Virology 378 (2008) 118–122

Contents lists available at ScienceDirect

Virology

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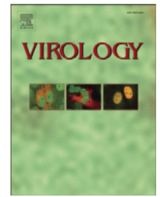

Cleavage of eukaryotic initiation factor eIF5B by enterovirus 3C proteases

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ARTICLE INFO

Article history:
Received 9 March 2008
Returned to author for revision
25 March 2008
Accepted 16 May 2008
Available online 24 June 2008

Keywords:
Coxsackievirus
eIF5B
Poliovirus
Protease
Rhinovirus
Translation shutoff

ABSTRACT

The enteroviruses poliovirus (PV), Coxsackie B virus (CVB) and rhinovirus (HRV) are members of *Picornaviridae* that inhibit host cell translation early in infection. Enterovirus translation soon predominates in infected cells, but eventually also shuts off. This complex pattern of modulation of translation suggests regulation by a multifactorial mechanism. We report here that eIF5B is proteolytically cleaved during PV and CVB infection of cultured cells, beginning at 3 hours post-infection and increasing thereafter. Recombinant PV, CVB and HRV $3C^{pro}$ cleaved purified native rabbit eukaryotic initiation factor (eIF) 5B *in vitro* at a single site (VVEQ↓G, equivalent to VMEQ↓G_{479} in human eIF5B) that is consistent with the cleavage specificity of enterovirus 3C proteases. Cleavage separates the N-terminal domain of eIF5B from its essential conserved central GTPase and C-terminal domains. $3C^{pro}$-mediated cleavage of eIF5B may thus play an accessory role in the shutoff of translation that occurs in enterovirus-infected cells.

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Introduction

Infection by enteroviruses such as poliovirus (PV), Coxsackie B virus (CVB) and human rhinovirus (HRV), members of the family *Picornaviridae*, leads to inhibition of host cell protein synthesis. During PV infection, shutoff begins within 2h, is complete with 4h, and is followed by a phase during which viral translation is also progressively inhibited (Etchison et al., 1982). The biphasic nature of these changes suggests that shutoff occurs by a multifactorial mechanism, so that factors required for translation of cellular mRNAs (predominantly following cap-dependent initiation) are targeted during the first phase of shutoff and factors that are also required for translation of viral mRNAs may be inactivated later. Enterovirus mRNAs all contain an internal ribosomal entry site (IRES) that promotes initiation by cap-independent ribosomal binding to the mRNA (Hellen and Sarnow, 2001), unlike most cellular mRNAs, which are translated following cap-dependent initiation.

Whereas the mechanism of initiation on enterovirus IRESs is incompletely characterized, many details of the cap-dependent initiation mechanism have been elucidated (Pestova et al., 2007). First, eIF1, eIF1A and eIF3 promote binding of eIF2·GTP·Met-$tRNA^{Met}_i$ to a 40S ribosomal subunit to form a 43S preinitiation complex. Its attachment to the capped 5′ end of mRNA is mediated by eIF4F (which consists of the eIF4E cap-binding subunit, the eIF4A RNA helicase and eIF4G), and is enhanced by eIF4B and the poly(A) binding protein (PABP). eIF4G coordinates ribosomal recruitment to mRNAs by binding PABP, mRNA and eIFs 3, 4E and 4A. 43S complexes scan to the initiation codon, forming 48S complexes in which the Met-$tRNA^{Met}_i$ anticodon is base-paired to the initiation codon in the ribosomal peptidyl (P) site. In the final subunit-joining stage, eIF5 induces hydrolysis of eIF2-bound GTP, releasing Met-$tRNA^{Met}_i$ into the P site, and eIF5B mediates displacement of eIF2 GDP and other factors from the 48S complex and joining of a 60S subunit to yield an 80S ribosome.

