



HAL
open science

HIV protease cleaves poly(A)-binding protein

Enrique Álvarez, Alfredo Castelló, Luis Menéndez-Arias, Luis Carrasco

► **To cite this version:**

Enrique Álvarez, Alfredo Castelló, Luis Menéndez-Arias, Luis Carrasco. HIV protease cleaves poly(A)-binding protein. *Biochemical Journal*, 2006, 396 (2), pp.219-226. 10.1042/BJ20060108. hal-00478519

HAL Id: hal-00478519

<https://hal.science/hal-00478519>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

HUMAN IMMUNODEFICIENCY VIRUS PROTEASE CLEAVES POLY(A) BINDING
PROTEIN

Enrique Álvarez*, Alfredo Castelló, Luis Menéndez-Arias and Luis Carrasco
Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Facultad de Ciencias,
Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain.

* Corresponding author: Enrique Álvarez. Present address, Centro Nacional de Biotecnología
(CSIC), Cantoblanco, 28049 Madrid, Spain, Tel, 34-915854526; Fax, 34-915854506; e-mail:
ealvarez@cnb.uam.es

Page heading title: PABP cleavage by retroviral proteases

SYNOPSIS

The poly(A) binding protein (PABP) is able to interact with the 3' poly(A) tail of eukaryotic mRNA, promoting its translation. Cleavage of PABP by viral proteases encoded by several picornaviruses and caliciviruses plays a role in the abrogation of cellular protein synthesis. We report that infection of MT-2 cells with HIV-1 leads to an efficient proteolysis of PABP. Analysis of PABP integrity was carried out in BHK-21 and COS-7 cells upon individual expression of the protease from several members of the *Retroviridae* family, e.g. Moloney murine leukemia virus (MoMLV), mouse mammary tumor virus (MMTV), human T-cell leukemia virus type I (HTLV-I), simian immunodeficiency virus (SIV), human immunodeficiency virus type 1 (HIV-1) and HIV-2. Moreover, protease activity against PABP was tested in a HeLa cell free system. Only MMTV, HIV-1 and HIV-2 proteases were able to cleave PABP in absence of other viral proteins. Purified HIV-1 and HIV-2 proteases directly cleave PABP1 at positions 237 and 477, separating the two first RNA recognition motifs from the C-terminal domain of PABP. An additional cleavage site located at position 410 was detected for HIV-2 protease. These findings indicate that some retroviruses may share with picornaviruses and caliciviruses, the capacity to proteolyze PABP.

Key words: translation; poly(A) binding protein; eukaryotic initiation factor; human immunodeficiency virus; protease.

INTRODUCTION

The initiation of translation is a multistep process, being a major regulatory target for translational control in animal virus infected cells. In the early steps of translation the 5' cap structure of the mRNA is recognized by eIF4F complex. eIF4F also binds to the small ribosome subunit by its interaction with eIF3 forming the 48S complex. Then, the small ribosomal subunit migrates along the 5'-untranslated region (5'UTR) until an AUG initiation codon is encountered in a favorable context. The eIF4F complex is composed of three polypeptides: the cap-binding protein eIF4E, the ATP-dependent RNA helicase eIF4A and the scaffolding protein eIF4G [1]. Another motif recognized by the translation machinery in the mRNA is the poly(A) tail, which is achieved by means of the poly(A) binding protein (PABP). In addition, PABP interacts with the N-terminal domain of eIF4G, promoting the circularization of the mRNA [2, 3]. The eIF4G-PABP interaction may induce several changes in the translation initiation complex that increase the eIF4E affinity for the cap structure [4]. In this regard, simultaneous interactions between the 5' cap structure and the 3' poly(A) tail of the mRNA synergistically stimulate translation, both *in vitro* and *in vivo* [5, 6]. In human cells there are three types of PABP proteins, with several isoforms encoded by different genes and with different localizations and functions: cytoplasmic PABPs (PABPC group), nuclear PABP (PABPN) and X-linked PABP. Cytoplasmic PABP is a highly conserved protein that contains two domains linked by an unstructured region rich in proline and methionine residues: an N-terminal domain with four conserved RNA recognition motifs (RRMs) and a C-terminal helical domain that is not required for RNA recognition, but is essential for PABP oligomerization and for the interaction with several regulatory proteins implicated in deadenylation of the poly(A) tail and the initiation and termination of translation [7, 8]. PABP interacts with translation initiation factors such as eIF4G and eIF4B [5, 9, 10], with the eukaryotic release factor eRF3 [11-13], and with two regulatory proteins termed as PABP-interacting proteins 1 and 2 (Paip1 and Paip2), which are implicated in regulation of translation [14, 15].

Animal viruses have evolved mechanisms to manipulate the host translational machinery in order to maximize efficient viral mRNA translation and facilitate the selective translation of viral mRNA. For example, in picornavirus-infected cells, the proteolytic cleavage of eIF4G by rhinovirus or poliovirus 2A proteases (2A^{pro}) or by the aptovirus protease L inhibits translation of capped cellular mRNAs [16]. In contrast, translation of the uncapped picornavirus RNA that occurs by a cap-independent mechanism involving the internal ribosome entry site (IRES) is not affected by eIF4G hydrolysis. This mechanism is not exclusive of some picornaviruses. Retroviral genomic RNAs are capped at their 5' ends and contain a 3' poly(A) tail. However, retroviral RNAs have a relatively long 5' UTR, and the presence of stable secondary structures between the cap and the initiation codon has been proposed to strongly interfere with the scanning mechanism that operates during the initiation of translation [17]. Interestingly, IRES elements have been found in several retroviruses, including HIV-1, and their effects promoting cap-independent translation of retroviral genomic RNAs have been demonstrated [18]. In addition,

recent reports have shown that eIF4G is cleaved by the proteases (PR) of several members of the family *Retroviridae*, including mouse mammary tumor virus (MMTV), Moloney murine leukemia virus (MoMLV), human T-cell leukemia virus type I (HTLV-I), simian immunodeficiency virus (SIV), human immunodeficiency virus type 1 (HIV-1) and HIV-2 [19, 20]. The hydrolysis of eIF4G leads to the inhibition of cap-dependent translation without affecting IRES-driven protein synthesis [19, 20].

PABP plays an important role in translation and it is not surprising that certain viruses have developed mechanisms to target this protein to abrogate cellular translation. Rotavirus nonstructural protein 3 (NSP3) interacts with the N-terminus region of eIF4G, replacing PABP. This interaction leads to the shut-off of cellular protein synthesis, whereas viral mRNA translation is maintained unaffected by circularization via NSP3 interaction with the 3'UTR of viral transcripts [21]. Several reports have shown that PABP is cleaved by 2A^{pro} and 3C protease (3C^{pro}) during enterovirus infection [22-24]. Poliovirus 2A^{pro} bisects PABP, while poliovirus 3C^{pro} cleaves this factor at three sites separating its C-terminal and N-terminal domains. It has been proposed that the removal of the C-terminal domain inhibits cellular translation without affecting binding of the N-terminal domain of PABP to the poly(A) tail and eIF4G [25]. A similar strategy has recently been described in caliciviruses, in which the 3C-like protease (3CL^{pro}), like poliovirus 3C^{pro}, cleaves PABP during infection [26]. In the present work, we present evidence showing that retroviral PRs such as these from MMTV, HIV-1 and HIV-2 are able to hydrolyze PABP.

