Purification of Two Picornaviral 2A Proteinases: Interaction with eIF-4γ and Influence on *in Vitro* Translation†

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ABSTRACT: A mammalian cell infected with a human rhinovirus or enterovirus has a much reduced capability to translate capped mRNAs (the host cell shutoff), while still allowing translation of uncapped viral RNA. Biochemical and genetic evidence suggests that the viral proteinase 2A induces cleavage of the eukaryotic initiation factor (eIF) 4γ (also known as p220) component of eIF-4 (formerly called eIF-4F). However, neither the mechanism underlying the specific proteolysis of eIF-4γ nor the influence of this cleavage on the translation of capped mRNAs has been clarified. Such studies have been hampered by a lack of large quantities of a purified 2A proteinase. Therefore, the mature proteinases 2A of human rhinovirus 2 and coxsackievirus B4 were expressed in soluble form in *Escherichia coli*. A four-step purification protocol was developed; 1 mg of highly purified 2A proteinase per gram wet weight of *E. coli* was obtained. Both enzymes cleaved directly eIF-4γ as part of the purified eIF-4 complex. Addition of HRV2 2A proteinase to HeLa cell cytoplasmic translation extracts resulted in eIF-4γ cleavage and drastically reduced the translation of capped mRNA; addition of purified eIF-4 restored translation to the initial level. However, translation of a reporter gene driven by the 5′-untranslated region of human rhinovirus 2 was translated 2–3-fold more efficiently in the presence of HRV2 2A proteinase.

Human rhinoviruses (HRVs)[1] and coxsackieviruses, members of the picornavirus family, are both capable of causing the common cold (Stott & Killington, 1972; Grist & Ried, 1988). The existence of more than 100 HRV and 29 CV serotypes precludes successful vaccine development, as has been the case for other picornaviruses, such as poliovirus or foot and mouth disease virus (Rueckert, 1990). Antiviral strategies have concentrated on virally encoded proteinases as they are essential for viral replication (Kraeusslich & Wimmer, 1988; Hellen *et al.*, 1989) and are particular in utilizing cysteine as the active-site nucleophile surrounded by residues typically found in serine proteinases. Such proteinases have not as yet been observed in the host cell (Argos *et al.*, 1984; Gorbalenya *et al.*, 1986; Bazan & Fletterick, 1988).

Picornaviral genomes possess at least two such proteinases which carry out the processing of the viral polyprotein into the mature viral proteins (Hanecak *et al.*, 1982; Toyoda *et al.*, 1986; Kraeusslich & Wimmer, 1988). The polyprotein is contained in a single open reading frame on the RNA genome; this single-stranded RNA molecule of positive polarity is around 7500 nt in length [for a review, see Stanway (1990)]. The derived polyprotein of about 2000 amino acids is normally not observed; in rhinoviruses and enteroviruses (i.e., polioviruses and CVs), the 2A proteinase cleaves during translation between the C-terminus of VP1 and its own N-terminus (Butterworth & Korant, 1974; Korant *et al.*, 1980). The 2A proteinases of rhinoviruses and enteroviruses also modify the host cell, leading to loss of the ability to translate capped RNAs; this phenomenon is known as the host cell shutoff. Picornaviral RNAs are not affected as they initiate internally (Sonenberg, 1990). The primary target of 2A proteinases has remained obscure; its action leads, however, to cleavage of the polypeptide eIF-4γ,[2] part of the cap-binding complex eIF-4 (Kraeusslich *et al.*, 1987; Wyckoff *et al.*, 1990a, 1992). However, the relevance of eIF-4γ cleavage to host cell shutoff and the involvement of 2A proteinases is still a subject of much dispute (Bonneau & Sonenberg, 1987; Perez & Carrasco, 1992).

Recently, a scheme to enrich HRV2 2A proteinase with an additional N-terminal methionine residue from bacterial extracts was presented (Sommergruber *et al.*, 1992). We report here the purification to homogeneity of recombinant HRV2 and CVB4 2A proteinases possessing natural N-termini. Experiments with purified material provided evidence for the direct *in vitro* cleavage of eIF-4γ in purified preparations of rabbit eIF-4 and in HeLa cell extracts. Furthermore, incubation of purified HRV2 2A with a HeLa cell cytoplasmic translation extract reduced translation of a capped mRNA and increased the translation efficiency of an

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[1] Abbreviations: HRV, human rhinovirus; CV, coxsackievirus; E64, 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane; nt, nucleotide or nucleotides; IPTG, isopropyl thiogalactoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; 5′UTR, 5′ untranslated region.

