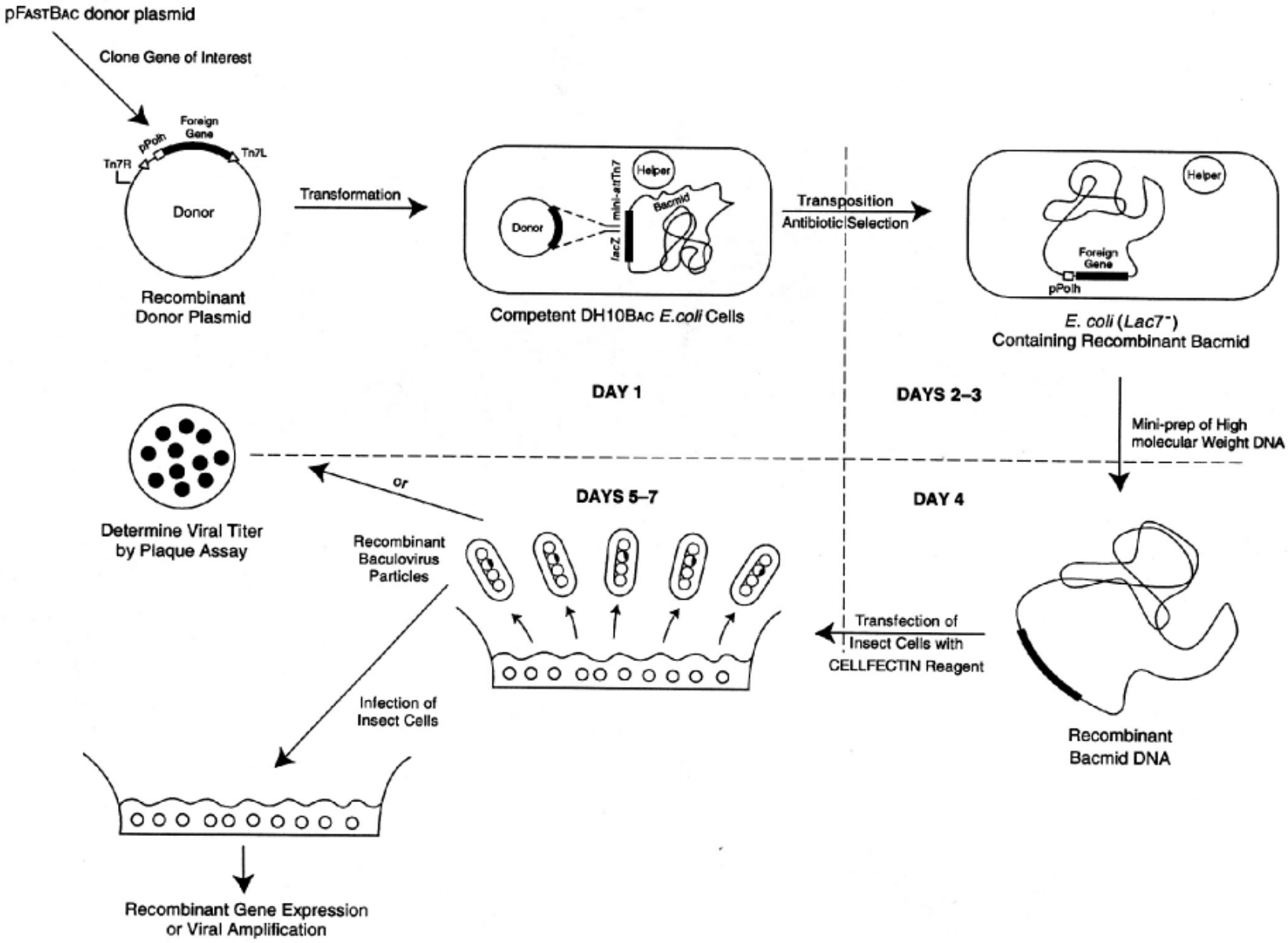

pFastBac donor plasmid
Clone Gene of Interest
Foreign Gene
pPolh
Tn7R
Tn7L
Donor
Recombinant Donor Plasmid
Transformation
Donor
mini-attTn7
lacZ
Helper
Bacmid
Competent DH10Bac E.coli Cells
Transposition
Antibiotic Selection
Helper
Foreign Gene
pPolh
E. coli (Lac7⁻) Containing Recombinant Bacmid
DAY 1
DAYS 2–3
Mini-prep of High molecular Weight DNA
or
DAYS 5–7
DAY 4
Determine Viral Titer by Plaque Assay
Recombinant Baculovirus Particles
Transfection of Insect Cells with CELLFECTIN Reagent
Recombinant Bacmid DNA
Infection of Insect Cells
Recombinant Gene Expression or Viral Amplification