The earliest changes in the translation apparatus during enterovirus infection involve cleavage by the virus-encoded 2A protease ($2A^{pro}$) of eIF4GI (which precedes the shutoff of host protein synthesis), and with slightly slower kinetics, of the less abundant eIF4GII (Etchison et al., 1982; Gradi et al., 1998). eIF4GI/eIF4GII are cleaved at a single site, splitting domains that bind PABP and eIF4E from those that bind eIF3 and eIF4A (Lamphear et al., 2005), thereby abrogating eIF4G's function in bridging factors that bind capped mRNA, that unwind mRNA and that bind the ribosome. PABP is also cleaved in enterovirus-infected cells, predominantly by the 3C protease ($3C^{pro}$), which separates PABP into an N-terminal fragment that binds eIF4G and the 3′ poly(A) tail of mRNA, and a C-terminal fragment that binds eIF4B and the termination factor eRF3 (Joachims et al., 1999; Kerekatte et al., 1999; Kuyumcu-Martinez et al., 2002). In addition, the translation regulator 4E-BP1 is dephosphorylated during infection. This event sequesters eIF4E and abrogates eIF4F's cap-binding function, which contributes to the

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doi:10.1016/j.virol.2008.05.019

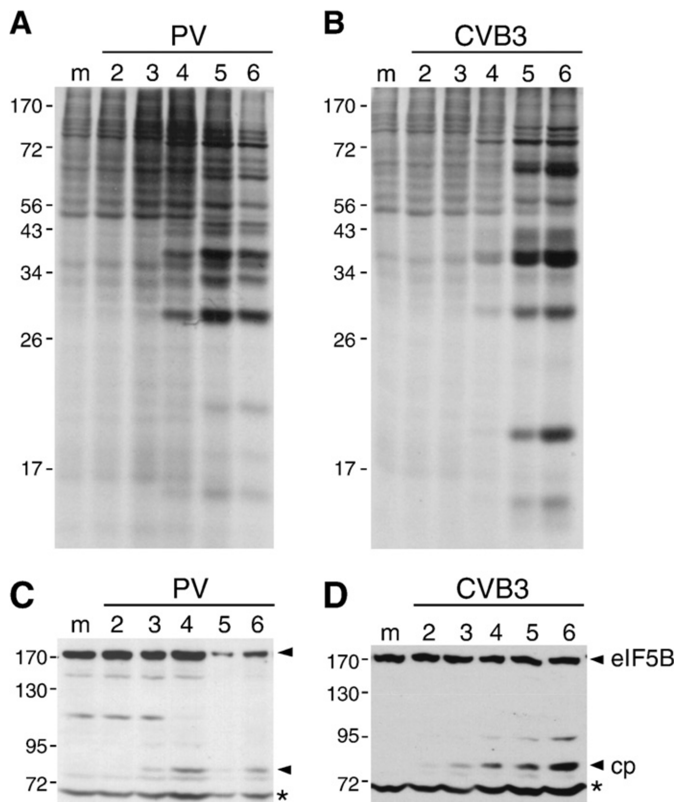

Fig. 1. Cleavage of eIF5B during enterovirus infections. (A, B) 293 T cells were mock infected (M) or infected with (A) poliovirus or (B) Coxsackievirus B3 (CVB3) and pulse labeled with [^{35}S]Trans label. Samples from mock-infected cells were pulse-labeled at 1 hour p.i. and harvested at 2 hours p.i.; samples for infected cells were harvested at the indicated hours p.i. after pulse-labeling for 1 h. Aliquots were then analyzed by SDS-PAGE. (C, D) Aliquots of samples of mock-infected 293T (m) or 293T cells infected with (C) poliovirus and (D) Coxsackievirus B3 and harvested at the indicated times (hours) p.i. were subjected to SDS-PAGE and immunoblotted with a polyclonal antibodies to eIF5B. A ~70 kDa host protein present in mock-infected cells that cross-reacted with these antibodies is indicated by asterisks (*) and served as an internal loading control. The positions of molecular weight markers, of intact eIF5B and of a cleavage product (cp) are indicated.

shutoff of host protein synthesis and activation of IRES-mediated translation (Gingras et al., 1996; Svitkin et al., 2005), but cannot fully account for shutoff because it lags behind this process.

