Cleavage of eIF4G by HIV-1 protease: effects on translation

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Abstract **We have recently reported that HIV-1 protease (PR) cleaves the initiation factor of translation eIF4GI [Ventoso et al., Proc. Natl. Acad. Sci. USA 98 (2001) 12966–12971]. Here, we analyze the proteolytic activity of HIV-1 PR on eIF4GI and eIF4GII and its implications for the translation of mRNAs. HIV-1 PR efficiently cleaves eIF4GI, but not eIF4GII, in cell-free systems as well as in transfected mammalian cells. This specific proteolytic activity of the retroviral protease on eIF4GI was more selective than that observed with poliovirus 2A^{pro}. Despite the presence of an intact endogenous eIF4GII, cleavage of eIF4GI by HIV-1 PR was sufficient to impair drastically the translation of capped and uncapped mRNAs. In contrast, poliovirus IRES-driven translation was unaffected or even enhanced by HIV-1 PR after cleavage of eIF4GI. Further support for these in vitro results has been provided by the expression of HIV-1 PR in COS cells from a Gag-PR precursor. Our present findings suggest that eIF4GI intactness is necessary to maintain cap-dependent translation, not only in cell-free systems but also in mammalian cells.**

Key words: HIV-1 protease; eIF4GI; eIF4GII; Regulation of translation; Viral protease; Translation initiation factor

1. Introduction

Translation in eukaryotes begins with the recruitment of mRNAs by the 4F group of initiation factors (IF), which recognize the cap structure present in mRNAs to promote ribosome entry [1–3]. The eIF4F complex is composed of three subunits: eIF4E, which binds to the cap structure of mRNAs, the RNA helicase, eIF4A, which unwinds the eventual secondary structure present in mRNAs, and the eIF4G subunit, which interacts with a number of proteins and initiation factors, including PABP (poly(A)-binding protein), eIF3, eIF4E and eIF4A [2,4,5]. Thus, the N-terminal half of eIF4G contains the binding domains for eIF4E and PABP, whereas the central part of eIF4G is responsible for the interaction with eIF4A and eIF3 [6–11]. Finally, the C-terminal region seems to be involved in regulating eIF4G activity [12,13]. A novel form of eIF4G (eIF4GII) has been found that shares ~50% sequence homology with eIF4GI [14]. Data from in vitro experiments suggested that eIF4GI and II factors could be functionally exchangeable. Notably, translational shut-off induced by poliovirus and rhinovirus appears to correlate with cleavage of eIF4GII, rather than with the proteolysis of eIF4GI [15,16]. eIF4GII is less abundant in the cell than eIF4GI and its exact contribution to the overall control of cell translation remains to be explored [16].

eIF4F has emerged as a key target in translational control [2]. One of the best illustrated examples of this central role of eIF4G comes from the proteolytic degradation of this factor in cells subjected to conditions of stress, such as viral infections or apoptosis [1,17,18]. Caspase-mediated cleavage of eIF4G precedes the inhibition of translation in cells undergoing apoptosis [19,20]. In addition, some members of the picornavirus group encode viral proteases (enterovirus 2A^{pro} and aphthovirus L^{pro}) that cleave and inactivate eIF4GI leading to the shut-off of host translation [21,22]. These proteases also bisect eIF4GII, but with delayed kinetics [15,16]. Recently, eIF4GI has also been described as a target for HIV-1 protease (PR) [23,24]. While eIF4GI is cleaved once at positions 641 and 643 by 2A^{pro} and L^{pro}, respectively, HIV-1 PR cleaves at positions 678/681 and 1086 to separate this factor into three moieties [24]. In all these instances, cleavage of eIF4G by the viral protease uncouples the activity of the factor domains, leading to the blockade of translation initiation.

The impact of eIF4G cleavage on translation has been extensively analyzed, mainly using in vitro translation systems programmed with synthetic mRNAs. Cleavage of eIF4G by picornaviral proteases impairs translation of capped mRNAs, whereas internal initiation of translation directed by IRES elements is unaffected or even enhanced by eIF4G cleavage [25,26]. The effect of eIF4G cleavage on translation of artificially uncapped mRNAs is controversial. Some reports have shown that translation of both capped and uncapped mRNAs is inhibited to a similar extent, whereas other studies have claimed that translation of uncapped mRNAs is not only resistant, but is even increased upon cleavage of eIF4G [27–30].