[2] The cap-binding complex containing eIF-4E, eIF-4A, and eIF-4γ was originally called eIF-4F (Grifo *et al.*, 1983), but more recently the name eIF-4 has been proposed (Safer, 1989). The latter is used here, as is the designation eIF-4γ for the polypeptide previously referred to as p220 (Safer, 1989; Yan *et al.*, 1992).

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mRNA bearing the 5′-untranslated region of HRV2. The translation of the capped RNA could be restored to normal levels by the addition of purified eIF-4.

MATERIALS AND METHODS

Enzymes, Reagents, and Chemicals. Restriction endonucleases and DNA modifying enzymes were from New England Biolabs (Beverly, MA); reactions were performed as described by the supplier. The purification of T7 RNA polymerase and its use were as described (Grodberg & Dunn, 1988). Micrococcal nuclease was from Boehringer Mannheim (Mannheim, FRG). Oligonucleotide and oligopeptide syntheses were as described (Sommergruber *et al.*, 1989, 1992). [^{35}S]Trans label (1100 Ci/mmol) was from American Radiolabelled Chemicals, St. Louis, MO; ^{14}C-labeled protein markers were from Amersham (Amersham, U.K.). Ammonium sulfate ("suprapur grade") was from Merck, Darmstadt, FRG, and proteinase inhibitors were from Sigma; all other chemicals were of reagent grade.

Bacterial Strains and Plasmids. pET expression plasmids were maintained in the *Escherichia coli* K strain HMS174 (F^- *recA* $r^-_{K12}m^+_{K12}Rif^R$) and expressed in the pLysE derivative of *E. coli* B strain BL21(DE3) (F^- *ompT* $r^-_Bm^-_B$) (Studier *et al.*, 1990). Integrated into the chromosome of this strain is one copy of the T7 RNA polymerase gene under the control of the *lac* operon. In addition, the T7 lysozyme protein, the gene of which is carried episomally, is expressed constitutively. LB medium containing ampicillin (100 μg/mL) and chloramphenicol (30 μg/mL) as required was used throughout.

Plasmid pET8c/HRV2 2A. The expression vector pET8c/HRV2 2A (Figure 1A) contains the HRV2 cDNA nt 3075–3586 which code for the C-terminal 28 amino acids of VP1 and all 142 amino acids of HRV2 2A. This sequence was introduced in-phase into the *Bam*HI site of pET8c as the 0.52-kb *Sal*I/*Hin*dIII fragment of M13/2A described in Liebig *et al.* (1991). All protruding ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I before ligation. On transformation into *E. coli* BL21(DE3)pLysE cells and induction with IPTG, a fusion protein comprising 16 plasmid-derived amino acids, 28 amino acids of VP1, and 142 amino acids of 2A is produced. The 2A molecule cleaves itself off the growing peptide chain, resulting in the accumulation of mature HRV2 2A proteinase in the bacteria.

Plasmid pET8c/CVB4 2A. pET8c/CVB4 2A contains the CVB4 nucleotides 3189–3740 [amplified by PCR from the plasmid pT7FLCB4 (Jenkins *et al.*, 1987)] followed by a stop codon cloned into the *Bam*HI site of pET8c as for pET8c/HRV2 2A. On induction with IPTG, a fusion protein comprising 13 amino acids of the T7 gene 10 protein, 34 amino acids of CVB4 VP1, and 150 amino acids of the 2A proteinase is produced. Once again, the 2A proteinase cleaves itself from the growing peptide chain to give the mature proteinase.

Plasmid pHRV2/5′UTR-2C. Nt 19–611 of the HRV2 cDNA were fused to a fragment containing nt 3871–5220 of the HRV2 cDNA (Skern *et al.*, 1985), in which the two G residues at 4838 and 4839 had been mutated to T and A, respectively. A stop codon is thus present at the end of the 2C gene. This HRV2 expression block was then introduced between the *Hin*dIII and *Pst*I sites of pGEM2 (Promega, Madison, WI), such that nt 19 was proximal to the T7 RNA polymerase promoter (Figure 1B).

