Eukaryotic initiation factor 4GI is a poor substrate for HIV-1 proteinase

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Abstract Eukaryotic initiation factor (eIF) 4GI is efficiently cleaved during picornaviral replication. eIF4GI processing has also recently been observed during HIV-1 replication. We have compared the efficiency of eIF4GI proteolysis in rabbit reticulocyte lysates during translation of mRNAs encoding the foot-and-mouth disease virus leader proteinase (L^{pro}) or the HIV-1 proteinase (HIV-1^{pro}). L^{pro} cleaved 50% eIF4GI within 12 min whereas HIV-1^{pro} required 4 h; the concentrations were 2 pg/μl (0.1 nM) for L^{pro} and 60 pg/μl (2.66 nM) for HIV-1^{pro}. HIV-1^{pro} processing of eIF4GI is therefore not quantitatively analogous to that of L^{pro}, suggesting that the primary function of eIF4GI cleavage in HIV-1 replication may not be protein synthesis inhibition.

Key words: Retrovirus; Human immunodeficiency disease virus; Picornavirus; Foot-and-mouth disease virus; eIF4G cleavage; Initiation of translation

1. Introduction

In eukaryotic cells, mRNA is recruited to the 40S ribosomal subunit by the eIF4 (eukaryotic initiation factor 4) proteins [1]. Members of the eIF4 group of initiation factors play a central role in the initiation of protein synthesis in eukaryotes [2]. These factors comprise the cap-binding protein eIF4E, the RNA helicase eIF4A, the RNA binding protein eIF4B and the adaptor protein eIF4G which occurs in two isoforms, eIF4GI and eIF4GII [3]. In mammalian systems, eIF4E, eIF4A and eIF4G can be isolated as a complex designated eIF4F. eIF4F, together with eIF4B and eIF3, recognise the cap structure at the 5′-end of the cellular mRNA, unwind RNA secondary structure in an ATP-dependent manner and enable binding of the 40S ribosomal subunit to the mRNA [4–6].

The eIF4F complex is an important control point for the overall rate of protein synthesis [7,8]. During the replication of certain picornaviruses such as foot-and-mouth disease virus (FMDV), human rhino- (HRV) and enteroviruses, translation of capped cellular mRNA in the infected cell is shut down by specific cleavage of eIF4G isoforms [9–11]. Initiation of protein synthesis from viral mRNA is unaffected as it initiates internally and does not require a cap-structure [12].

Recently, experiments have shown that eIF4GI is cleaved late in HIV-1-infected cells [13] and that purified HIV-1^{pro} can cleave eIF4GI in rabbit reticulocyte lysates (RRLs) [14]. The proteolytic products of HIV-1^{pro} are different to those of the FMDV leader proteinase (L^{pro}). However, neither groups reported on the efficiency of the eIF4GI cleavage by HIV-1^{pro}. We show here that there is not only a qualitative difference, but also a significant quantitative difference in the cleavage of eIF4GI by FMDV L^{pro} and HIV-1^{pro}.

2. Materials and methods

2.1. Plasmids

The plasmid pCITE FMDV Lb^{pro}VP4VP2 containing the FMDV nucleotides 892–1411 of the FMDV $O1_k$ cDNA [15] was described in [16]. The plasmid encodes all 173 amino acids of the Lb form of L^{pro}, all 85 amino acids of VP4 and 78 amino acids of VP2. The plasmid pET11cHIV-1^{proD} contains the cDNA for two HIV-1^{pro} (BH10 strain) monomers linked via a short hinge region of seven amino acids (AlaGlyAlaMetGlyGlyAla) and was derived from plasmid pT7-2PR [17]. This covalently linked HIV-1^{pro} dimer was cloned into pCITE (Novagen) to generate pCITE HIV-1^{proD}. pCITE Δp6HIV-1^{proD} was constructed as pCITE HIV-1^{proD} except that it contains in addition the sequence encoding the 20 amino acids from the C-terminus of the p6 transframe domain, the protein preceding the HIV-1^{pro} in the genome.