In this report, we have analyzed the proteolytic activity of HIV-1 PR on eIF4GI and eIF4GII and its effect on translation of synthetic mRNAs in cell-free systems and in transfected mammalian cells.

2. Materials and methods

2.1. Plasmid construction and in vitro synthesis of mRNAs

pKS-Luc and pT7 IRES Polio-Luc plasmids were described previously [31]. To construct pKS-5′LHIV-Luc plasmid, the entire 5′ leader region of HIV-1 (clone pNL4.3) was amplified by PCR using the primers 5′ (CGACGCGGCCGCGGTCTCTCTGGTTAGACC) and 3′ (CGCAGACTAGTATCTCTCTCCTTCTAGCCTCC). The result-

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ing DNA fragment of 335 nucleotides was digested with *Not*I and *Spe*I and cloned into pKS-Luc plasmid previously digested with the same enzymes. pcDNA-GAG-PR was constructed by subcloning the 5′LGAGPR fragment from pKS-5′LGAGPR plasmid into pcDNA3.0 plasmid using *Not*I and *Stu*I/*Sma*I enzymes [24]. In vitro transcription was carried out with T7 RNA polymerase according to the manufacturer's instructions (Promega). For capping of mRNAs, 0.5 mM of CAP analogue (^{7}mGTP, New England) was included in the reactions and the concentration of GTP was reduced to 0.05 mM. Transcription reactions were digested with DNase RQ1 and mRNAs were purified through chroma-spin columns (Clontech) and analyzed by agarose gel electrophoresis.

2.2. In vitro translations

Translation in HeLa cell extracts was carried out as described previously [32]. Briefly, translation mixtures containing 70% (v/v) of HeLa S10 extract were programmed with 10–100 ng of mRNA in a final volume of 12.5 μl and incubated at 30°C for 90 min. Luciferase activity was measured in a Monolight luminometer. Translation in rabbit reticulocyte lysates (RRL) (Promega) was performed according to the manufacturer's instructions, except that concentration of KAcOH was optimized to 120 μM. 50 μCi of [^{35}S]Met/Cys (Translabel, Amersham) was added and lysates were programmed with the indicated amounts of mRNAs. The reaction was stopped by addition of 2× sample buffer and analyzed by SDS–PAGE and autoradiography.

2.3. Immunoblotting

50 μg of total protein was analyzed by SDS–PAGE, transferred to nitrocellulose membrane (Bio-Rad) and probed with the following antibodies: anti-eIF4GI (1:1000 dilution), anti-eIF4GII (1:1000 dilution, a gift from N. Sonenberg) or anti-p24 (1:500 dilution, EV Pro-