To obtain a more complete overview of the shut-off process, the status in PV and CVB type 3 (CVB3)-infected cells of factors involved in subunit joining was analyzed. eIF5B was found to be cleaved at a single site during enterovirus infection. Cleavage *in vitro* was mediated by 3C^{pro} alone and was specific, because eIF1, eIF1A, eIF4B, eIF4F and eIF5 were not substrates for 3C^{pro}. The time course of eIF5B proteolysis suggests that its cleavage could contribute to the shutoff of host and viral translation observed in enterovirus-infected cells.

Results and discussion

Cleavage of eIF5B in enterovirus-infected cells

To determine whether the abundance or integrity of eIF5B was altered during enterovirus infection, cytoplasmic lysates from poliovirus type 1 (PV1) (Mahoney)- and Coxsackievirus B3 (CVB3)-infected 293T cells harvested at various times p.i. (Figs. 1A, B) were immunoblotted using polyclonal antibodies directed against the carboxy-terminal amino acid residues 921–1220 of human eIF5B (Wilson et al., 1999). A ~ 70kDa protein present in mock-infected cells that cross-reacted with these antibodies is indicated by asterisks (Figs. 1C, D) and served as an internal loading control. The origin of this and another cross-reactive protein of ~ 110kDa (Fig. 1C) have not been established. A ~ 83kDa putative eIF5B cleavage product first appeared at 3h p.i., a time point that coincided with the initial appearance of viral proteins (Figs. 1A, B), and subsequently increased in prominence. Cleavage of eIF5B therefore begins early in enterovirus infection, at a similar time to cleavage of eIF4G and PABP (Etchison et al., 1982; Gradi et al., 1998; Kerekatte et al., 1999). Full length eIF5B with an apparent molecular weight in these gel/buffer conditions of 175kDa (Pestova et al., 2000) was readily detected by immunoblotting in lysates of 293T and HeLa cells (Fig. 1 and data not shown), but its abundance during the course of infection suggested that only partial cleavage of eIF5B had occurred. The level of cleavage varied between experiments done with PV1 and CVB3-infected 293T cells, in some instances being lower than shown in Fig. 1, and did not correlate with the multiplicity of infection (data not shown). The transient and variable appearance of additional smaller immunoreactive bands in some infections (data not shown), suggested that further cleavage or degradation of cleavage products occurred in infected cells, possibly due to proteasome or caspase activation, a characteristic of PV-infected cells (Belov et al., 2003; Barral et al., 2007).

The observations that the level of eIF5B cleavage in enterovirus-infected cells was variable suggested that eIF5B might under some circumstances be protected from 3C^{pro}-mediated cleavage that we reasoned might result either from post-transcriptional modification, such as phosphorylation (e.g. Beausoleil et al., 2004) or from interaction with other proteins. The kinases that phosphorylate eIF5B are not known and phospho-specific eIF5B antibodies are not available, so the influence of phosphorylation on eIF5B's susceptibility to cleavage could not be characterized. eIF5B's interacting partners include eIF1A, 40S and 60S ribosomal subunits (Marintchev et al., 2003; Olsen et al., 2003; Unbehaun et al., 2007), but their presence did not affect cleavage of eIF5B by HRV or PV 3C^{pro} *in vitro* (data not shown). The reason for the variable level of cleavage of eIF5B in infected cells therefore remains to be determined.