EXPERIMENTAL

Plasmids and in vitro Transcription – The plasmids pT7SV-HIV-1PR and pT7SV-2A^{pro} and pT7SVwt were described previously [27].

The pTM1-derived plasmids containing the PR-coding regions of several retroviruses were described in detail earlier [19, 20]. The pTM1-Luc plasmid, which contains the luciferase gene, and the plasmid pTM1-2A^{pro} have also been described [28, 29].

The plasmid pGEX-2T-PABP1 containing the sequence encoding the human PABP1, lacking the first nine amino acids and fused to the glutathione S-transferase (GST) gene, was obtained as previously described [30] and kindly provided by Amelia Nieto, Centro Nacional de Biotecnología, CSIC, Madrid, Spain.

Capped genomic SV mRNAs were synthesized *in vitro* using the T7 RNA polymerase kit (Promega). Plasmids used as DNA templates in these assays were linearized with *Xho*I.

Cell Culture, Virus Infection and Transfection - MT-2 cells were grown in RPMI medium 1640 containing 10 % fetal calf serum. MT-2 cells were infected with HIV-1 virus (NL3.4 strain) by using a multiplicity of infection of ~ 1 plaque-forming units per cell, and at three days post-

infection (dpi) cells were lysed in sample buffer as described previously [29]. HIV-1-infected cells were provided by Balbino Alarcón, Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Madrid, Spain .

BHK-21 cells were electroporated with 30 µg of recombinant SV genomic RNAs in final volume of 50 µl as described [27]. Electroporated-cells were grown in DMEM (Dulbecco's modified Eagle's minimal essential medium) containing 10 % fetal calf serum and non-essential amino acids, and at 8 hours post electroporation (hpe) cells were lysed in sample buffer.

COS-7 cells were grown in DMEM containing 10% fetal calf serum and non-essential amino acids. Coupled infection/DNA transfection of COS-7 cells with recombinant vaccinia virus (vvT7) and pTM1-derived plasmids have been described in detail previously [28]. Transfection efficiencies were determined by immunofluorescence using an anti-luciferase antiserum (Promega) after transfecting the cells with the plasmid pTM1-Luc as described previously [19].

Western Blotting - Proteins were transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad) for Western blot analysis. Western blots were developed with the following antibodies: mouse monoclonal anti-PABP antibody (Abcam) at a 1:250 dilution; anti-eIF4GI antisera raised against peptides derived from the N- and C-terminal regions of the human eIF4GI [28] at a 1:1000 dilution; or rabbit antisera against the C-terminal region of eIF4GII (a gift from N. Sonenberg, McGill University, Montreal, Canada) at 1:500 dilution; hybridoma supernatant of a monoclonal antibody against eIF4A factor at a 1:50 dilution (a gift from H. Trachsel, Institute for Biochemistry and Molecular Biology, University of Berne, Switzerland) and mouse ascites of a monoclonal antibody against HIV-1 p24 antigen (Centralised Facility for AIDS reagents) was used at a 1:100 dilution. Goat anti-rabbit IgG antibody coupled to peroxidase and goat anti-mouse IgG antibody coupled to peroxidase (Pierce) were used at a 1:10000 dilution.

Protein Purification - HIV-1 PR was purchased from the Centralised Facility for AIDS reagents, from Dr. I. Pichova. Purified HIV-2 PR [31] was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from B. Shirley and M. Cappola (Boehringer Ingelheim Pharmaceuticals). MoMLV PR was expressed and purified as previously described [32]. The chimeric maltose binding protein (MBP)-2A^{pro} was purified by affinity chromatography, as described previously [33]. The pGEX-2T-PABP1 plasmid was used to purify the GST-PABP1 fusion protein by affinity chromatography, using a glutathione-agarose 4B resin (Pharmacia Biotechnology) as described previously [30].

Protease Cleavage Assays - To detect PABP processing by HIV PRs in cell-free systems, crude HeLa S10 extracts were incubated with 2.5 ng/µl of recombinant HIV-1 PR, HIV-2 PR, or MBP-2A^{pro}, and at indicated times, reactions were stopped by adding sample buffer. Lysates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Degradation kinetics of translation factors were determined by densitometric scanning of the protein band

corresponding to each factor analyzed using a GS-710 calibrated Imagin Densitometer (Bio-Rad). In order to map the cleavage sites of HIV-1 PR and HIV-2 PR on PABP, 50 µg of recombinant GST-PABP1 protein were incubated with 1 µg of recombinant HIV-1 PR or HIV-2 PR in a total volume of 200 µl for 3 h at 30°C in a buffer containing 50 mM Na₂HPO₄, pH 6.0, 25 mM NaCl, 5 mM EDTA, and 1 mM DTT. Cleavage products were separated by SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon polivinylidene difluoride membrane (Bio-Rad) and then subjected to automated Edman degradation with an Applied Biosystems Procise Sequencer in the Proteomics Service of Centro de Investigaciones Biológicas CSIC, Madrid, Spain.

RESULTS

Cleavage of PABP in HIV-1-Infected Cells - The hydrolysis of both PABP and eIF4G could contribute to the drastic shut-off of host translation in poliovirus-infected cells. In order to investigate the susceptibility of PABP to proteolysis after HIV-1 infection, MT-2 cells were infected with HIV-1 at a multiplicity of ~1 plaque-forming units per cell. The integrity of PABP was assessed by Western blotting at 3 dpi using anti-PABP antiserum (Figure 1A, upper panel). Proteolytic cleavage of PABP was clearly apparent in MT-2 cells infected with HIV-1. Thus, intact PABP decreased considerably, while a smaller immunoreactive protein band with molecular mass of about 53 kDa appeared. To analyze the integrity of other initiation factors these samples were blotted using antibodies against eIF4GI, eIF4GII and eIF4A (Figure 1). The amount of eIF4A remained constant after infection, indicating that this protein was not modified by HIV-1 and that similar amounts of proteins were loaded in all cases (Figure 1A, lower panel). Cleavage of eIF4GI was detected in MT-2 infected-cells, using antibodies that recognize epitopes at the N-terminal (Nt) or C-terminal (Ct) regions of the initiation factor. (Figure 1B). Hydrolysis of eIF4GI was estimated to be around 50-60%. In agreement with previous reports, eIF4GII was much less affected by HIV-1 infection [20]. Finally, the activity of HIV-1 PR in infected cells was also tested by Western blotting using anti-p24 antiserum (Figure 1C). The Gag (p55) precursor was processed to intermediate CA-MA product and to CA (p24) by HIV-1 PR.