Expression of 2A Proteinases. For expression, a 400-mL stationary-phase culture of *E. coli* BL21(DE3)pLysE bearing pET8c/HRV2 2A or pET8c/CVB4 2A was diluted 1:12 in 5 L of fresh medium. After 3-h incubation with shaking (160 rpm) at 34 °C (OD_{590} 0.4–0.5), IPTG was added to a final concentration of 0.3 mM; the cultures were then incubated for a further 5 h at 34 °C. The bacteria were harvested by centrifugation at 6000*g*; after being rinsed once with 200 mL of washing buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA), the cell pellets were stored at −80 °C.

Purification of 2A Proteinases. Typically, the cell pellet from 5 L of culture was resuspended in 30 mL of buffer A [50 mM NaCl, 50 mM Tris-HCl (pH 8.0 at 20 °C), 1 mM EDTA, 5 mM DTT, and 5% glycerol]; cell lysis was completed by sonication using three to six bursts of 5 s each. Insoluble material was removed by centrifugation (39000*g*, 30 min; all centrifugation steps were performed at 4 °C) to give fraction 1; 8 mL of a saturated ammonium sulfate solution (adjusted to pH 8.0 with Tris-HCl) was added with stirring to the 32 mL of supernatant (20% saturation). Precipitation was for 12 h at 4 °C. Precipitated proteins were removed by centrifugation (39000*g*, 30 min), the supernatant (40 mL) was saved, and a further 13 mL of ammonium sulfate was added (40% saturation). After at least 4 h at 4 °C, the precipitated proteins were collected by centrifugation at 15000*g* for 30 min and were dissolved in 31 mL of buffer A (fraction 2). This suspension was loaded onto a Pharmacia FPLC HR 10/10 Mono-Q column which had been preequilibrated with buffer A (all chromatography steps were performed at room temperature). After the column was washed with 4 bed volumes of buffer A, the following gradient composed of buffer A and buffer B (same as buffer A, except for 1 M NaCl) was applied: 20 mL of 0–30% B, 120 mL of 30–60% B, and 20 mL of 60–100% B. HRV2 2A proteinase elutes at around 370–400 mM NaCl whereas the CVB4 2A enzyme elutes at 300 mM NaCl. Fractions containing proteinase were identified by SDS–PAGE and cleavage activity (as detailed below), pooled (fraction 3), and applied directly to a Pharmacia Superdex 75 Hiload 26/60 column preequilibrated with buffer A. The gel filtration column was developed with buffer A. HRV2 2A proteinase elutes with an apparent molecular mass of 30 kDa whereas the CVB4 2A elutes at 17 kDa. Fractions from the A_{280} peak were examined by SDS–PAGE, and those containing 2A proteinase at a purity of more than 98% (estimated after staining with Coomassie brilliant blue R250) were pooled (total volume 18 mL) and concentrated about 30-fold (fraction 4) in an Amicon Centriprep 10 cell.

The protein concentrations of 2A fractions were measured using the method of Bradford (1976); the concentrations of purified 2A proteinases were determined using the absorbance at 280 nm. For HRV2 2A, the 12 tyrosine and 1 tryptophan residues give a molar extinction coefficient at 280 nm of about 20 000 M^{-1} cm^{-1}; for CVB4, the value is 21 900 M^{-1} cm^{-1} (9 tyrosine and 2 tryptophan residues) (Fasman, 1977).

HRV2 2A Proteinase Assays. HRV2 2A proteinase activity was measured using the peptide P8-P8′, corresponding to the eight C-terminal residues of VP1 and the eight N-terminal residues of 2A proteinase, as described (Skern *et al.*, 1985; Sommergruber *et al.*, 1989). Briefly, aliquots for assay were incubated in a final volume of 100 μL with 0.25 μmol of peptide in buffer A at 25 °C. Aliquots were removed after 1, 2, 3, 4, 5, 7, 10, 15, and 30 min; the samples were processed and analyzed by reversed-phase HPLC on a C_{18} column as described (Sommergruber *et al.*, 1989). One unit of HRV2 2A proteinase is that amount of enzyme which cleaves 1 μmol of peptide P8–P8′ per minute at 25 °C.

Purification of eIF-4. eIF-4 was prepared from rabbit reticulocyte high-salt washes using the procedure of Lamphear and Panniers (1990). The polypeptide pattern of such a preparation analyzed by SDS–PAGE was previously published by De Benedetti *et al.* (1991) (Figure 7, panel C).

2A Proteinase Cleavage of eIF-4γ. Incubation of purified eIF-4 with 2A proteinases was performed in buffer A in a final volume of 20 μL at 30 °C for 10 min with 0.1 μg of eIF-4 and varying amounts of 2A proteinase.