Fig. 6-1. Generation of recombinant baculoviruses and protein expression with the Bac-to-Bac Expression System. The gene of interest is cloned into a pFastBac transfer vector, and this recombinant donor plasmid is used to transform DH10Bac competent cells containing a bacmid with a mini-*att*Tn7 target site and a helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid is able to transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins expressed by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZ*α gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells. Transfected insect cells are analyzed by expression of the recombinant protein. The medium containing viral particles is used to amplify viral stock or to infect insect cells. (This figure is reproduced from the instruction manual for Bac-To-Bac Baculovirus Expression Systems, Life Technologies.)

elements on the pFastBac vector can transpose to the mini-*att*Tn7 target site on the bacmid with the transposition proteins expressed from the helper plasmid. Colonies containing recombinant bacmids are identified by antibiotic selection and blue/white screening, since the transposition results in disruption of the *lac*Zα gene on the bacmid.

Using site-specific transposition to insert foreign genes into a bacmid propagated in *E. coli* has a major advantage over the generation of recombinant baculoviruses in insect cells by homologous recombination. Recombinant virus DNA isolated from selected colonies is not mixed with parental, nonrecombinant virus, eliminating the need for multiple rounds of plaque purification.

In this chapter, we describe the use of the Bac-to-Bac expression system to express eIF4G-1 isoforms. To facilitate purification after expression, we incorporated a His-tag at the N-terminus of eIF4G-1.

B. Experimental Procedures

1. Construction of the transfer vector pFastBac4G-1a^{DM} and pFastBac4G-1e^{DM}

pFastBac4G-1a^{DM} was constructed by ligating the pFastBac HTb vector, digested with *Stu*I and *Xho*I, to an insert obtained from pCITE4G-1a^{DM} by digestion with *Eco*RV and *Xho*I. PFastBac4G-1e^{DM} was constructed by ligating the pFastBac HTb vector, digested with *Stu*I and *Xho*I, to an insert obtained from pCITE4G-1e^{DM} by digesting with *Sgr*AI, treating with the Klenow fragment of DNA polymerase I (Promega), and then cutting with *Xho*I. Competent *E. coli* cells were transformed with the ligation reaction

mixtures. The subcloned pFastBac4G-1a^{DM} and pFastBac4G-1e^{DM} were first screened by restriction analysis, and then their structures were confirmed by DNA sequencing.

2. Isolation of the recombinant bacmid DNAs

The plasmids pFastBac4G-1a^{DM} and pFastBac4G-1e^{DM} were cultured, purified, and then used to transform competent DH10Bac cells. The transformed DH10Bac cells were incubated on Luria Agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG for 48 h. White colonies were selected and cultured overnight. Recombinant bacmid DNAs were isolated by using a Concert High Purity Plasmid Miniprep System (Life Technologies).

3. Transfection of Sf9 cells with recombinant bacmid DNAs

Sf9 cells were seeded in 6-well plates in 2 ml of Sf-900II SFM medium containing 50 μg of penicillin and 50 μg streptomycin and allowed to attach at 27°C for 1 h. Buffer D (5 μl of mini-prep bacmid DNA in 100 μl Sf-900 II SFM without antibiotics) and Buffer E (6 μl CellFECTIN Reagent in 100 μl Sf-900 II SFM without antibiotics) were mixed gently and incubated for 15-45 min at room temperature. Sf9 cells were washed once with 2 ml of Sf-900 II SFM without antibiotics. For each transfection, 0.8 ml of SF-900 II SFM was added to the 200-μl mixture of Buffer D and E and mixed gently. Wash media was aspirated and the diluted lipid-DNA transfection mixture was overlaid onto the cells. Cells were incubated for 5 h in a 27°C incubator. Transfection mixtures were removed from the wells, 2 ml of Sf-900 II SFM containing antibiotics were added, and cells were incubated for 72 h at 27°C. Viruses were harvested from the cell culture medium at 72 h post-transfection. Cells were washed with PBS and lysed by adding 50 μl cell lysis buffer on the top of the monolayer and incubating for 5 min. Cell

lysates were centrifuged at 10,000 X g for 10 min to remove the cell debris. Cell lysates were treated or mock-treated with 2A protease and subjected to SDS-PAGE. The samples were transferred to PVDF membranes and probed with anti-eIF4G antibody.