2.2. In vitro transcription and translation

Plasmid pCITE FMDV Lb^{pro} was linearised with *Sal*I; plasmids pCITE HIV-1^{proD} and pCITE Δp6HIV-1^{proD} were linearised with *Bam*HI. In vitro transcription with T7 RNA polymerase and in vitro translation were as described in [16,18]. In vitro translation reactions (typically 50 μl) contained 70% RRL (Promega), 20 μCi of [^{35}S]methionine (1000 Ci/mmol, American Research Company) and amino acids (except methionine) at 20 μM. After preincubation for 2 min at 30°C, translation was started by addition of RNA to a concentration of 15 ng/μl unless stated otherwise. Aliquots (10 μl) were removed at the designated time points and the reaction was stopped by immediate transfer to ice, the addition of unlabelled methionine and cysteine to a final concentration of 2 mM and Laemmli sample buffer.

2.3. Electrophoresis and immunoblotting

The polyacrylamide gel electrophoresis system described by Dasso and Jackson [19] was used to separate translation products (gels contained 15% acrylamide) and to monitor the state of eIF4GI (gels contained 6% acrylamide). Translation products were detected by fluorography (exposure to film was for 20 h); the state of eIF4GI was determined by immunoblotting using the anti-(eIF4GI peptide 7) antiserum as described in [16,18].

2.4. Quantification of protein synthesis

Quantification of protein synthesis using an Instant Imager (Canberra Packard) was as described previously, except that calculations were adjusted for the use of [^{35}S]methionine alone [16,18].

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Abbreviations: eIF4G, eukaryotic initiation factor 4G; FMDV, foot-and-mouth disease virus; L^{pro}, leader proteinase; PAGE, polyacrylamide gel electrophoresis; RRL, rabbit reticulocyte lysate

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3. Results

L^{pro} is the first protein encoded on the FMDV genome. As protein synthesis initiates from one of two AUG codons, two forms of L^{pro}, Lab^{pro} and Lb^{pro} can be generated [20]. Both forms have the same enzymatic activities [21]. Based on evidence that the 28 amino acid shorter Lb^{pro} form is the biologically relevant one [22], we have consistently investigated this form.

We have recently developed an RRL assay for the in vitro proteolysis of eIF4GI by the FMDV Lb^{pro} or the $2A^{pro}$ of rhino- and enteroviruses [18]. In this system, the picornaviral proteinases are translated from a suitable mRNA, transcribed in vitro and their synthesis detected by labelling with [^{35}S]methionine. As eIF4GI is abundant in the RRL, processing of this molecule can be easily followed by immunoblotting. Cleavage of eIF4GI by FMDV Lb^{pro} is very efficient; for example, eIF4GI is cleaved to 50% after 8 min and to 90% after 12 min at an Lb^{pro} concentration of 20 pg/μl (1 nM) at the 12 min time-point [18].

The efficiency of this reaction is underlined in Fig. 1 in which the concentration of RNA (transcribed from pCITE Lb^{pro}VP4VP2) has been reduced by five fold compared to that previously employed [18]. Self-processing of Lb^{pro} between its own C-terminus and the N-terminus of VP4 is very efficient. No uncleaved precursor is visible. Lb^{pro} itself is just visible after 12 min (Fig. 1A); the VP4VP2 is less intense, as it contains only two methionine codons compared to the four in Lb^{pro}. Despite the very low concentration of Lb^{pro}, cleavage of eIF4GI begins at 8 min; 50% processing is observed at 12 min at an Lb^{pro} concentration of about 2 pg/μl (0.1 nM) (Fig. 1B). eIF4GI itself migrates with a molecular weight of 220 kDa as a series of bands [23]. The bands have different N-termini which arise from the use of different AUG codons during synthesis of eIF4GI [24]. Cleavage of eIF4GI by Lb^{pro} at its single recognition site between Gly^{674}*Arg (numbering according to [24]) generates a series of N-terminal cleavage products which are detected by the N-terminal antiserum used here (cp_N, Fig. 1B). The single C-terminal cleavage fragment is not detected by this antiserum. In summary, the cleavage of eIF4GI by Lb^{pro} is an extremely efficient reaction which can take place in RRLs at the low concentrations of Lb^{pro} found in FMDV-infected cells.