Direct cleavage of eIF5B by enterovirus 3C protease

The analysis of virus-infected cell lysates suggested that enteroviruses encode a protease that cleaved eIF5B, yielding at least one fragment detectable by immunoblotting with antibodies directed against its C-terminal region. Incubation with purified recombinant HRV type 14 (HRV14) 3C^{pro} resulted in partial cleavage of purified native rabbit eIF5B, yielding fragments (cp1 and cp2) with apparent molecular weights of ~ 85kDa and ~ 68kDa (Fig. 2A, lane 2). cp1 was recognized by mouse polyclonal antibodies directed against eIF5B$_{1121–1219}$ (data not shown). In similar experiments carried out with purified CVB3 3C^{pro}, purified native eIF5B was also partially cleaved (Fig. 2C, lane 2), whereas purified PV 3C^{pro} cleaved purified eIF5B to near-completion, in both instances yielding cp1 and cp2 cleavage fragments with electrophoretic mobilities like those of the HRV 3C^{pro} cleavage products (Fig. 2A, lanes 2 and 3). These cleavage reactions were performed overnight at 4°C, but the specificity of cleavage was not changed on incubation for shorter periods at 37°C (data not shown). We cannot rule out the possibility that 3CD^{pro}, the major form of 3C^{pro} in infected cells (Ypma-Wong et al., 1988) cleaves eIF5B with different kinetics than 3C^{pro}.

Cleavage of eIF5B by HRV14 and PV 3C^{pro} was specific, because whereas a control substrate was cleaved to completion after incubation with HRV14 3C^{pro} (Fig. 2D, lane 2), the integrity and abundance of purified recombinant eIF1, eIF1A, eIF4B and eIF5, and of purified native eIF4F were unaltered after incubation with HRV14 and PV 3C^{pro} in identical conditions (Fig. 2A, lanes 5 and 6; Fig. 2B, lanes 2, 3, 5 and 6; Fig. 2D, lane 4; Fig. 2E, lane 4). A recombinant fragment of eIF5B corresponding to amino acid residues 587–1220 ("eIFΔ5B") was not cleaved by HRV14 3C^{pro} in these conditions (Fig. 2E, lanes 1 and 2), indicating that cleavage likely occurred upstream of a.a. 587. These observations extend earlier reports that eIF2, eIF3, eIF4A, eIF4B and eIF4E are not cleaved during enterovirus infections (Duncan et al.,