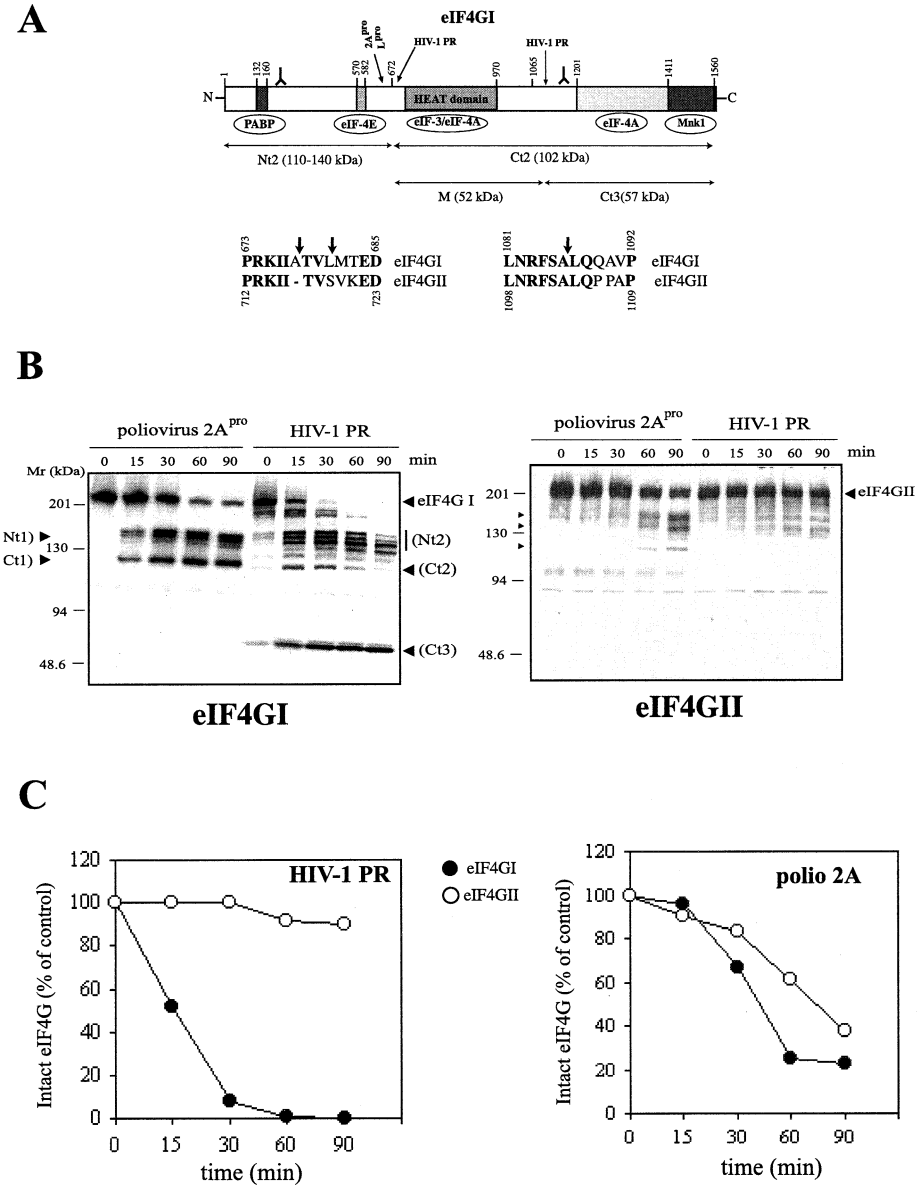

Fig. 1. Kinetics of eIF4GI and eIF4GII cleavage by HIV-1 PR in HeLa cell extracts. A: Schematic diagram of eIF4GI depicting the domains involved in the interaction with other initiation factors. The cleavage sites for HIV-1 PR and poliovirus 2A^{pro} are also denoted, as well as the main fragments of eIF4GI generated upon treatment with these proteases. The sequence alignments of cleavage sites of HIV-1 PR in eIF4GI and eIF4GII are also indicated. B: HeLa S10 extracts (50 μg) were incubated with 25 ng of HIV-1 PR or 2 μg of MBP-2A^{pro} for the indicated time periods and analyzed by Western blot against eIF4GII. The membranes were then stripped and reprobed with anti-eIF4GI antibodies. Cleavage products derived from eIF4GI and eIF4GII are indicated (arrowheads). C: Quantification of eIF4GI and eIF4GII cleavage from the experiment shown in B. Bands corresponding to intact eIF4GI and eIF4GII were quantified by computer densitometry and expressed as a percentage of the control (no protease treatment).

gramme EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, UK).

2.4. Transfection of mammalian cells

COS-7 cells ($\sim 0.3\times10^5$) were transfected with 1 μg of pcDNA-GAG-PR plasmid by coupled transfection/infection with recombinant vT7-3, as described previously [31]. 16 h post treatment cells were lysed in sample buffer and analyzed by Western blot. In cotransfection experiments with pKS-Luc plasmid, cells were lysed in luciferase buffer (25 mM glycylglycine, pH 7.8, 1 mM dithiothreitol, 0.5% Triton X-100) for luciferase activity measurements.