4. Infection of Sf9 cells with recombinant baculovirus particles

For amplifying viral stocks, monolayer cultures were infected at a moi of 0.01 to 0.1. For expression of recombinant protein, monolayer or suspension cultures were infected at a moi of 5 to 10. Infected cells were harvested at 48 h.

5. Purification of eIF4G-1a^{DM} and eIF4G-1e^{DM} from infected Sf9 cells

Sf9 cells were harvested 48 h pi by centrifugation for 5 min at 500 X g and washed twice with PBS. Cell pellets were lysed in 5 volumes of lysis buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethyl sulfonyl fluoride, 1% Nonidet P-40, 20 μg/ml Leupeptin, 50 μg/ml Aprotinin, and 10 μg/ml Pepstatin A at 4°C by inverting the tube end-over-end for 5 min. Cell debris was removed by centrifugation at 10,000 X g for 10 min. The lysates were added to Ni-NTA agarose affinity columns (Qiagen) pre-equilibrated with Buffer F containing 20 mM Tris-HCl (pH 8.5), 500 mM KCl, 20 mM imidazole, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol at 4°C. The column was washed with 10 volumes of Buffer F, two volumes of Buffer G containing 20 mM Tris-HCl (pH 8.5), 1 M KCl, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol at 4°C, and then two volumes of Buffer F. Protein was eluted with 5-10 volumes of Buffer H, consisting of 20 mM Tris-HCl (pH 8.5), 100 mM KCl, 100 mM imidazole, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol at 4°C. Fractions of 0.5 ml were collected and combined. The eluted fractions were separated by ion exchange chromatography on a Mono Q column with a salt gradient

from 0 to 1 M KCl in Buffer I, which contains 20 mM Tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol, and 10 % (v/v) glycerol (Lamphear and Panniers 1990). The Mono Q chromatography was performed by Tol Fowler.

C. Results

1. eIF4G-1a^{DM} and eIF4G-1e^{DM} are expressed in Sf9 cells transfected with recombinant bacmid DNAs

To obtain the recombinant viral stock, insect cells were transfected with recombinant bacmids in 6-well plates. Three days after transfection, cells were collected. The cell lysates were either treated or mock-treated with 2A protease. The expression of eIF4G-1a^{DM} and eIF4G-1e^{DM} was monitored by western blotting. Full-length eIF4G-1a^{DM} and eIF4G-1e^{DM} were expressed (Fig. 6-2, lanes 1-8) and were resistant to 2A protease (lanes 2, 4, 6, and 8). One culture failed to express eIF4G-1e^{DM} (lanes 9 and 10). The exogenous eIF4G-1a^{DM} and eIF4G-1e^{DM} can be distinguished from endogenous eIF4G in insect cell by electrophoretic mobility (lanes 11 and 12).

2. Purification of eIF4G-1a^{DM} and eIF4G-1e^{DM} from infected Sf9 cells

Cells infected with recombinant baculovirus were collected and cell lysates were purified by Ni-NTA agarose affinity chromatography. The major purification products are the full-length eIF4G-1a^{DM} and eIF4G-1e^{DM} (Fig. 6-3, panel A, lanes 6 and 12). However, some smaller proteins are also present, presumably degradation products of eIF4G-1a^{DM} and eIF4G-1e^{DM}. The yield after Ni-NTA agarose affinity