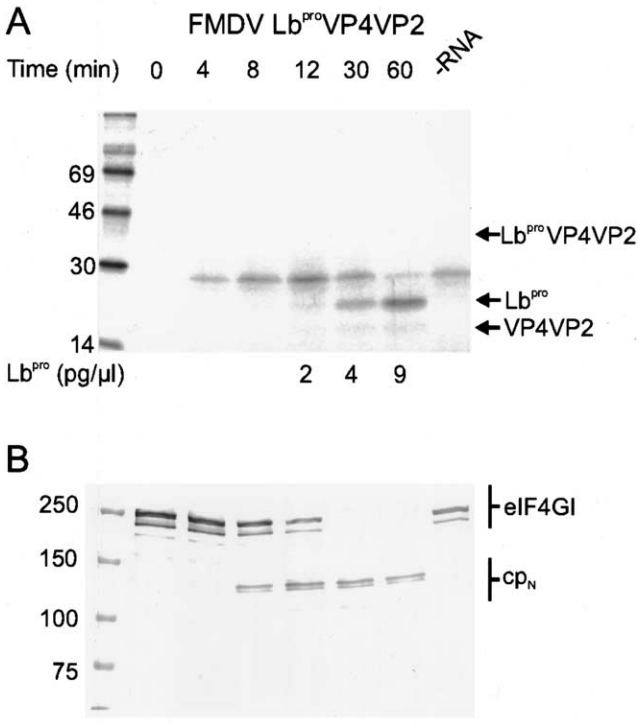

Fig. 1. Cleavage of eIF4GI during in vitro translation of FMDV Lb^{pro}. RRLs were incubated with or without the mRNA of FMDV Lb^{pro} (0.2 ng/μl) as described in Section 2; protein synthesis was terminated at the times given by placing the samples on ice followed by the addition of unlabelled methionine and cysteine to 2 mM and Laemmli sample buffer. Aliquots (2 and 3 μl, respectively) were analysed on 15% polyacrylamide gels followed by fluorography for the synthesis of Lb^{pro} (A) and on 6% polyacrylamide gels followed by immunoblotting for the status of eIF4GI (B). The positions of the uncleaved Lb^{pro}VP4VP2 and the cleavage products Lb^{pro} and VP4VP2 are marked in (A); the concentration (in pg/μl) of Lb^{pro} reached at each time point is indicated underneath the lane. In (B), intact eIF4GI and the cleavage product cp_N are marked. Protein standards (in kDa) are indicated in both panels.