3. Results

3.1. Differential cleavage of HIV-1 PR on eIF4GI and eIF4GII in vitro

To assay the proteolytic activity of HIV-1 PR on eIF4GI and eIF4GII, HeLa S10 extracts were incubated with purified HIV-1 PR and the integrity of eIF4G factors was analyzed at different times. For comparison, cell extracts were also incubated with poliovirus $2A^{pro}$. As shown in Fig. 1B, both poliovirus $2A^{pro}$ and HIV-1 PR efficiently cleaved eIF4GI giving rise to the previously characterized band pattern of proteolytic products [24]. However, the kinetics of eIF4GI hydrolysis by these two proteases differed. HIV-1 PR hydrolyzed eIF4GI more rapidly than poliovirus $2A^{pro}$. Thus, an almost complete cleavage of eIF4GI was achieved for HIV-1 PR after 30 min of incubation, whereas 70% of the initiation factor remained intact at this time when incubated with poliovirus $2A^{pro}$ (Fig. 1C). More extensive proteolysis of eIF4GI by poliovirus $2A^{pro}$ required longer incubation times (90 min), although it did not yield the thorough degradation observed with HIV-1 PR.

Next, the kinetics of eIF4GII proteolysis by these two viral proteases were examined. Cleavage of eIF4GII by poliovirus $2A^{pro}$ was slightly delayed in comparison with eIF4GI (Fig. 1C). This result agrees closely with previous reports showing some preferential activity of picornaviral $2A^{pro}$ on eIF4GI [15]. Notably, no gross modification of eIF4GII was observed after incubation of HeLa extracts with HIV-1 PR. Densitometric quantification revealed neither a significant reduction of intact eIF4GII nor the appearance of smaller products related to this factor.

3.2. Effects of HIV-1 PR on translation of capped, uncapped and poliovirus IRES-containing mRNAs

Given the preferential activity of HIV-1 PR on eIF4GI, we focussed our interest on the impact of HIV-1 PR on translation in the presence of intact eIF4GII. To this end, several mRNAs bearing different 5′ non-coding sequences were synthesized (Fig. 2A). HeLa cell extracts were preincubated with the indicated amounts of HIV-1 PR or poliovirus $2A^{pro}$. The integrity of eIF4GI and eIF4GII was analyzed before addition