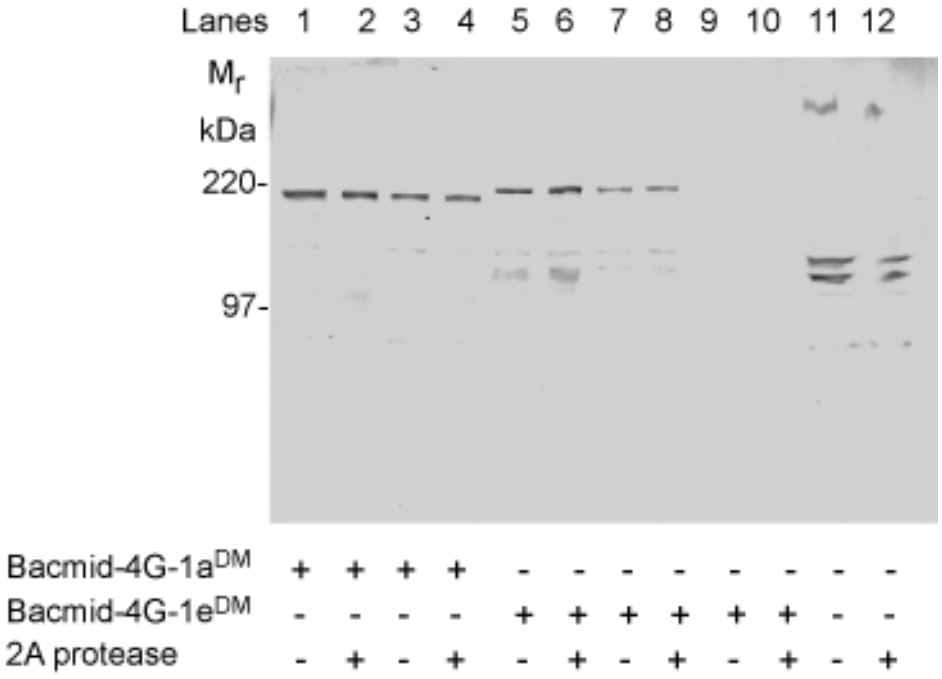
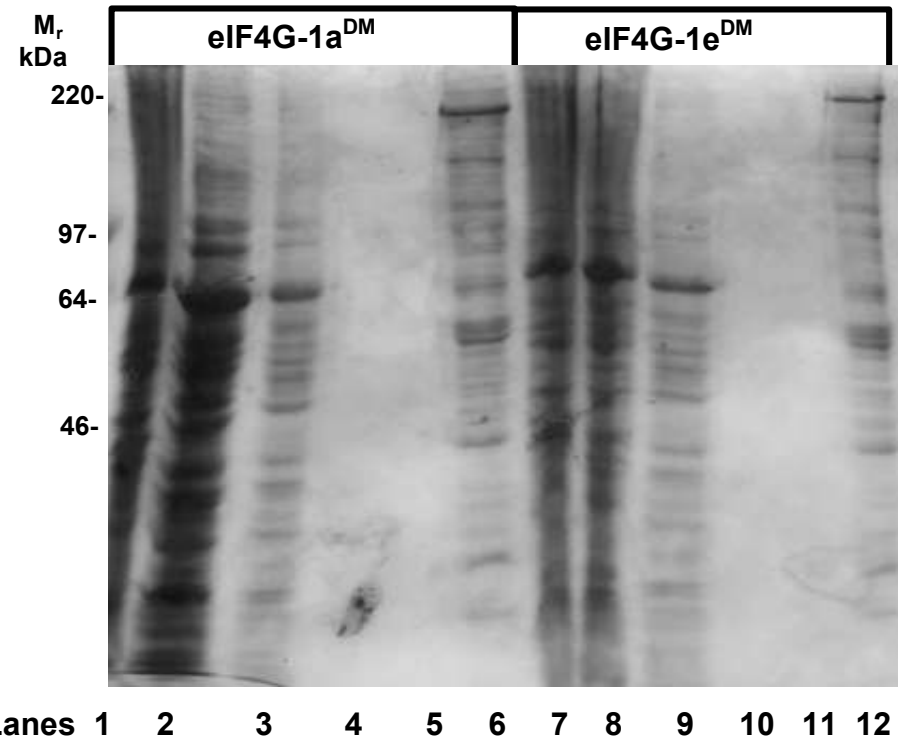

Lanes 1 2 3 4 5 6 7 8 9 10 11 12
M_r
kDa
220-
97-
Bacmid-4G-1a^{DM} + + + + - - - - - - - -
Bacmid-4G-1e^{DM} - - - - + + + + + + - -
2A protease - + - + - + - + - + - +

Fig. 6-2. Expression of eIF4G-1a^{DM} and eIF4G-1e^{DM} in Sf9 cells after transfection with recombinant baculovirus DNAs. Sf9 cells were cultured in 6-well plates and transfected with recombinant baculovirus DNAs expressing eIF4G-1a^{DM} and eIF4G-1e^{DM}. Three days after transfection, cells were lysed, and the lysates were treated or mock-treated with 2A protease, subjected to SDS-PAGE, transferred to PVDF, and probed with anti-eIF4G-1 antibodies.