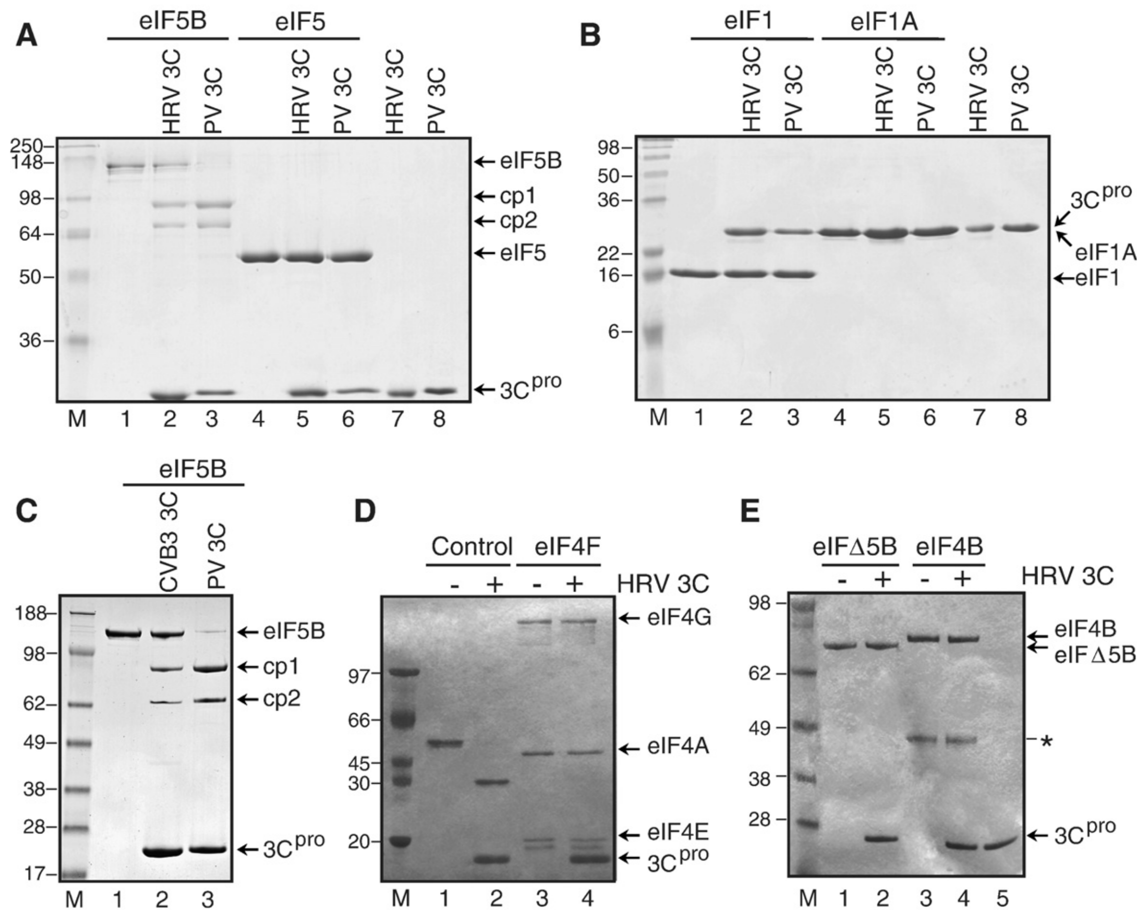

Fig. 2. Specific cleavage of eIF5B by CVB3, HRV14 and PV1 3C proteases. (A) Purified native rabbit eIF5B (lanes 1–3) and purified recombinant human eIF5 (lanes 4–6), (B) purified recombinant human eIF1 (lanes 1–3) and purified recombinant human eIF1A (lanes 4–6), (C) purified native rabbit eIF5B (lanes 1–3), (D) control protease substrate (lanes 1 and 2) and purified native rabbit eIF4F, and (E) purified recombinant human $eIF5B_{587–1220}$ (eIFΔ5B) (lanes 1 and 2) and purified recombinant human eIF4B (lanes 3 and 4) were incubated alone, with purified recombinant CVB3 $3C^{pro}$, HRV 14 $3C^{pro}$ or PV1 $3C^{pro}$, as indicated. Reaction mixtures were then analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining. The positions of 3C proteases, of initiation factors and, in the case of eIF5B, of their cp1 and cp2 cleavage products on gels are indicated to the right of each panel. The asterisk (*) indicates a contaminant co-purifying with recombinant eIF4B (c.f. Pisarev et al., 2007).

1983; Etchison et al., 1984; Lee et al., 1985; O'Neill and Racaniello, 1989).

To map the cleavage site precisely, the N-terminal sequence of the cp1 fragment of native rabbit eIF5B was determined by Edman sequencing to be [G/S]GQEK[E/S]DTPP. This peptide sequence aligns precisely with the deduced sequence of a fragment of rabbit genomic DNA (Fig. 3B) and aligns well with a single conserved region of human eIF5B, which is consistent with cleavage of human eIF5B at Q478–G479 (Fig. 3B). The molecular weight of the C-terminal cp1 cleavage fragment of human eIF5B is 83.8 kDa, which is consistent with the molecular weight of the cleavage fragment identified in enterovirus-infected 293T cells and in *in vitro* reactions done using purified eIF5B and CVB3, HRV14 and PV1 3C proteases (e.g. Figs. 1 and 2A, C). The highly charged amino-terminal domain of eIF5B is responsible for its anomalous mobility in SDS-PAGE (Wilson et al., 1999; Pestova et al., 2000), and accordingly, the molecular weight of the N-terminal cp2 cleavage fragment of human eIF5B (55.1 kDa) is less than that predicted on the basis of its mobility in SDS-PAGE (~68 kDa). Cleavage at VVEQ↓G in rabbit eIF5B and likely at VMEQ↓G_{479} in human eIF5B is consistent with the cleavage specificity of enterovirus 3C proteases, for which the principal determinants for cleavage are Gln-Gly (Q–G) amino acid pairs flanking the scissile bond, and any of a small subset of