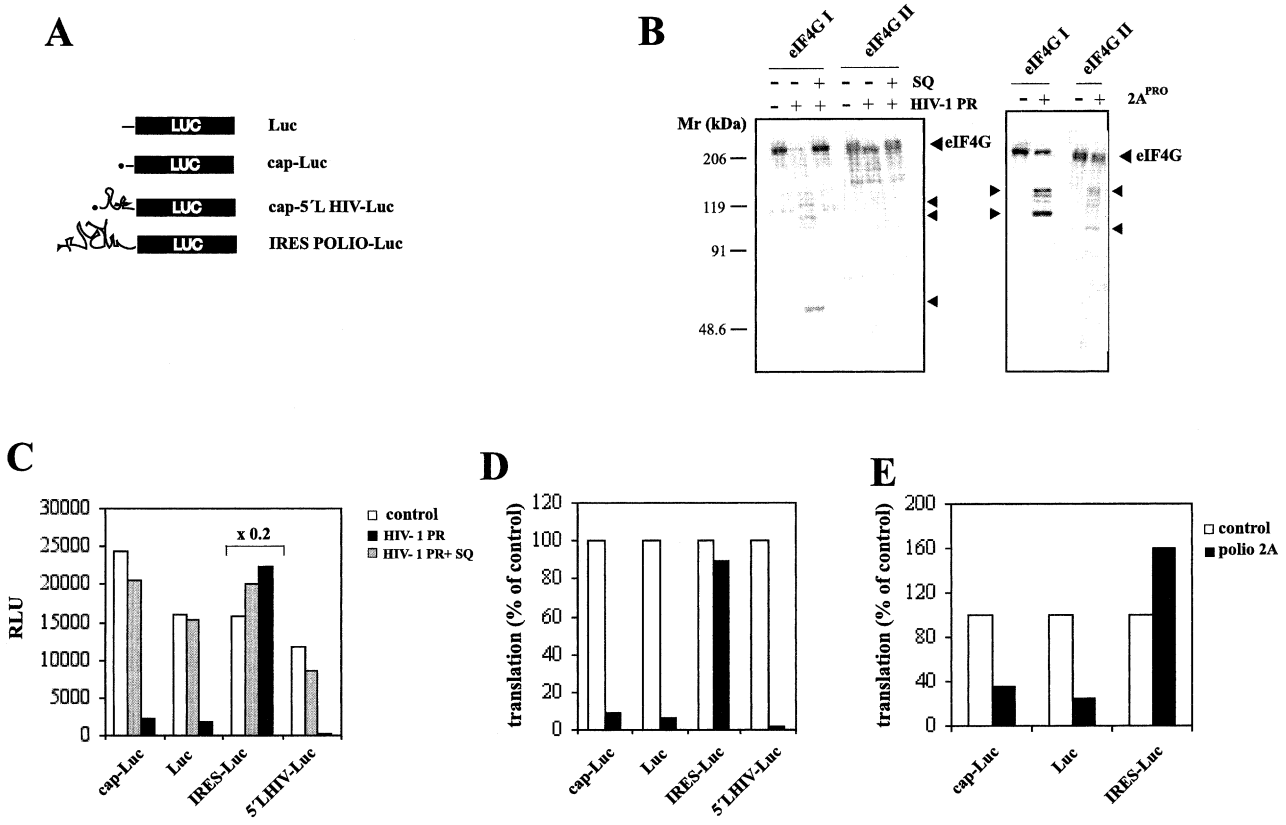

Fig. 2. Effect of HIV-1 PR-mediated cleavage of eIF4GI on translation in HeLa cell extracts. A: Diagram of synthetic mRNAs used to program the in vitro translations. B: HeLa cell extracts were preincubated with 25 ng HIV-1 PR (with or without 1.5 μM saquinavir (SQ)) or 2 μg MBP-$2A^{pro}$ for 30 min and 2 h, respectively. Afterwards, 1.5 μM of saquinavir was added to all samples to neutralize the activity of HIV-1 PR and an aliquot was taken to analyze the integrity of eIF4G factors before the addition of mRNAs. C: Luciferase activities were measured after programming extracts with 200 ng of cap-Luc, 200 ng of uncapped Luc or 10 ng of IRES Polio-Luc mRNAs and incubation at 30°C for 90 min. Since poliovirus IRES-driven translation was much more efficient than cap-dependent translation in these extracts, values of luciferase activity obtained for IRES Polio-Luc mRNAs were divided by 5. RLU: relative light units. D: Means of three independent experiments, expressed as percentage inhibition with respect to control samples. E: Effect of MBP-$2A^{pro}$ treatment on translation of the indicated mRNAs. Data were calculated as described in D.