The determination of the inhibitor profile of eIF-4γ cleavage in HeLa cell cytoplasmic extracts was performed as follows. Forty-two micrograms of HeLa cell cytoplasmic extract (prepared as described below, but without micrococal nuclease treatment) was incubated with 8 μg of HRV2 2A proteinase in buffer A in a final volume of 70 μL for 30 min at 30 °C to ensure maximal possible cleavage of eIF-4γ and to activate possible cellular proteinases. Various concentrations of the inhibitors elastatinal, antipain, and E64 were then added to 10-μL aliquots and the mixtures allowed to stand for 10 min on ice. To examine whether any proteinases had been activated which possessed a different inhibitor profile than that of HRV2 2A, a further 6 μg of HeLa cell cytoplasmic extract was then added to each aliquot followed by incubation at 30 °C for 30 min.

Cleavage of eIF-4γ was analyzed by SDS–PAGE on gels containing 6% acrylamide. The gels were blotted electrophoretically onto nitrocellulose filters which were subsequently probed with either the rabbit anti-eIF-4γ peptide 7 (raised against amino acids 327–342) or the rabbit anti-eIF-4γ peptide 6 antiserum (raised against amino acids 1230–1249) as described by Yan *et al.* (1992). Blotting conditions, the concentration of the second antibody, and its detection were essentially as described (Liebig *et al.*, 1991).

In Vitro Translation Using HeLa Cell Cytoplasmic Extracts. Micrococcal nuclease treated HeLa cell cytoplasmic extracts were prepared, and translations were carried out as described (Lawson & Semler, 1991; Molla *et al.*, 1991), except that cells were lysed mechanically with a Dounce homogenizer in isotonic buffer (Hartmuth & Barta, 1987) and dialysis was performed against buffer D of Dignam *et al.* (1983). Translation reactions contained in a final volume of 20 μL either 1 μg of rabbit globin mRNA [prepared by oligo(dT) selection of RNA isolated from rabbit reticulocytes] or 1 μg of RNA (prepared by *in vitro* transcription of the plasmid pHRV2/5′UTR-2C). Where stated, extracts were preincubated for 5 min at 30 °C with 2 μg (18 mU) of purified HRV2 2A proteinase (or buffer A).

To examine the restoring activity of purified rabbit eIF-4, translation extract (55 μL) was preincubated with 40 μg (360 milliunits) of HRV2 2A for 1 min at 30 °C followed by the addition of elastatinal to 500 μM. After 10 min on ice, five 10-μL aliquots were taken, and eIF-4 was included as indicated; as eIF-4 preparations were stored in 100 mM KCl, the ionic strength of the individual reactions was adjusted accordingly.

Reactions were quenched by the addition of unlabeled methionine to 40 mM. For the analysis of translation products, 7-μL aliquots of the translation reactions were added to 13 μL of Laemmli sample buffer (Laemmli, 1970) and heated to 95 °C for 5 min, and the mixture was analyzed by SDS–PAGE on gels containing 15% acrylamide. For fluorography, gels were fixed, soaked in 1 M sodium salicylate, dried, and exposed to Kodak XAR-5 X-ray film. The state of eIF-4γ at the end of the reaction was analyzed with 7-μL aliquots as described above.