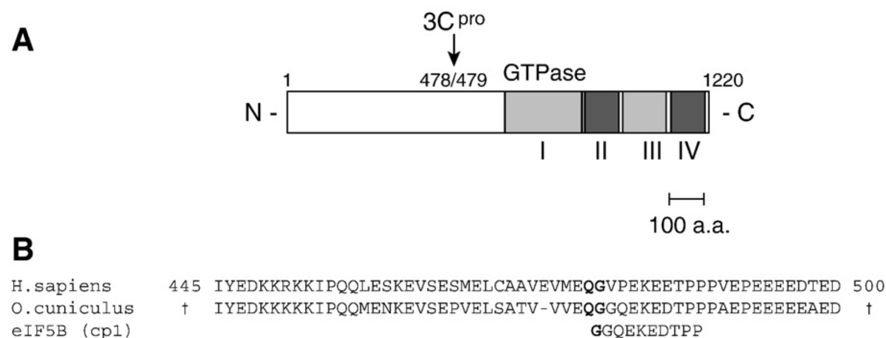

Fig. 3. (A) Diagrammatic representation of human eIF5B. The site of cleavage by HRV14 $3C^{pro}$ is shown relative to the positively charged N-terminal domain and the four conserved structural domains (I–IV) (54). (B) CLUSTAL-W alignment of the N-terminal sequence of the $3C^{pro}$ cleavage product cp1 of rabbit (*Oryctolagus cuniculus*) eIF5B, the corresponding eIF5B coding sequence, deduced from an *O. cuniculus* genomic fragment (Genbank: AAGW01235144.1), and the sequence of human eIF5B (Genbank: NM_015904).

amino acids (usually Ala or Val, but also other amino acids with small, aliphatic side chains) at the P4 position (Cordingley et al., 1990). It is also consistent with the cleavage specificity of enterovirus 3C proteases for cellular substrates such as TATA-binding protein, PABP, and NF-κB (p65-RelA) in which the principal 3C cleavage sites are ASPQ↓G (Das and Dasgupta, 1993), VHVQ↓G (Kuyumcu-Martinez et al., 2002), and LLNQ↓G (Neznanov et al., 2005), respectively. Despite the structural homology of enteroviral 3C proteinases, sequence variation between the three proteinases tested here (42%–59% pairwise amino acid identity) includes residues that determine substrate specificity at P2 and P2′ positions relative to the scissile bond (Matthews et al., 1999; Binford et al., 2005; Lee et al., 2007). These differences may account for the different extents of cleavage observed here.

The large amino-terminal domain (NTD) that is cleaved from mammalian eIF5B by $3C^{pro}$ does not significantly influence nucleotide binding by this factor (Pisareva et al., 2007), but the kinetics of other individual steps in the working cycle of mammalian eIF5B have not yet been characterized. eIF5B is one of the least abundant initiation factors (Pisarev et al., 2007), so that any change in its activity caused by proteolytic cleavage might therefore contribute to enterovirus-induced shutoff of translation. There are several indications how eIF5B's activity might be altered by $3C^{pro}$-mediated cleavage, which separates the N-terminal domain of eIF5B from its essential conserved central GTPase and C-terminal domains. First, the GTP-dependency of the activity of eIF5B in ribosomal subunit joining is altered in some circumstances by deletion of an N-terminal fragment from eIF5B that is similar in size to that cleaved off by $3C^{pro}$ (Pestova et al., 2000). Second, the NTD of yeast eIF5B binds directly to eIF1A (Olsen et al., 2003), a component of 43S complexes that binds directly to the 40S subunit, so that deletion of the eIF5B NTD might affect the kinetics of binding of eIF5B to 48S complexes or of its release from assembled 80S ribosomes. Third, by analogy with the corresponding domain in eIF5B's prokaryotic homologue IF2, which is required for maximal growth, the NTD could interact with or induce conformational changes in the small ribosomal subunit (Moreno et al., 1999; Caserta et al., 2006).