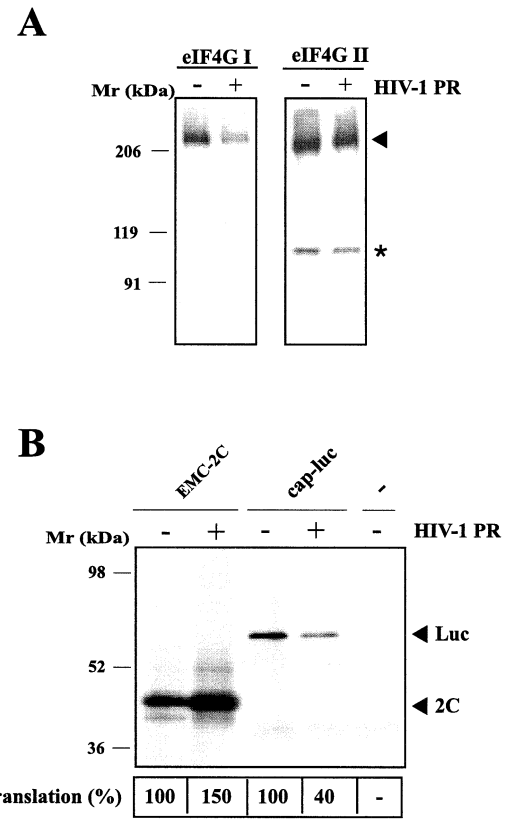

Fig. 3. Effect of HIV-1 PR on translation in RRL. Lysates were treated or not with 25 ng of HIV-1 PR for 30 min, followed by the addition of 1.5 μM of saquinavir. An aliquot of lysates was analyzed by Western blot against eIF4GI and eIF4GII (A) and the remaining lysate was used to program translations with 10 ng of EMC-2C or 50 ng of cap-Luc mRNAs (B). Quantification of bands was carried out by computer densitometry and data are expressed as percentage of control with respect to untreated lysates.

of different mRNAs. Cleavage of eIF4GI by HIV-1 PR was almost complete, whereas 20% of eIF4GI remained intact upon treatment with poliovirus $2A^{pro}$ (Fig. 2B). In addition, poliovirus $2A^{pro}$ also degraded eIF4GII by 50%. No reduction of the intact form of eIF4GII was observed upon incubation with HIV-1 PR. However, it should be noted that treatment with HIV-1 PR induced the disappearance of the lowest mobility band of eIF4GII.

Preincubation of extracts with HIV-1 PR largely prevented the translation of capped and uncapped luciferase mRNAs (90% inhibition), but no decrease of luciferase synthesis was found with the mRNA bearing the poliovirus IRES sequence (Fig. 2C,D). Notably, HIV-1 PR-mediated inhibition of translation was even stronger (98%) in the case of luciferase mRNAs bearing the 5′ leader of HIV-1. It should be noted that when 5′ leader of HIV-1 was placed before the luciferase coding region, translation of this mRNA was three- to five-fold inhibited compared with capped mRNA (Fig. 2D). In contrast, translation of luciferase mRNAs driven by poliovirus IRES was resistant to cleavage of eIF4GI. Similar results were obtained for poliovirus $2A^{pro}$, although the extent of translational inhibition obtained was lower than with HIV-1 PR (Fig. 2E).

In addition, the effect of HIV-1 PR was tested on the translation of synthetic mRNAs in RRL. As shown in Fig. 3A, intact eIF4GI was reduced by 70% upon incubation with HIV-1 PR, although the resulting cleavage fragments of eIF4GI were not detected. Our antibodies raised against human eIF4GI also failed to detect HIV-1 PR-induced cleavage products of rabbit eIF4GI, as described previously [29]. In contrast, no cleavage of eIF4GII by HIV-1 PR was apparent in RRL (Fig. 3A) even at the highest concentration of protease tested (data not shown). Nevertheless, translation of capped luciferase mRNAs was inhibited by 60% upon HIV-1 PR treatment, which correlates with the extent of eIF4GI cleavage (Fig. 3A). As was the case with HeLa cell extracts, translation driven by IRES of EMC virus was slightly stimulated by HIV-1 PR treatment (Fig. 3B).

3.3. Effect of HIV-1 PR on translation in mammalian cells

To analyze the activity of HIV-1 PR on translation in intact cells, COS-7 cells were transfected with a plasmid encoding the entire Gag precursor of HIV-1 followed by the PR gene. HIV-1 PR was active in transfected cells as evidenced by the processing of the Gag precursor to yield CA, MA and CA–MA proteins, as occurs in HIV-1-infected cells (Fig. 4A). The action of HIV-1 PR and the subsequent processing of Gag precursor were completely prevented by addition of 2 μM saquinavir to the cell culture. Notably, expression of Gag-PR in transfected cells induced the cleavage of eIF4GI. In addition, a slight modification in the mobility of the eIF4GII band was observed, but no cleavage products derived from this factor could be detected.