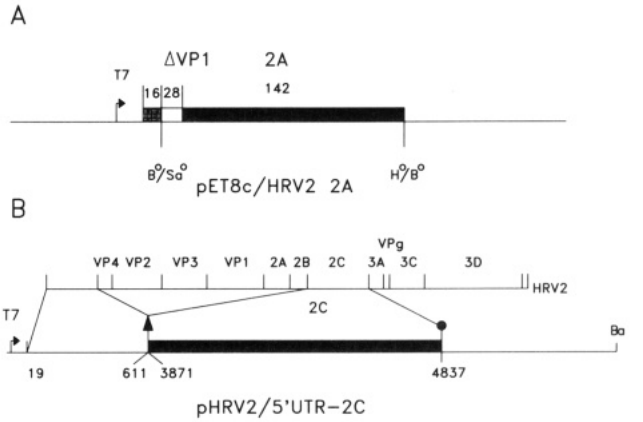

FIGURE 1: (A) Expression block of the plasmid pET8c/HRV2 2A. The T7 RNA polymerase promoter (T7) is followed by 16 plasmid-derived amino acids (hatched block), the 28 amino acids of the C-terminus of VP1 (white block), and all 142 amino acids of HRV2 2A proteinase (black block). Restriction sites used to introduce the HRV2 sequence are shown. Abbreviations: B, *Bam*HI; H, *Hin*dIII; Sa, *Sal*I. The circle indicates the loss of the site during construction. (B) Expression block of the plasmid pHRV2/5′UTR-2C. The nt 19–611 of the HRV2 5′UTR are fused to the HRV2 2C gene (black block); the nomenclature of the viral proteins is that of Rueckert and Wimmer (1984). Translation of an RNA produced by *in vitro* transcription with T7 RNA polymerase (the promoter is indicated) following linerization with *Bam*HI (Ba) starts at the AUG at 611 (large, closed arrow) and ends at the synthetic stop codon at 4837 (filled circle), giving a mature 2C protein with a molecular mass of 37 kDa.

RESULTS

Expression of 2A Proteinases. To prepare a system producing soluble, mature 2A proteinases, we made use of the T7 expression system of Studier *et al.* (1990) and of the ability of 2A proteinases to cleave themselves out of a growing peptide chain. For example, the plasmid pET8c/HRV2 2A (described under Materials and Methods; Figure 1) allows prokaryotic signals (from the T7 gene 10 sequence) for initiation of translation of the fusion protein to be used; the 16 plasmid-derived amino acids are followed by only 28 amino acids from the C-terminus of VP1. These 28 amino acids are sufficient to allow HRV2 2A proteinase to cleave itself off the peptide chain, but not to cause the fusion protein to become insoluble, as with pEx2A which contains the entire VP1 protein (Skern *et al.*, 1991). For expression of CVB4 2A proteinase, the plasmid pET8c/CVB4 2A was similarly designed, except that 34 amino acids of CVB4 VP1 are present. This strategy should provide a maximum accumulation of soluble, mature 2A proteinases. The two plasmids were used to transform the pLysE derivative of the *E. coli* strain BL21(DE3).

Purification and Characterization of Recombinant 2A Proteinases. Recombinant 2A proteinases were then purified using the four-step purification scheme described under Materials and Methods; the results of a typical experiment for HRV2 2A are shown in Table I and Figure 3. The use of the pLysE derivative of the *E. coli* strain BL21(DE3) was of importance for bacterial lysis; on warming to 4 °C after storage at −80 °C, the bacteria were almost completely lysed. Thus, only a few short bursts of sonication were necessary to degrade high molecular weight DNA and reduce viscosity, minimizing the risk of protein denaturation.

Purification was simplified in that after ion-exchange chromatography on the Mono-Q column, the 2A proteinases were the dominant proteins present in fraction 3 with a molecular mass of under 40 kDa. Thus, upon gel filtration of this material on a Superdex 75 Hiload 26/60 column, the

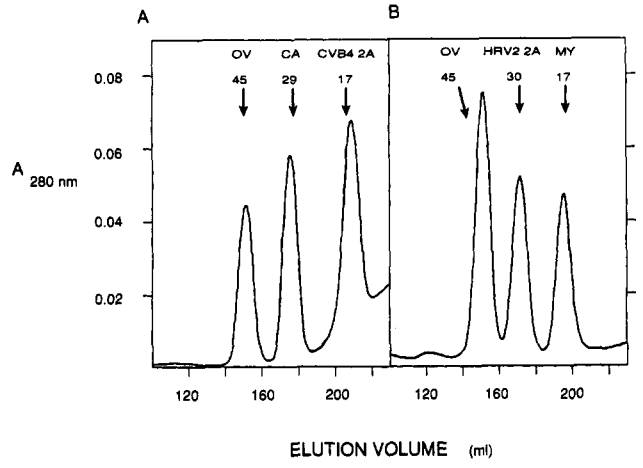

FIGURE 2: Gel exclusion chromatography of HRV2 and CVB4 2A proteinases on Superdex 75 Hiload 26/60. The elution profiles of the following mixtures are shown: (A) CVB4 2A proteinase (750 μg), chicken ovalbumin (OV, 1 mg), and bovine erythrocyte carbonic anhydrase (CA, 1 mg). (B) HRV2 2A proteinase (750 μg), chicken ovalbumin (OV, 1.5 mg), and horse heart myoglobin (MY, 1 mg). Samples (8 mL) were applied at a flow rate of 1.5 mL min^{-1} in buffer A. The apparent molecular masses (in kilodaltons) are shown above the peaks. CVB4 2A elutes with theoretical molecular mass of 16.7 kDa, whereas HRV2 2A (theoretical molecular mass of 16.2 kDa) elutes with an apparent molecular mass of 30 kDa.

high molecular mass contaminants were readily removed. Figure 2 shows the characterization of the behavior of the two proteinases during gel filtration. The CVB4 2A proteinase elutes at the position corresponding to the predicted molecular mass of 16.7 kDa (Figure 2A); in contrast, HRV2 2A elutes with an apparent molecular mass of 30 kDa, compared to the theoretical mass of 16.2 kDa (Figure 2B).