HIV-1 PR cleaves PABP - Next, the ability of HIV-1 PR to cleave PABP was tested when this protease was expressed alone in culture cells. For this purpose, the HIV-1 PR-coding region within the *pol* gene was placed under the control of a duplicated late promoter in a Sindbis virus (SV) construct in order to generate a recombinant SV that expresses the HIV-1 PR in absence of other HIV-1 proteins (SV-PR) [27]. In addition, the poliovirus 2A gene was cloned using a similar strategy to generate a recombinant SV expressing poliovirus 2A^{pro} (SV-2A) [27]. The transcribed RNAs corresponding to wt SV, SV-PR and SV-2A were electroporated in BHK cells, and the integrity of PABP was examined at 8 hpe (Figure 2A). The expression of HIV-1 PR led to over 60% cleavage of PABP, a result similar to that found after expression of poliovirus 2A^{pro}. A polypeptide of around 53 kDa, which could be a proteolytic product of PABP, was detected in cells electroporated with SV-PR RNA. This band has only been detected sporadically, and in low

abundance, in over-exposed immunoblots, but it is identical in size to those observed in HIV-1-infected cells. Moreover, cleavage products were not detected in cells expressing the poliovirus 2A^{pro}, most probably due to a small amount of proteolytic products that are not well detected by the monoclonal antibody against PABP. In this regard, in a previous report the authors could not detect PABP cleavage products using this antibody both in poliovirus-infected cells and in cell free systems[22]. The decrease of PABP induced by expression of HIV-1 PR was abolished when 12 μ M saquinavir, a specific inhibitor of the HIV PR, was present (Figure 2A). Furthermore, the 53kDa cleavage product is not detectable in SV-PR-expressing cell when SQ is present (Figure 2A). Cell extracts were further analyzed to determine the cleavage of eIF4GI and eIF4GII (Figures 2B and 2C). Previous findings revealed the presence of two forms of eIF4G of 220 and 150 kDa in BHK cells [27, 28]. These different eIF4G forms may be the result of post-translational modifications. The 150 kDa product may be a breakdown product of eIF4GI. Both polypeptides of 220 and 150 kDa are absent from cells expressing 2A^{pro} and HIV-1 PR. In agreement with our previously reported data, the expression of HIV-1 PR induces an effective cleavage of eIF4GI, while eIF4GII remained intact (Figures 2B and 2C) [19, 20]. The kinetics of the hydrolysis of PABP, eIF4GI and eIF4GII by HIV-1 PR were analyzed after electroporation of BHK cells (Figure 2D). The cleavage of PABP by HIV-1 PR was similar to that observed with eIF4GI proteolysis. In addition, cleavage of eIF4GII by HIV-1 PR was very inefficient in this assay.

Other retroviral PRs cleave PABP in Transfected Cells - The susceptibility of PABP to proteolysis mediated by different retroviral PRs encoded by MoMLV, MMTV, HTLV-I, SIV, HIV-1 and HIV-2 was tested in transfected cells. For this purpose, the pTM1-derived plasmids containing the corresponding retroviral PR-coding sequences were transfected in COS-7 cells with the vaccinia virus vvT7 system. Poliovirus 2A^{pro} was expressed in parallel to serve as a control of PABP cleavage. The integrity of PABP was assessed by Western blot at 16 h post-transfection (hpt) (Figure 3A). Expression of HIV-1, HIV-2 and MMTV PR induced a substantial decrease in intact PABP comparable to that obtained after transfection of poliovirus 2A^{pro}. Cleavage products of PABP were not detected by Western blot in these cells, in a similar fashion to that observed in cells electroporated with SV-2A mRNA. The addition of 2 μ M saquinavir prevented a loss of PABP caused by HIV-1 and HIV-2 PRs, suggesting that the lower level of intact PABP in HIV PR-transfected cells was due to their proteolytic activity. On the other hand, a decrease in PABP was not detectable in cells transfected with plasmids containing the retroviral PR-coding regions of MoMLV, HTLV-I and SIV. The integrity of eIF4GI and eIF4GII was also analyzed in PR-transfected cells (Figures 3B and 3C). In agreement with previous data, all retroviral PRs were able to cleave eIF4GI (Figure 3B), while the PRs of MMTV, MoMLV, HTLV-I and SIV cleaved eIF4GII in transfected cells (Figure 3C). The efficiency of transfection in these experiments was around 70%, as determined by immunofluorescence of cells transfected with the pTM1-Luc plasmid, using an anti-luciferase antiserum (data not shown).

In vitro cleavage of PABP by HIV-1 and HIV-2 PRs - The proteolysis of PABP was further studied in cell-free systems using purified recombinant PRs from HIV-1, HIV-2 and MoMLV. The recombinant MBP-2A^{pro} was included as a control. HeLa cell extracts were incubated for 90 min at 30°C with 3 ng/μl of each purified PR. Under the experimental conditions used, complete proteolytic processing of intact PABP by HIV-1 and HIV-2 PRs occurs, while some intact PABP still remains upon incubation with MBP-2A^{pro} (Figure 4A). This result indicates that the purified HIV-1 and HIV-2 PRs hydrolyze PABP more efficiently than MBP-2A^{pro}. On the other hand, the MoMLV PR was unable to cleave PABP in this assay. The specificity of these cleavages was evidenced by using an HIV-1 PR inhibitor such as saquinavir, which blocked cleavage of PABP1 by both HIV-1 and HIV-2 PRs (Figure 4A). Consistent with the above results, the purified HIV-1 and HIV-2 PRs proteolyzed eIF4GI *in vitro*, while eIF4GII was very inefficiently degraded by both PRs (data not shown). In addition, the integrity of other initiation factors such as eIF4E, eIF4A and the 100 kDa subunit of eIF3 was analyzed (Figure 4B) upper, middle and lower panels, respectively). The amount of these translation factors remained constant after incubation with recombinant HIV-1 and HIV-2 PRs.

To analyze the kinetics of proteolysis of PABP, eIF4GI and eIF4GII, HeLa cell extracts were incubated with recombinant HIV-1 PR and HIV-2 PR, and at the indicated times the reactions were stopped (Figures 5A and 5B). As observed with SV-PR in culture cells, the kinetics of cleavage of PABP by HIV-1 and HIV-2 PRs were very similar to those observed for eIF4GI (Figures 5C and 5D). Consistent with previous observations using HIV-1 and HIV-2 PRs [19, 20], hydrolysis of eIF4GII does not occur with the retroviral proteases throughout the assay.

Identification of the cleavage sites of HIV-1 and HIV-2 PRs on PABP1 - To determine if the cleavage of HIV-1 and HIV-2 PRs on PABP was the result of a direct or indirect activation of cellular proteases, the fusion protein GST-PABP1 (9-636) was incubated with the recombinant HIV-1, HIV-2 and MoMLV PRs. In some assays, 2 μM saquinavir (in the case of HIV-1 PR or HIV-2 PR) and 20 μM ritonavir (in the case of MoMLV PR) were added. The staining of SDS-polyacrylamide gels with Coomassie brilliant blue revealed the generation of several cleavage products (Figure 6A). Incubation of GST-PABP1 (9-636) with both HIV-1 PR and HIV-2 PR revealed a similar degradation pattern but with some slight differences (Figure, 6A). Cleavage of GST-PABP1 by HIV-1 and HIV-2 PRs was inhibited by addition of 2 μM saquinavir. In addition, the purified MoMLV PR was unable to cleave the recombinant GST-PABP1 under the conditions used in this assay (Figure 6A).