We next analyzed the effect of HIV-1 PR on the expression of luciferase gene from a pKS-Luc plasmid. Cotransfection of pcDNA-Gag-PR with pKS-Luc inhibited luciferase synthesis by 70%. Notably, this inhibition was prevented when saquinavir was added to the cell cultures (Fig. 4B). This finding indicates that HIV-1 PR is responsible for the inhibitory effect on luciferase synthesis. Consistent with our in vitro results, the synthesis of poliovirus 2C protein driven by EMC virus IRES was not affected by Gag-PR expression (Fig. 4C).

4. Discussion

Our initial finding that HIV-1 PR had the capacity to proteolyse the initiation factor of translation eIF4G provided new clues for the investigation of the regulation of translation in retroviruses [24]. Now we demonstrate that HIV-1 PR selectively proteolyses eIF4GI very efficiently in cell-free systems. In contrast, eIF4GII seems not to suffer any major degradation, since no cleavage products were identified under our experimental conditions. However, there is evidence that the electrophoretic mobility of eIF4GII changes slightly. eIF4GII usually runs in polyacrylamide gels as a close doublet; after HIV-1 PR incubation, the upper form disappears, while the amount of the lower form increases. A partial differential sensitivity of eIF4GI and eIF4GII to cleavage by picornaviral proteases has been reported before [15,16]. We now document that the preferential proteolysis of the eIF4GI form over the degradation of eIF4GII is exacerbated in the case of HIV-1 PR. Notably, some findings presented here agree with recently published data [23]. Several explanations can be put forward to account for the preferential cleavage of the eIF4GI form by HIV-1 PR. The distal and proximal cleavage sites for HIV-1 PR surrounding position 680 in the eIF4GI sequence are poorly conserved in eIF4GII. Thus, the Ala^{678} at the N-ter-