A. Purification of eIF4G-1^{DM} by Ni-NTA agarose affinity chromatograph



B. Purification of eIF4G-1a^{DM} by Mono Q column chromatograph

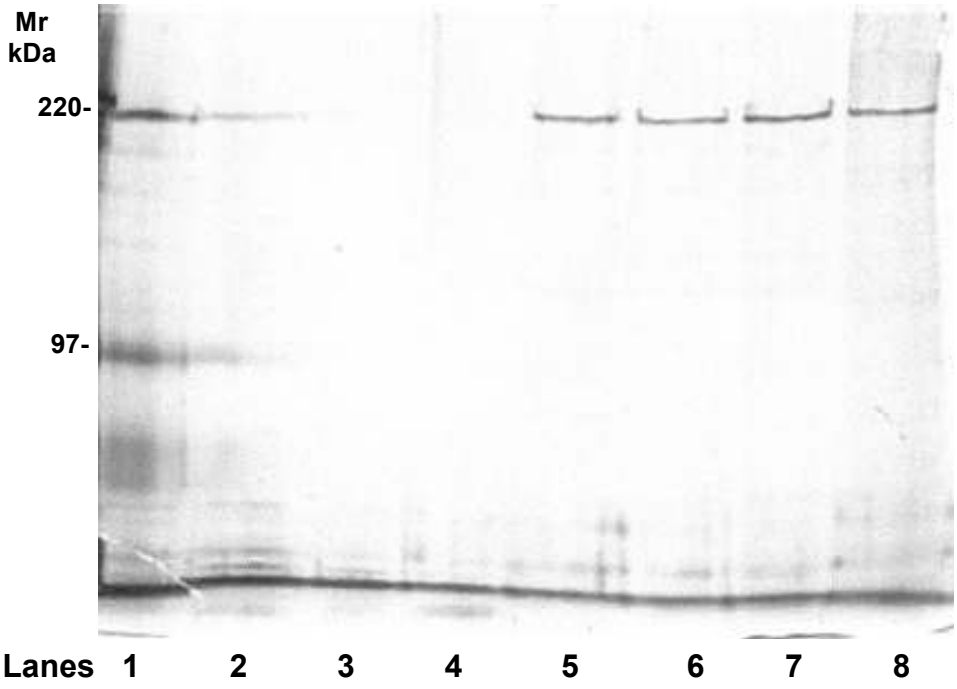


Fig. 6-3. Purification of eIF4G-1a^{DM} and eIF4G-1e^{DM} from baculovirus-infected Sf9 cells. Cells were cultured in flasks and infected at 5-10 moi with recombinant baculoviruses. The infected cells were collected after 2 d. Cells were lysed and His-tagged eIF4G-1 purified by Ni-NTA agarose affinity chromatography. (A) The fractions from each of the procedures were subjected to SDS-PAGE on an 8% gel and stained with Coomassie blue. Whole cells (lanes 1 and 7); cell lysates (lanes 2 and 8); flowthrough (lanes 3 and 9); wash (lanes 4-5 and 10-11); and eluted proteins (lanes 6 and 12). (B) The eluted fractions from the Ni-NTA affinity chromatography were applied to a Mono Q column. The peaks were collected and subjected to SDS-PAGE on a 6% gel and visualized by silver staining.

chromatography is about 10 mg/l. In order to remove these other proteins, we used ion-exchange chromatography after this treatment; apparently pure eIF4G-1a^{DM} can be obtained (panel B).