In order to precisely identify the PR proteolysis sites, the cleavage products were sequenced by the Edman degradation method. N-terminal sequencing of the polypeptides of ~27 and 20 kDa rendered FERHED, which corresponds to a cleavage site located between positions 237 and 238 of PABP1 (Figure 6B). Sequencing analysis of the 18 kDa polypeptide rendered a S, T, Q, R, V and A in sequencing cycles 1 to 6, which correspond to the cleavage site located at positions 477/478 of PABP1. In addition, AAIPQT was identified as the N-terminal sequence of

the ~8 kDa fragment detected after incubation of GST-PABP1 with HIV-2 PR, which reveals a processing site located between positions 410 and 411 of PABP1 (Figure 6B). Edman degradation of polypeptides of ~72 and 52 kDa rendered a sequence that correspond with the six first amino acids of GST (Figure 6A, denoted by asterisks). These findings reflect that both HIV-1 and HIV-2 PRs cleave PABP1 around the same sites (Figure 6C). The presence of additional cleavage sites on PABP1, such as the cleavage site located at position 410/411, could also explain in part the variability observed among the degradation products rendered by HIV-1 and HIV-2 PRs. The relative conservation of the PABP1 cleavage sites of viral PRs supports the view that this protein is a genuine substrate for the HIV-1 and HIV-2 PRs.

DISCUSSION

A variety of viral products are responsible for cell damage that occurs during virus infection. Animal viruses encode PRs that cleave polyprotein precursors to render the mature viral proteins. Apart from those substrates, a number of host proteins are also cleaved by viral PRs. The hydrolysis of the host proteins alters different cellular functions. Thus, PRs of several viruses cleave protein factors of the cellular translational machinery, causing a down-regulation of cellular mRNA translation, while increasing the synthesis of viral proteins. Thus, cleavage of the initiation factor eIF4G by proteases of several picornaviruses leads to the inhibition of translation directed by cellular capped mRNAs [16]. Moreover, bisection of eIF4G by picornavirus PRs enhances the translation of the virus mRNA genome that contains an IRES motif at its 5' end. Recent reports have shown that eIF4G is also a target of retroviral PRs [19, 20]. In some retroviruses including HIV-1, viral PR-mediated cleavage of eIF4G leads to the inhibition of cap-dependent translation, without affecting the IRES-driven protein synthesis directed by retroviral mRNAs [19, 20, 34].

Previous reports have shown that PABP is also cleaved by the PRs of several picornaviruses and caliciviruses during infection [22-24, 26]. Its degradation, together with the cleavage of eIF4G, contribute to the observed inhibition of cellular translation that occurs during viral infection. The cleavage of PABP by the poliovirus 3C^{pr^o} occurs at the C-terminal domain of the protein. As a result, the C-terminal portion, which is implicated in interaction with other proteins, is separated from the RNA-interacting N-terminal domain [24]. This cleavage may selectively inhibit poly(A)-dependent translation [24, 25]. The C-terminal domain of PABP interacts with several proteins that are implicated in mRNA translation (i.e. eIF4B and eRF3) [5, 9-13]. The cleavage of PABP by 3C^{pr^o} disrupts its interaction with eIF4B and eRF3, probably affecting late events in translation, such as ribosome recycling. Our results show that HIV PRs also cleaved PABP within its C-terminal domain. The identified cleavage sites (at positions 410-411 and 477-478) were relatively close to those recognized by poliovirus 3C^{pr^o}. In addition, HIV PRs also hydrolyze PABP between residues 237 and 238. This cleavage leads to the removal of the first two RNA recognition motifs (RRM1 and RRM2) of PABP, which may retain some of the functions of the protein.

All of the RRM motifs of PABP possess RNA-binding ability [35], although the first two domains together (RRM1-RRM2) are the ones with the highest affinity for the poly(A) tail [36]. The RRM2 motif is involved in direct interaction with eIF4G [9, 37]. It has been shown that translation can be rescued in PABP-depleted Krebs-2 cell extracts by the addition of purified recombinant PABP fragments such as RRM1-RRM2 or RRM1-RRM4, although less efficiently than with intact PABP [38]. These observations suggested that the interaction of PABP and eIF4G is essential for efficient translation, although additional interactions of the C-terminal domain of PABP with other factors would be necessary for a complete stimulation of translation [38]. In agreement with that proposal, cleavage of PABP within the RRM3 motif by HIV PR may abolish in part, but not completely, the function of its N-terminal domain, since the RRM1-RRM2 region still retains the eIF4G binding site and the ability to bind RNA.

Infection of C8166 cells by HIV-1 led to a drastic decline in host translation coincident with the decrease of intact eIF4GI [20]. However, total proteolysis of eIF4GI is not sufficient to block translation of cellular mRNAs engaged in the translational machinery [39, 40]. The shut-off of host protein synthesis is coincident with the hydrolysis of eIF4GII in poliovirus- and rhinovirus-infected cells, as well as in apoptotic cells [41, 42]. PABP hydrolysis by poliovirus 3C^{pro} inhibits the translation of endogenous mRNAs in HeLa cell extracts completely [25]. Our present findings indicate that cleavage of both eIF4GI and PABP occur with similar kinetics in cell culture and in a cell-free system, suggesting that PABP proteolysis may also contribute to host cell translation inhibition and could be responsible for the shut-off of host protein synthesis, observed in some cell lines upon HIV-1 infection [20]. In HIV-1-infected cells, the correlation observed between eIF4GI hydrolysis and the inhibition of cellular protein synthesis could be attributed to the viral PR-cleavage of both eIF4GI and PABP.

Interestingly, retroviral PRs such as those from MoMLV, HTLV-I or SIV-1 are able to hydrolyze eIF4GI and eIF4GII but not PABP, while inhibiting protein synthesis in transfected COS-7 cells [19]. Although both HIV-1 and HIV-2 PRs are able to proteolyze eIF4GI and PABP very efficiently, eIF4GII remains as a poor substrate of both enzymes [19, 20]. Taken together, available data suggest that, at least two of those factors must be inactivated to efficiently inhibit cap- and poly(A)-dependent translation of cellular mRNAs.

Apart from factors implicated in translation, other host proteins have been identified as substrates of HIV PRs and the potential role of cleavage of cellular proteins in the cytotoxic effect inflicted by HIV has been pointed out by several laboratories [43, 44]. Examples of cell proteins cleaved by HIV PRs are microtubule-associated proteins, cytoskeletal proteins and nuclear factor- κ B, among others [45, 46]. Nevertheless, further investigations are required to define the precise role of each of those proteolytic cleavages in the cytopathogenicity provoked by HIV infection.