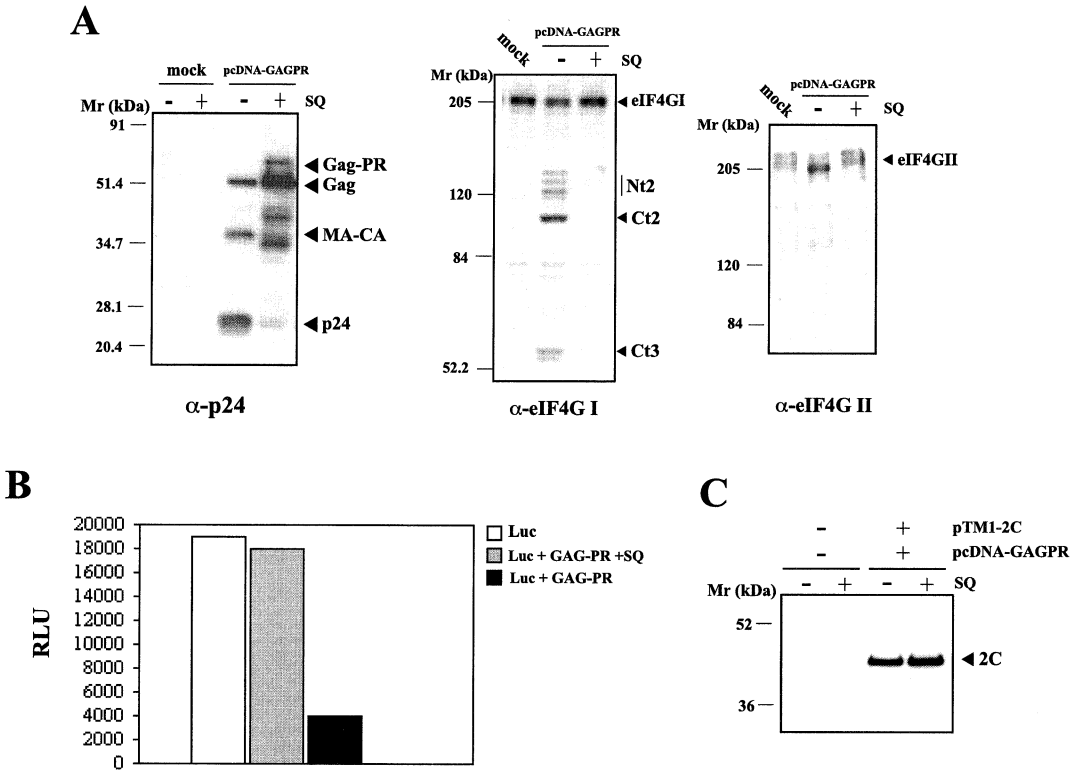

Fig. 4. Effect of HIV-1 Gag-PR expression on translation in transfected cells. COS cells were cotransfected with 0.5 μg of pcDNA-GAG-PR and 0.15 μg of pKS-Luc plasmids as described in Section 2. Where indicated, cell cultures were treated with 2 μM saquinavir (SQ) and extracts were obtained at 16 h post treatment. A: An aliquot was analyzed by Western blot against HIV-1 p24 (left), eIF4GI (middle) and eIF4GII (right). B: Luciferase activity of extracts. C: Western blot against poliovirus 2C protein.

minus of the distal hydrolyzable peptide bond is absent in eIF4GII (Fig. 1A). In addition, the proximal dipeptide L-M[681] in eIF4GI that is also cleaved by HIV-1 PR has no counterpart in eIF4GII, where a dipeptide S-V is present instead. In fact, the flanking sequences present at the cleavage sites are conserved in both eIF4GI and eIF4GII. Therefore, the differences in primary sequence probably make eIF4GII refractory to cleavage by HIV-1 PR at positions 678/681. Less clear is the lack of HIV-1 PR cleavage at position 1086 of eIF4GII, since this site and the flanking sequences are conserved in both factors. Therefore, in this case the lack of cleavage of the eIF4GII form may be ascribed to conformational constraints that make this site inaccessible to HIV-1 PR. The differential activity of HIV-1 PR on eIF4GI has provided a system with which to test the specific role that this form of eIF4G plays in translation in cell-free systems and in transfected cells. Our data indicate that in vitro translation of capped and uncapped luciferase mRNAs is strictly dependent on eIF4GI integrity. Translation was almost completely inhibited in HeLa cell extracts when eIF4GI was proteolysed by HIV-1 PR even though eIF4GII remained undegraded. This dependence of protein synthesis on eIF4GI was even higher when a luciferase mRNA bearing the 5′ leader region of HIV-1 was used to program cell-free systems. This result agrees with the notion that translation of mRNAs bearing highly structured regions, like that present in the 5′ leader of HIV-1 genome, were more dependent on eIF4F activity [33,34]. However, translation driven by picornaviral IRES was not affected by eIF4GI cleavage, thus agreeing well with previous findings [25,26].

The differential activity of HIV-1 PR on the two forms of eIF4G was also observed in RRL, although cleavage of eIF4GI by HIV-1 PR was less efficient than in HeLa cell extracts. This observation correlates with the lower inhibition of luciferase translation in RRL. Thus, there is a close parallel between the intactness of eIF4GI and the extent of cap-dependent translation. These results agree with recently published data from Ohlman et al. [23] showing a poor inhibition (30–40%) of cap-dependent translation in RRL upon HIV-1 PR treatment. Surprisingly, FMDV IRES-driven translation was also weakly compromised (20–30% inhibition) in HIV-1 PR-treated RRL extracts. In our opinion, RRL do not represent a good system to analyze the HIV-1 PR-mediated cleavage of eIF4G on translation.

Taken together, our results illustrate that eIF4GI supports most of the initiation events in cell-free translation systems. These data were also corroborated in transfected cells, and are consistent with the fact that eIF4GI is more abundant in the cell than eIF4GII [16]. However, our results partially contrast with those reported by Gradi et al. [14]. These authors suggested that eIF4GI and eIF4GII were functionally interchangeable, based on the observation that addition of recombinant eIF4GII restored translation in extracts depleted of eIF4GI in cell extracts. However, the role that eIF4GII plays in the control of translation in intact cells has not yet been elucidated, perhaps because picornaviral proteases discriminate poorly between eIF4GI and eIF4GII cleavage. The finding that HIV-1 PR specifically targets eIF4GI raises the possibility of dissecting the separate roles that eIF4GI and eIF4GII play in translation.

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