In summary, these studies have identified eIF5B as a novel enterovirus $3C^{pro}$ substrate, and the time course of eIF5B proteolysis in infected cells suggests that it could contribute to the virus-induced shutoff of host and viral translation. $3C^{pro}$ impairs cap-mediated initiation (Kuyumcu-Martinez et al., 2004) and initiation mediated by PV1 and CVB3 IRESs (our unpublished data). Future studies will be directed to identifying the contribution of eIF5B cleavage to shutoff relative to the effects of $3C^{pro}$-mediated cleavage of other factors involved in enterovirus translation, such as PABP and the IRES *trans*-acting factors PTB and PCBP2 (Back et al., 2002; Perera et al., 2007).

Methods

Cells and virus infection

293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glutamine and 10% fetal bovine serum (FBS). Poliovirus (Type 1, Mahoney) and Coxsackievirus B3 were grown in HeLa cells and purified on cesium chloride gradients (Jones and Ehrenfeld, 1983) and from infected HeLa supernatants by polyethylene glycol precipitation (Kuyumcu-Martinez et al., 2002), respectively. 293T cells or HeLa cells were infected with PV1 at MOI=30 or CVB3 at MOI=10 in serum-free DMEM. At 1 hour post-infection, media were removed and replaced with DMEM containing 2% FBS. Metabolic labeling was done by incubating cells for 1 h in (–met, –cys) DMEM containing 2% dialyzed FBS and ^{35}S-Trans label (MP Biomedicals) at 30 μCi/mL.

Purification of ribosomal subunits and eukaryotic initiation factors

Native eIF4F and eIF5B were purified from rabbit reticulocyte lysate (Pisarev et al., 2007). Recombinant eIF1, eIF1A, eIF4B, eIF5 and $eIF5B_{587-1220}$ (eIFΔ5B) were overexpressed in *Escherichia coli* and purified (Pisarev et al., 2007). The integrity and activity of these factors and ribosomal subunits were assessed by polyacrylamide gel electrophoresis and using functional assays dependent on each of them (Pisarev et al., 2007).

eIF5B cleavage assays, sequencing, and blotting

Recombinant PV $3C^{pro}$ and CVB3 $3C^{pro}$ were expressed in *E. coli* and purified as described (Joachims et al., 1999; Zell et al., 2002); recombinant HRV14 $3C^{pro}$ was from Novagen (Madison, WI). To assay cleavage of individual initiation factors, 2 μg eIF5B, eIFΔ5B, eIF1, eIF1A, eIF5, eIF4B or eIF4F was incubated overnight at 4 °C in cleavage buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5) and with 1 μg HRV $3C^{pro}$, 1 μg CVB3 $3C^{pro}$, 0.5 μg PV $3C^{pro}$ or without added protease. Cleavage products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE); gels were stained with Coomassie blue. Immunoblotting of eIF5B and its cleavage products was done using polyclonal rabbit antibodies directed against amino acid residues 921–1220 of human eIF5B (Wilson et al., 1999) or polyclonal mouse antibodies directed against amino acid residues 1121–1219 of human eIF5B (Abnova). The eIF5B cp1 cleavage product was resolved by SDS-PAGE and transferred to PVDF membrane for amino-terminal sequencing.

Acknowledgments

This work was supported by PHS grant AI-51340 and by award 0755900T from the American Heart Association (Heritage Affiliate) (to C. U.T.H.) and by PHS grant AI-50237 (to R.E.L.). We thank Tatyana Pestova for the gift of native eIF5B, Stuart Wilson for the anti-eIF5B antibodies, Roland Zell for the CVB3 $3C^{pro}$ expression vector and Irina Abaeva for purifying recombinant CVB3 $3C^{pro}$. Protein sequence analysis was provided by The Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment.

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