It is widely accepted that most retroviruses do not affect cellular protein synthesis after infection. This is not the case for HIV, which blocks cellular protein synthesis more or less efficiently depending on the viral strain and cell line analyzed [47, 48]. In HIV-1, the Vpr

protein blocks proliferation of CD4⁺ T cells at the G₂ cell cycle checkpoint [18, 49]. At this stage, the initiation of cap-dependent translation is suppressed, probably due to the cleavage of both eIF4GI and PABP, while the initiation of IRES-containing mRNAs ensures Gag and Gag-Pol synthesis. In this scenario, cleavage of both PABP and eIF4GI could facilitate viral gene expression, while contributing to the inhibition of host protein synthesis. The finding that many retroviral and picornaviral PRs target eIF4G, and in some cases PABP, indicates that those viruses may share a common mechanism of translational control.

ABBREVIATIONS FOOTNOTE

The abbreviations used are: PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's Medium; BHK-21, baby hamster kidney; PBS, phosphate buffered saline; PR, protease; MBP, maltose binding protein; GST, glutathione S-transferase; PABP, poly(A) binding protein; Paip, PABP-interacting protein; eIF, eukaryotic initiation factor; RRM, RNA recognition motif; MoMLV, Moloney murine leukemia virus; MMTV, mouse mammary tumor virus; HTLV-I, human T-cell leukemia virus type I; SIV, simian immunodeficiency virus; HIV, human immunodeficiency virus type 1; SQ, saquinavir; RTV, ritonavir; IRES, internal ribosome entry site; UTR, untranslated region; NSP3, nonstructural protein 3; dpi, days post-infection; hpe, hours post-electroporation; hpt, hours post-transfection.

ACKNOWLEDGEMENTS

Financial support from Grant BMC2003-00494 from the DGICYT and an Institutional Grant to the Centro de Biología Molecular "Severo Ochoa" from the Fundación Ramón Areces, are acknowledged. E.A. and A.C. held fellowships from the Ministerio de Educación y Ciencia.

REFERENCES

- 1 Hentze, M. W. (1997) eIF4G: a multipurpose ribosome adapter? *Science* **275**, 500-501
- 2 Jacobson, A. and Favreau, M. (1983) Possible involvement of poly(A) in protein synthesis. *Nucleic Acids Res.* **11**, 6353-6368
- 3 Jacobson, A. (1996) in *Translational control* (Hershey, J. W., Mathews, M. B. and Sonenberg, N., eds.), pp. 451-480, Cold Spring Harbor Press
- 4 Borman, A. M., Michel, Y. M. and Kean, K. M. (2000) Biochemical characterisation of cap-poly(A) synergy in rabbit reticulocyte lysates: the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5'-end. *Nucleic Acids Res.* **28**, 4068-4075

- 5 Tarun, S. Z., Jr., Wells, S. E., Deardorff, J. A. and Sachs, A. B. (1997) Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation. *Proc. Natl. Acad. Sci. USA* **94**, 9046-9051
- 6 Gallie, D. R. (1991) The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev.* **5**, 2108-2116
- 7 Adam, S. A., Nakagawa, T., Swanson, M. S., Woodruff, T. K. and Dreyfuss, G. (1986) mRNA polyadenylate-binding protein: gene isolation and sequencing and identification of a ribonucleoprotein consensus sequence. *Mol. Cell. Biol.* **6**, 2932-2943
- 8 Sachs, A. B., Bond, M. W. and Kornberg, R. D. (1986) A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression. *Cell* **45**, 827-835
- 9 Imataka, H., Gradi, A. and Sonenberg, N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.* **17**, 7480-7489
- 10 Le, H., Tanguay, R. L., Balasta, M. L., Wei, C. C., Browning, K. S., Metz, A. M., Goss, D. J. and Gallie, D. R. (1997) Translation initiation factors eIF-iso4G and eIF-4B interact with the poly(A)-binding protein and increase its RNA binding activity. *J. Biol. Chem.* **272**, 16247-16255
- 11 Hoshino, S., Imai, M., Kobayashi, T., Uchida, N. and Katada, T. (1999) The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) carrying the translation termination signal to the 3'-Poly(A) tail of mRNA. Direct association of erf3/GSPT with polyadenylate-binding protein. *J. Biol. Chem.* **274**, 16677-16680
- 12 Kozlov, G., Trempe, J. F., Khaleghpour, K., Kahvejian, A., Ekiel, I. and Gehring, K. (2001) Structure and function of the C-terminal PABC domain of human poly(A)-binding protein. *Proc. Natl. Acad. Sci. USA* **98**, 4409-4413
- 13 Cosson, B., Couturier, A., Chabelskaya, S., Kiktev, D., Inge-Vechtomov, S., Philippe, M. and Zhouravleva, G. (2002) Poly(A)-binding protein acts in translation termination via eukaryotic release factor 3 interaction and does not influence [PSI(+)] propagation. *Mol. Cell. Biol.* **22**, 3301-3315
- 14 Craig, A. W., Haghghat, A., Yu, A. T. and Sonenberg, N. (1998) Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. *Nature* **392**, 520-523
- 15 Khaleghpour, K., Svitkin, Y. V., Craig, A. W., DeMaria, C. T., Deo, R. C., Burley, S. K. and Sonenberg, N. (2001) Translational repression by a novel partner of human poly(A) binding protein, Paip2. *Mol. Cell* **7**, 205-216
- 16 Prevot, D., Darlix, J. L. and Ohlmann, T. (2003) Conducting the initiation of protein synthesis: the role of eIF4G. *Biol. Cell* **95**, 141-156

- 17 Baudin, F., Marquet, R., Isel, C., Darlix, J. L., Ehresmann, B. and Ehresmann, C. (1993) Functional sites in the 5' region of human immunodeficiency virus type 1 RNA form defined structural domains. *J. Mol. Biol.* **229**, 382-397
- 18 Brasey, A., Lopez-Lastra, M., Ohlmann, T., Beerens, N., Berkhout, B., Darlix, J. L. and Sonenberg, N. (2003) The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle. *J. Virol.* **77**, 3939-3949
- 19 Alvarez, E., Menendez-Arias, L. and Carrasco, L. (2003) The eukaryotic translation initiation factor 4GI is cleaved by different retroviral proteases. *J. Virol.* **77**, 12392-12400
- 20 Ventoso, I., Blanco, R., Perales, C. and Carrasco, L. (2001) HIV-1 protease cleaves eukaryotic initiation factor 4G and inhibits cap-dependent translation. *Proc. Natl. Acad. Sci. USA* **98**, 12966-12971
- 21 Piron, M., Vende, P., Cohen, J. and Poncet, D. (1998) Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. *Embo J* **17**, 5811-5821
- 22 Joachims, M., Van Breugel, P. C. and Lloyd, R. E. (1999) Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. *J. Virol.* **73**, 718-727
- 23 Kerekatte, V., Keiper, B. D., Badorff, C., Cai, A., Knowlton, K. U. and Rhoads, R. E. (1999) Cleavage of Poly(A)-binding protein by coxsackievirus 2A protease in vitro and in vivo: another mechanism for host protein synthesis shutoff? *J. Virol.* **73**, 709-717
- 24 Kuyumcu-Martinez, N. M., Joachims, M. and Lloyd, R. E. (2002) Efficient cleavage of ribosome-associated poly(A)-binding protein by enterovirus 3C protease. *J. Virol.* **76**, 2062-2074
- 25 Kuyumcu-Martinez, N. M., Van Eden, M. E., Younan, P. and Lloyd, R. E. (2004) Cleavage of poly(A)-binding protein by poliovirus 3C protease inhibits host cell translation: a novel mechanism for host translation shutoff. *Mol. Cell. Biol.* **24**, 1779-1790
- 26 Kuyumcu-Martinez, M., Belliot, G., Sosnovtsev, S. V., Chang, K. O., Green, K. Y. and Lloyd, R. E. (2004) Calicivirus 3C-like proteinase inhibits cellular translation by cleavage of poly(A)-binding protein. *J. Virol.* **78**, 8172-8182
- 27 Castelló, A., Sanz, M. A., Molina, S. and Carrasco, L. (2006) Translation of Sindbis Virus 26S mRNA does not require intact eukaryotic initiation factor 4G. *J. Mol. Biol.* **355**, 942-956
- 28 Aldabe, R., Feduchi, E., Novoa, I. and Carrasco, L. (1995) Efficient cleavage of p220 by poliovirus 2A^{Pro} expression in mammalian cells: effects on vaccinia virus. *Biochem. Biophys. Res. Commun.* **215**, 928-936