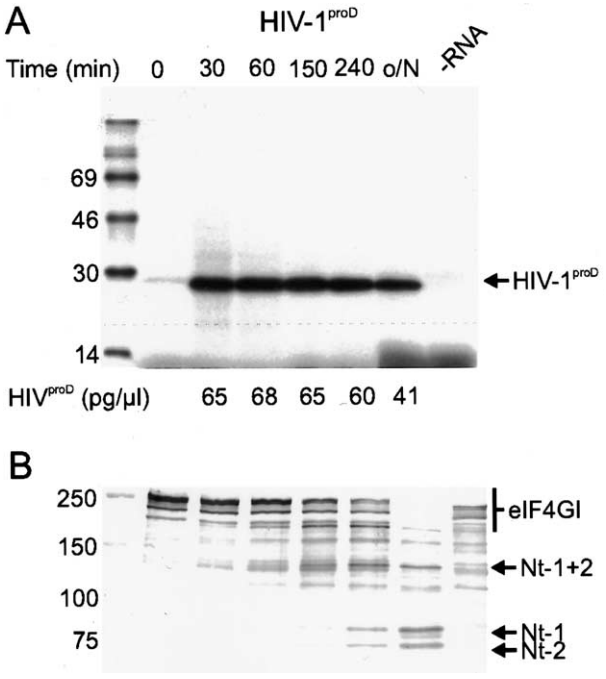

Fig. 2. Cleavage of eIF4GI during in vitro translation of the HIV-1^{proD}. mRNA was transcribed and used at a concentration of 15 ng/μl to programme HIV-1^{proD} protein synthesis (A) as described in Fig. 1. The positions of HIV-1^{proD} are marked and the corresponding concentrations (in pg/μl) reached at each time point are indicated underneath the lane. The cleavage of eIF4GI is analysed in (B). o/N, over night (20 h).

To compare the ability of eIF4GI in RRLs to serve as a substrate for HIV-1^{pro}, we synthesised RNA from plasmid pCITE HIV-1^{proD}, which contains two copies of part of the HIV-1 cDNA encoding the proteinase gene product linked in phase by a small oligonucleotide [17]. Translation of this mRNA generates a tethered dimer which is immediately active as dimerisation is not required. This strategy was followed as it is believed that dimerisation is slow at pH 7 [25], the approximate pH of the RRL.

The expression of the HIV-1^{proD} in RRLs was efficient as shown in Fig. 2A. However, cleavage products of eIF4GI were not visible until 150 min after HIV-1^{proD} synthesis had

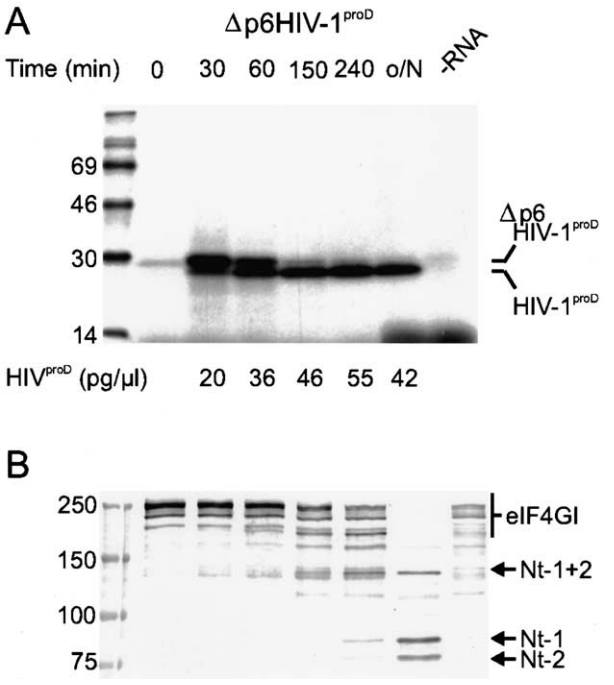

Fig. 3. HIV-1^{proD} self-processing and cleavage of eIF4GI during in vitro translation. Protein synthesis of Δp6HIV-1^{proD} (15 ng/μl) was as described in Fig. 1, as was the analysis of the synthesis of Δp6HIV-1^{proD} (A) and the cleavage of eIF4GI (B). The concentration (in pg/μl) of mature HIV-1^{proD} reached at each time point is indicated underneath the lane. o/N, over night (20 h).

begun and cleavage was not complete until after 20 h of incubation. The HIV-1^{proD} cleavage products of eIF4GI are different to those of Lb^{pro}, as observed by Ohlmann et al. [14]. Three products are visible for HIV-1^{proD}, migrating at 150 kDa, 80 kDa and 70 kDa. As the 150 kDa species seems to become less intense during the time-course of the incubation, it probably represents the precursor of the 80 kDa and 70 kDa fragments. These fragments are very similar to those observed by Ohlmann [14]. It should be noted that some non-specific degradation of eIF4GI also took place during this period of incubation (Fig. 2, no RNA lane). Further experiments used an RNA which only encoded the HIV-1^{pro} monomer; however, no processing of eIF4GI was observed, suggesting that the monomer indeed cannot dimerise in the RRLs (data not shown).