D. Discussion

We wished to obtain pure proteins expressed from Sf9 cells infected with recombinant baculovirus through a one step Ni-NTA agarose affinity chromatography. In our construction, we therefore incorporated a His-tag at the N-terminus of eIF4G-1a^{DM} and eIF4G-1e^{DM}. However, we encountered two problems when we used Ni-NTA agarose affinity chromatography. First, some full-length eIF4G-1^{DM} was eluted by buffer containing 20 mM imidazole. It is possible that the His-tag at the N-terminus does not bind with sufficient avidity to retain the protein because of the high molecular weight of eIF4G-1. Alternatively, the His-tag may be embedded inside of the eIF4G-1 folded molecule. We reduced the imidazole concentration in the washing buffer and obtained a better yield of protein. Second, eIF4G-1a^{DM} and eIF4G-1e^{DM} obtained from Ni-NTA agarose affinity chromatography were not pure. They contained degraded eIF4G-1^{DM} products as well as Sf9 cellular proteins. eIF4G-1 is a very unstable protein. Degraded products are observed in most mammalian cells. It is likely that Sf9 cellular proteases cleave eIF4G-1^{DM}, especially at the late stages of baculoviral infection when there is a host response causing apoptosis. It is known that some caspases cleave eIF4G (Bushell and others 2000; Clemens and others 2000). It is also possible that the contaminating Sf9 cellular proteins have some regions similar to the properties of the His-tag and are thus co-purfied.

We used a Mono Q column to further purify the eluate from Ni-NTA agarose affinity chromatography with a salt gradient from 0-500 mM KCl. We expected that eIF4G-1^{DM} would elute at 40 min as observed for eIF4F and cp$_C$. However, our eIF4G-1^{DM} eluted at the end of the salt gradient. This is probably due to the fact that full-length eIF4G-1 is an acidic protein, with clustered stretches of glutamate residues at the N-terminus (Yan and others 1992). These residues may be masked in eIF4F, due to eIF4E binding, and are not present at all in cp$_C$. This may make free, full-length eIF4G bind more tightly to the Mono Q column and require higher salt to be eluted. We were able to purify eIF4G-1^{DM} by increasing the salt gradient to 0-1 M KCl.

The ability to produce and purify full-length eIF4G-1 isoforms from Sf9 cells infected with recombinant baculoviruses provides a far more efficient way to study the function of individual eIF4G-1 isoforms *in vitro* than the two-phase *in vitro* translation system. The function of specific domains in eIF4G-1 can be studied by engineering eIF4G-1 isoforms lacking these domains. Possible *cis*-acting elements of mRNAs and *trans*-acting factors that interact with individual eIF4G-1 isoforms can be explored with full-length eIF4G-1. Also, the interaction of eIF4G-1 with various translation initiation factors and their influence on the binding of other initiation factors can be more accurately studied by using full-length eIF4G-1.

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CHAPTER 7

CONCLUDING REMARKS AND FUTURE PROSPECTS

A. Significance and Concluding Remarks

The studies presented in this dissertation touch on the function of two different isoforms of eIF4G-1, the mechanism of host protein synthesis shutoff during picornaviral infection, and the enhancement of viral protein synthesis.

Creation of an isoform of eIF4G-1 that is highly resistant to 2A protease cleavage provides us a useful tool to study the function of individual eIF4G-1 isoforms *in vitro* and *in vivo*. This enabled us to demonstrate higher translational activity of eIF4G-1e over eIF4G-1a in a two-phase *in vitro* translation system in Chapter 3. Different *in vivo* translational properties of two isoforms of eIF4G-1, eIF4G-1a and eIF4G-1e, were demonstrated in Chapter 4 by cotransfection with plasmids expressing 2A protease and GFP. Both eIF4G-1a^{DM} and eIF4G-1e^{DM} could rescue cap-dependent translation, whereas eIF4G-1e^{DM} but not eIF4G-1a^{DM} could suppress internal initiation.

The importance of the integrity of eIF4G-1 for maintaining host protein synthesis and restricting viral protein synthesis is demonstrated in Chapter 5. The expression of eIF4G-1e^{DM} results in prolonged host protein synthesis and delayed viral protein synthesis, which further leads to significantly reduced virus production. This protective effect is likely to be much more significant in human picornaviral diseases, in which viral infection occurs at lower moi than in our laboratory studies (about 10 moi). The delay of viral particle release could give more time for the host (patient) to win the battle against the virus.