- 29 Ventoso, I., Barco, A. and Carrasco, L. (1998) Mutational analysis of poliovirus 2A^{pro}. Distinct inhibitory functions of 2A^{pro} on translation and transcription. *J. Biol. Chem.* **273**, 27960-27967
- 30 Burgui, I., Aragon, T., Ortin, J. and Nieto, A. (2003) PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. *J. Gen. Virol.* **84**, 3263-3274
- 31 Rittenhouse, J., Turon, M. C., Helfrich, R. J., Albrecht, K. S., Weigl, D., Simmer, R. L., Mordini, F., Erickson, J. and Kohlbrenner, W. E. (1990) Affinity purification of HIV-1 and HIV-2 proteases from recombinant *E. coli* strains using pepstatin-agarose. *Biochem. Biophys. Res. Commun.* **171**, 60-66
- 32 Menendez-Arias, L., Gotte, D. and Oroszlan, S. (1993) Moloney murine leukemia virus protease: bacterial expression and characterization of the purified enzyme. *Virology* **196**, 557-563
- 33 Ventoso, I., Barco, A. and Carrasco, L. (1999) Genetic selection of poliovirus 2A^{pro}-binding peptides. *J. Virol.* **73**, 814-818
- 34 Perales, C., Carrasco, L. and Ventoso, I. (2003) Cleavage of eIF4G by HIV-1 protease: effects on translation. *FEBS Lett.* **533**, 89-94
- 35 Deo, R. C., Bonanno, J. B., Sonenberg, N. and Burley, S. K. (1999) Recognition of polyadenylate RNA by the poly(A)-binding protein. *Cell* **98**, 835-845
- 36 Burd, C. G., Matunis, E. L. and Dreyfuss, G. (1991) The multiple RNA-binding domains of the mRNA poly(A)-binding protein have different RNA-binding activities. *Mol. Cell. Biol.* **11**, 3419-3424
- 37 Kessler, S. H. and Sachs, A. B. (1998) RNA recognition motif 2 of yeast Pab1p is required for its functional interaction with eukaryotic translation initiation factor 4G. *Mol. Cell. Biol.* **18**, 51-57
- 38 Kahvejian, A., Svitkin, Y. V., Sukarieh, R., M'Boutchou, M. N. and Sonenberg, N. (2005) Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes Dev.* **19**, 104-113
- 39 Perez, L. and Carrasco, L. (1992) Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection. *Virology* **189**, 178-186
- 40 Irurzun, A., Sanchez-Palomino, S., Novoa, I. and Carrasco, L. (1995) Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage. *J. Virol.* **69**, 7453-7460
- 41 Gradi, A., Svitkin, Y. V., Imataka, H. and Sonenberg, N. (1998) Proteolysis of human eukaryotic translation initiation factor eIF4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. *Proc. Natl. Acad. Sci. USA* **95**, 11089-11094

- 42 Svitkin, Y. V., Gradi, A., Imataka, H., Morino, S. and Sonenberg, N. (1999) Eukaryotic initiation factor 4GII (eIF4GII), but not eIF4GI, cleavage correlates with inhibition of host cell protein synthesis after human rhinovirus infection. *J. Virol.* **73**, 3467-3472
- 43 Shoeman, R. L., Honer, B., Stoller, T. J., Kesselmeier, C., Miedel, M. C., Traub, P. and Graves, M. C. (1990) Human immunodeficiency virus type 1 protease cleaves the intermediate filament proteins vimentin, desmin, and glial fibrillary acidic protein. *Proc. Natl. Acad. Sci. USA* **87**, 6336-6340
- 44 Tomasselli, A. G., Hui, J. O., Adams, L., Chosay, J., Lowery, D., Greenberg, B., Yem, A., Deibel, M. R., Zurcher-Neely, H. and Heinrikson, R. L. (1991) Actin, troponin C, Alzheimer amyloid precursor protein and pro-interleukin 1 beta as substrates of the protease from human immunodeficiency virus. *J. Biol. Chem.* **266**, 14548-14553
- 45 Dunn, B. M. (1998) in *Handbook of proteolytic enzymes*. (Barret, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 919-928, Academic Press, London, United Kingdom
- 46 Wlodawer, A. and Gustchina, A. (2000) Structural and biochemical studies of retroviral proteases. *Biochim. Biophys. Acta* **1477**, 16-34
- 47 Agy, M. B., Wambach, M., Foy, K. and Katze, M. G. (1990) Expression of cellular genes in CD4 positive lymphoid cells infected by the human immunodeficiency virus, HIV-1: evidence for a host protein synthesis shut-off induced by cellular mRNA degradation. *Virology* **177**, 251-258
- 48 Somasundaran, M. and Robinson, H. L. (1988) Unexpectedly high levels of HIV-1 RNA and protein synthesis in a cytotoxic infection. *Science* **242**, 1554-1557
- 49 Goh, W. C., Rogel, M. E., Kinsey, C. M., Michael, S. F., Fultz, P. N., Nowak, M. A., Hahn, B. H. and Emerman, M. (1998) HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat. Med.* **4**, 65-71

FIGURE LEGENDS

Figure 1. PABP cleavage in HIV-1-infected cells. (A) MT-2 cells were infected with HIV-1 (multiplicity of infection 1). At 3 dpi the integrity of PABP was determined by Western blot (upper panel). The corresponding Western blot against eIF4A (lower panel) is shown. Mock, mock-infected cells; HIV-1, cells infected with HIV-1. Cleavage product derived from PABP in infected cells is indicated as c.p. An unknown anti-PABP-reactive band is also denoted by an asterisk. Mr, molecular mass. (B) Western blots against eIF4GI (upper panel) and eIF4GII (lower panel) at 3 dpi. (C) Western blot against the HIV-1 capsid protein (CA), p24 at 3 dpi. The position of the intact initiation factors is also indicated in the respective panel.