This slower rate of cleavage seemed to indicate that eIF4GI is a poor substrate for HIV-1^{pro}. To ensure that this result was not caused by the synthesis of an inactive HIV-1^{proD} and to show that the HIV-1^{proD} was active at the pH of the RRLs, we modified the DNA construction to include the 20 most C-terminal amino acids of the p6 'transframe' protein which precedes the HIV-1^{pro} in the gag reading frame of HIV-1 [26,27]. This enables the self-processing reaction to be monitored in RRLs. Accordingly, RNA from this plasmid (pCITE Δp6HIV^{proD}) was transcribed and used to drive protein synthesis. As can be seen in Fig. 3A, two forms of HIV-1^{proD} are visible after 30 min, representing the Δp6/HIV-1^{proD} and the mature dimer resulting from self-processing. After 2 h, all material was in the cleaved form, indicating that the HIV-1^{pro} was fully active in self-processing in RRLs. Nevertheless, analysis of the state of eIF4GI (Fig. 3B) shows that the kinetics are similar to those observed with the HIV-1^{proD} protein which did not require self-processing (compare Fig. 2B with Fig. 3B). No increase in cleavage rates was obtained by decreasing the pH of the RRL to pH 6, although interpretation was complicated by the apparent instability of eIF4GI under these conditions (data not shown).

No processing of eIF4GII was observed during the time courses used in these experiments (data not shown), confirming the observation of Ohlmann et al. [14] that eIF4GII is not a substrate for HIV-1^{pro}.

4. Discussion

The cleavage of the cellular protein eIF4GI by HIV-1^{pro} has been recently demonstrated. However, the role of eIF4GI cleavage in HIV-1 replication was not elucidated; it has been suggested that it may be involved in the ability of HIV-1^{pro} to inhibit host cell translation. As the HIV-1^{pro} is active at later times in HIV-1 infection, it is difficult to imagine a role for this cleavage in affecting viral translation. To understand cleavage of eIF4GI by HIV-1^{pro}, we quantitated the efficiency of this cleavage using the FMDV Lb^{pro} reaction as a benchmark.

The results show clearly that HIV-1^{proD} was much less efficient in cleaving eIF4GI in RRLs than FMDV Lb^{pro}. Even at extremely low rates of Lb^{pro} synthesis (2 pg/μl, 0.1 nM), eIF4GI cleavage was still 50% complete after 12 min. In contrast, even at much higher concentrations (65 pg/μl), cleavage by HIV-1^{proD} was not initiated until about 150 min and was still not complete after 20 h. This is also much slower than the reaction involving HRV2 2A^{pro}, which although requiring higher concentrations (140 pg/μl) than that found with L^{pro}, can still process eIF4GI to 50% within 15 min [18]. That the tethered dimer of HIV-1^{pro} was active under these conditions was demonstrated by including a piece of the p6 transframe protein, which thus allowed the efficiency of self-processing to be monitored. Despite the low efficiency of eIF4GI cleavage, this system is a simple way of measuring HIV-1^{pro} self-processing and intermolecular activity on a polypeptide substrate which is cleaved during infection.

Taken together, these results and those of Ohlmann [14] indicate that HIV-1^{pro} cleavage of eIF4GI is both quantitatively and qualitatively different from that of picornaviruses. This sheds doubt on whether the cleavage of eIF4GI by HIV-1^{pro} is related to an effect on protein synthesis during the HIV-1 replicative cycle.

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