B. Open Questions

eIF4G-1 runs as multiple protein bands ranging from 200-220 kDa on SDS-PAGE, as we observed in Chapter 2, 3, and 4. The relationship between

endogenous eIF4G-1 and eIF4G-1a or eIF4G-1e was demonstrated in Chapter 2 by comparing their migration on SDS-PAGE and by immunoreactivity. After these studies were completed, the endogenous eIF4G-1 protein isoforms were further characterized by mass spectrometry (Bradley and others 2002). However, eIF4G-1 is encoded by only one gene in mammals. There are several possibilities for how these protein isoforms are generated, such as alternate splicing, use of alternate translation initiation codon, protein degradation, and posttranslational modification. Evidence for one of these possible mechanisms, alternate translation initiation, has been presented (Byrd and others 2002).

The expression levels of eIF4G-1 isoforms may vary among different tissues and during different stages of the cell cycle, although this has not yet been systematically investigated. It would be interesting to know when, how, and why the different eIF4G-1 isoforms are expressed and regulated. The different activities of eIF4G-1a and eIF4G-1e illustrated in our studies might explain the abundance of eIF4G-1e, *i.e.*, its greater activity in translating polyadenylated mRNAs. Conceivably, there are tissues or developmental stages in which polyadenylation is relatively less important, and this may be reflected in higher levels of eIF4G-1a. The variation in eIF4G-1 levels during different stages of the cell cycle might be one mechanism to control cell cycle progression at the translational level. For instance, this could be a mechanism for controlling the synthesis of certain critical proteins encoded by mRNAs with different *cis*-acting elements, such as histones whose mRNAs lack a poly(A) tract. What are the *cis*-acting elements favored by different isoforms of eIF4G-1? Are there any *trans*-acting factors corresponding to the different isoforms of eIF4G-1 in addition to the canonical initiation factors that might be involved in selective synthesis of critical proteins? These

highly resistant to 2A protease eIF4G-1 forms might facilitate research to answer these questions in studies involving the expression of individual isoforms of eIF4G-1 *in vivo* using cells depleted of endogenous eIF4G by 2A protease or RNAi.

eIF4G-1e^{DM} eventually is cleaved during infection with MMd virus (Fig. 5-2). However, it maintains integrity until 5 h pi in GD cells and 3 h in G cells compared to only 2 h in OD and O cells. This result suggests that eIF4G-1e^{DM} might be cleaved by other proteases, some of which may be encoded by viruses and some of which may be induced by viral infection or apoptotic effects in the late stages of MMd infection. It has been shown that multiple eIF4G-1-specific protease activities exist in uninfected and infected cells (Zamora and others 2002). It is likely that late stages of MMd infection may activate certain cellular proteases to cleave eIF4G-1 in addition to 2A protease.

Interestingly, we observed that intact eIF4G-1e^{DM} persisted at all times during Mahoney virus infection (data not shown). Unfortunately, HeLa-G cells did not show significant host protein syntheses prolongation or viral protein synthesis delay during Mahoney virus infection (data not shown). On the other hand, eIF4G-1e^{DM} was eventually cleaved during MMd infection. This suggests either the 2A protease is significantly different in these two viruses or that eIF4G-1e^{DM} is cleaved by another mechanism in MMd-infected cells. Mahoney virus may have a different mechanism to control host protein synthesis other than cleavage of eIF4G-1, *i.e.,* the relative importance of eIF4G-1 may be greater in MMd infection than that of Mahoney. Viruses have developed elegant strategies to suppress host protein synthesis and promote viral protein synthesis (Schneider and Mohr 2003; Gale and others 2000). It is possible that two or more mechanisms are used for certain virus to control the host protein synthesis shutoff

and favor viral protein synthesis. If eIF4G cleavage is the only mechanism or the major mechanism for certain picornavirus, the virus may have developed additional ways to ensure the complete cleavage of eIF4G. Thus, MMd may utilize not only 2A protease but also cellular proteases or other viral proteases. If there are other mechanisms such as dephosphorylation of 4E-BP in addition to eIF4G cleavage occurring in certain picornavirus infection, then the complete cleavage of eIF4G may not be essential for this virus to survive. By using 2A protease-resistant eIF4G isoforms, we may be able to study the mechanisms by which picornaviruses suppress host protein synthesis in addition to eIF4G cleavage.

Prolonged host protein syntheses were observed in Fig. 5-4. We propose that this prolonged host protein synthesis may interfere with and restrict viral translation and replication. Using DNA microarray analysis, polysomal mRNAs actively engaged in translation could be identified. This and other methods could be used to identify these host proteins participating in the process of virus restriction, opening the way for new anti-viral strategies.

C. References

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