Figure 2. Cleavage of PABP in cells electroporated with recombinant SVs that express heterologous proteases. (A) Cells were electroporated with transcription buffer (BHK), wt SV,

SV-PR or SV-2A RNAs and incubated in the presence or absence of 12 μ M saquinavir. At 16 hpe, cell extracts were analyzed by Western blotting using specific antibodies against human PABP1. Cleavage product derived from PABP in SV-PR-infected cells is indicated as c.p.; SQ, saquinavir; Mr, molecular mass. The relative amount of intact PABP for each transfection experiment is indicated below each corresponding lane. **(B)** Detection of eIF4GI cleavage products by Western blotting using a mixture of antisera against its N- and C-terminal regions. c.p., cleavage fragments. The amount of intact eIF4GI for each transfection experiment is indicated below each corresponding lane. **(C)** Detection of eIF4GII cleavage products by Western blotting using an antisera against the C-terminal region. Ct, C-terminal fragments of eIF4GII. The amount of intact eIF4GII for each transfection experiment is indicated below each lane. **(D)** Cleavage kinetics of PABP, eIF4GI and eIF4GII by HIV-1 PR. BHK cells were electroporated as described in materials and methods and cell lysates were obtained at the indicated times. The values were obtained by densitometric scanning of the corresponding intact protein band at each time indicated.

Figure 3. Cleavage of PABP, eIF4GI and eIF4GII in transfected cells. **(A)** COS-7 cells were transfected with the empty pTM1 vector or with pTM1 carrying inserts containing the PR gene of several retroviruses or poliovirus 2A^{pro}. At 16 hpt, equal amounts of protein extract were loaded in a polyacrylamide gel and analyzed by Western blotting using a monoclonal antibody against PABP. Data are referred to control experiments carried out with the empty pTM1 vector, and the values were obtained by densitometric scanning of the protein band of \approx 70 kDa corresponding to PABP. **(B)** Western blot against eIF4GI. **(C)** Western blot against eIF4GII. The estimated amount of intact protein in cell extracts obtained from transfections with different constructs is indicated below each panel.

Figure 4. Cleavage of PABP *in vitro* by purified recombinant HIV-1, HIV-2 and MoMLV PRs. 50 μ g of crude HeLa S10 extracts were incubated with 3 ng/ μ l of each recombinant PR in a total volume of 20 μ l for 90 min and subjected to Western blotting analysis by using: **(A)** a monoclonal antibody against PABP; **(B)** a polyclonal antisera against 100-kDa subunit of eIF3 factor (upper panel), an antibody against PABP, an antibody against eIF4A (middle panel) or a monoclonal antibody raised against eIF4E factor (bottom panel).

Figure 5. Cleavage kinetics of PABP, eIF4GI and eIF4GII *in vitro* by purified recombinant HIV-1 and HIV-2 PRs. 50 μ g of crude HeLa S10 extracts were incubated with 3 ng/ μ l of HIV-1 PR **(A)** or HIV-2 PR **(B)** in a total volume of 20 μ l during the indicated times and subjected to Western blotting analysis by using a monoclonal antibody against PABP (upper panel), a mixture of antisera against N-terminal and C-terminal regions of eIF4GI (middle panel) or an antisera against the C-terminal region of eIF4GII (lower panel). Degradation kinetics of PABP, eIF4GI and eIF4GII in HeLa cell extracts incubated with HIV-1 PR **(C)** and HIV-2 PR

(D). Determinations were obtained by densitometric scanning of the corresponding intact protein band at the indicated times.

Figure 6. Identification of HIV-1 PR and HIV-2 PR cleavage sites on PABP1. (A) 5 μ g of recombinant GST-PABP1 protein were incubated with 100 ng of recombinant HIV-1 PR, HIV-2 PR or MoMLV PR in a total volume of 20 μ l as described in materials and methods. Cleavage products were separated by SDS-polyacrylamide gel electrophoresis and the hydrolysis fragments were stained by Coomassie blue. The sequenced polypeptides are indicated with a head arrow. Bands corresponding to GST N-terminal polypeptides are also denoted by asterisks (B) Aminoacid sequence of the PABP1 cleavage sites identified using HIV-1 and HIV-2 PRs. (C) Diagram showing the functional domains found in PABP1 based on the available data, including the position of mapped cleavage sites for HIV-1 and HIV-2 PRs. The RNA binding domains (RRM) and the regions involved in the binding to other translation factors are shown. PABP, poly(A) binding protein. Sequence numbering refers to the human PABP1 isoform.

FIGURE 1

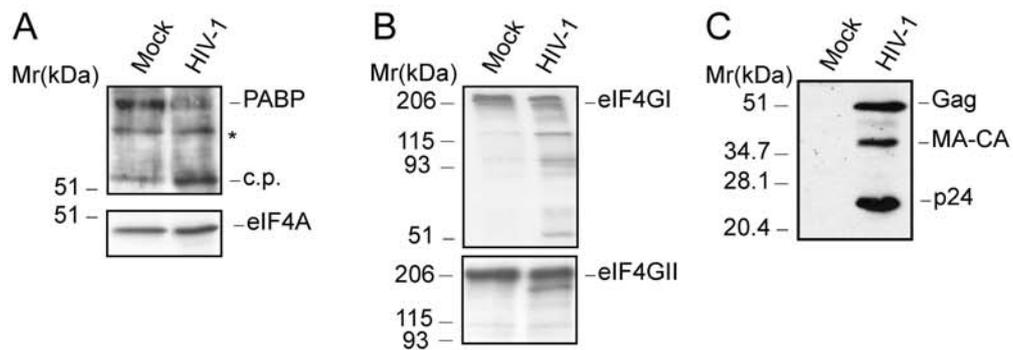


FIGURE 2

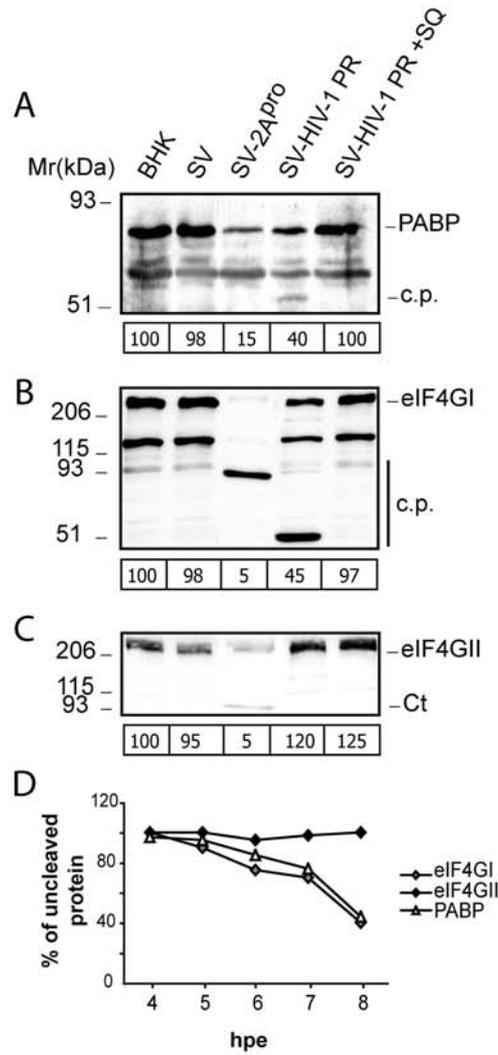


FIGURE 3

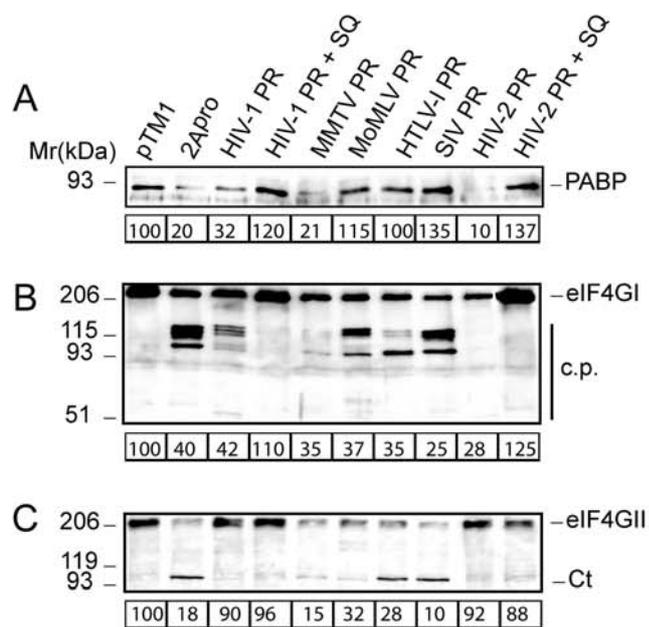


FIGURE 4

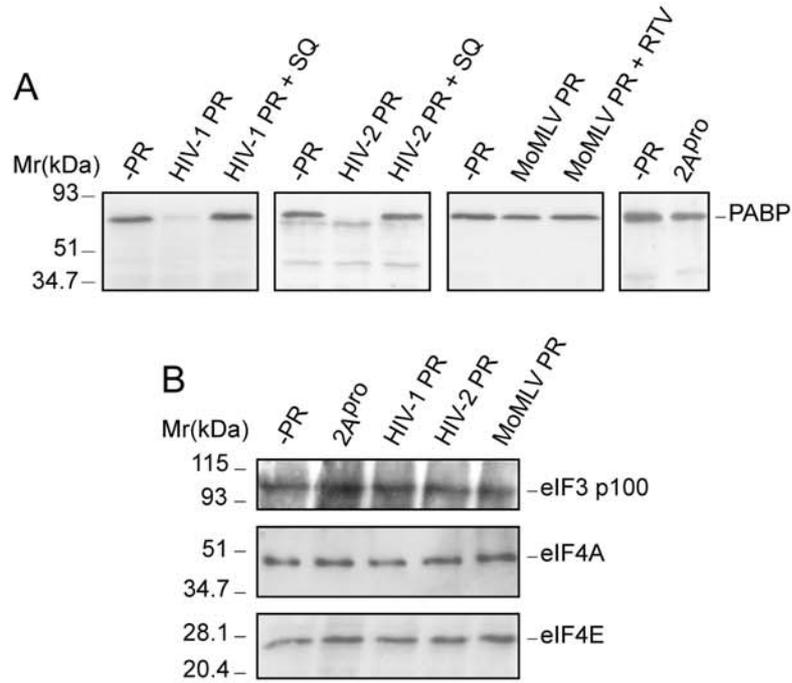


FIGURE 5

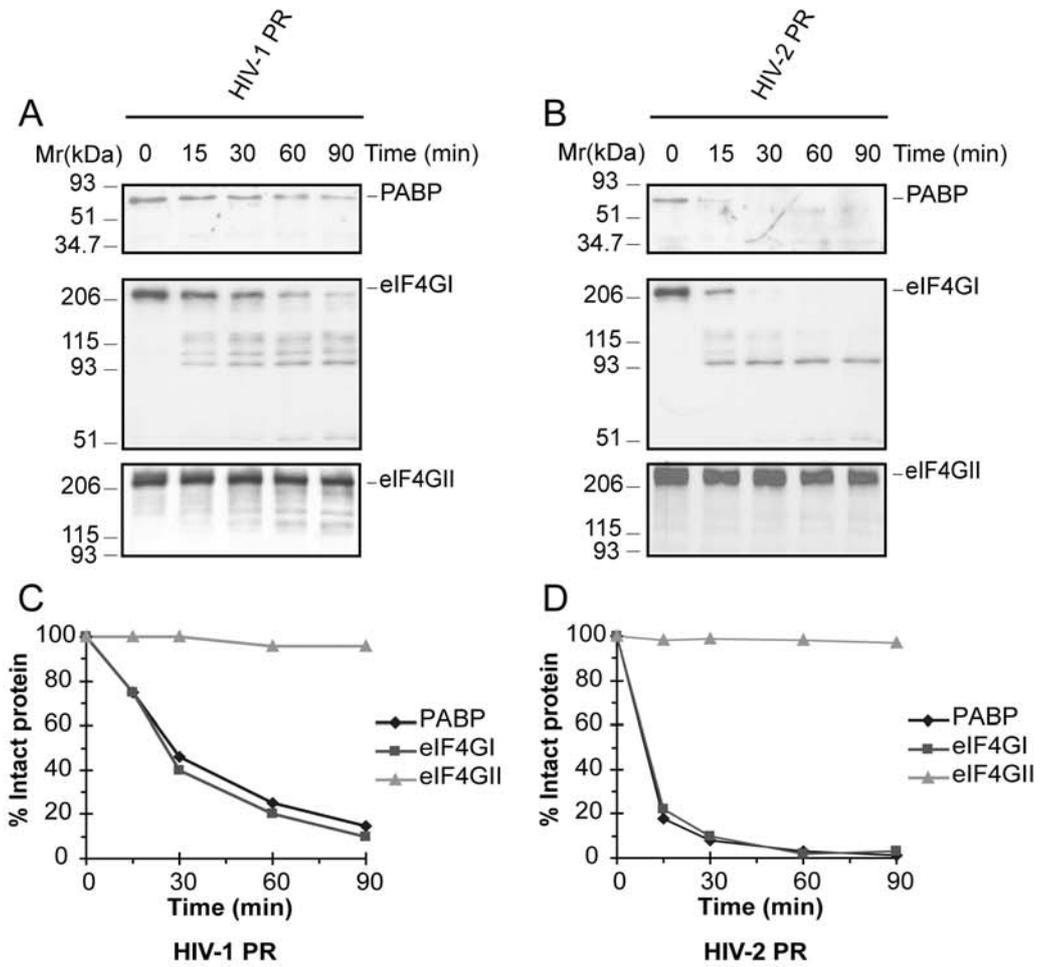


FIGURE 6