Table I summarizes the protein content (analyzed by SDS–PAGE in Figure 3A) and enzymatic activity in the pooled fractions of a typical purification of HRV2 2A. Yields of recombinant HRV2 2A proteinase were about 5 mg from 5 g of *E. coli* (wet weight); the specific activity was about 9 units/mg of HRV2 2A protein. SDS–PAGE (Figure 3A, lane 4), chromatofocusing on a Pharmacia Mono-P HR 5/20 column, and capillary electrophoresis (data not shown) indicated that the purity of the fractions of HRV2 2A was greater than 98%. Figure 3B shows a comparison of preparations of HRV2 2A proteinase and CVB4 2A proteinase; the eight extra amino acids of the CVB4 enzyme account for the difference in mobility.

Amino acid sequencing (determined by automated gas-phase Edman degradation) of the purified proteins revealed the N-termini to be those predicted from the nt sequence. The proteinases were stored at 4 °C at concentrations of 5 mg/mL and were stable for several months. The K_m for HRV2 2A proteinase was 5.2×10^{-4} M on the peptide TRPIITTAG*-GPSDMYVH (the asterisk indicates the position of cleavage), very similar to that (5.4×10^{-4} M) found using impure preparations of HRV 2A proteinase containing an extra N-terminal methionine residue (Sommergruber *et al.*, 1992); the K_m for CVB4 2A proteinase on its cognate peptide (ERASLITT*GPYGHQSG) was 2.5×10^{-4} M.

In Vitro Cleavage of eIF-4γ by 2A Proteinases. The availability of two highly purified 2A proteinases together with purified eIF-4 enabled us to investigate separately the cleavage of eIF-4γ and the effect of this cleavage on translation. To this end, we incubated purified HRV2 and CVB4 2A proteinases with purified eIF-4 as described under Materials and Methods; Figure 4A shows the state of eIF-4γ after incubation with 2A proteinases by SDS–PAGE followed by immunoblotting. Cleavage of the eIF-4γ by both proteinases is clearly visible. Figure 4B shows that cleavage was dependent on the 2A proteinase concentration, partial cleavage still being obtained with 28 ng of enzyme. As antiserum 7 is directed against amino acids 327–342 of eIF-4γ, the cleavage products detected in Figure 4A,B are N-terminal [designated cp_a in Yan *et al.* (1992)]; the C-terminal product [designated cp_b in Yan *et al.* (1992)] is revealed with antiserum 6 directed against amino acids 1230–1249 (Figure 4C). As reported (Yan *et al.*, (1992), cp_a is comprised of several bands, whereas cp_b appears to be a single entity.

Wyckoff *et al.* (1990a) demonstrated that eIF-3 was essential for cleavage of eIF-4γ by bacterial extracts containing poliovirus 2A proteinase. The presence of eIF-3 proteins in the eIF-4 preparations used here could not be demonstrated (data not shown).

Inhibition of in Vitro eIF-4γ Cleavage in HeLa Cell Cytoplasmic Extracts. The experiment described above provides clear evidence for direct cleavage of eIF-4γ by HRV2 2A proteinase in a system using purified components and no necessity for a second proteinase. To investigate the cleavage of eIF-4γ in a cell-free extract, use was made of proteinase inhibitors shown previously to inhibit HRV2 2A proteinase (Sommergruber *et al.*, 1992).

The experiment shown in Figure 5 was designed to test whether the cleavage of eIF-4γ was inhibited by the same concentrations of inhibitors as HRV2 2A proteinase. In order to allow the activation of any cellular proteinases, HeLa cytoplasmic cell extracts were first incubated with HRV2 2A proteinase for 30 min at 30 °C; Figure 5A shows that the specific cleavage of eIF-4γ was complete (compare lanes 1 and 2). Various concentations of inhibitors were then added and the mixtures allowed to stand for 10 min on ice. If a quiescent proteinase capable of cleaving eIF-4γ had been activated during this time, the inhibitor profile of the cleavage of any material added afterward should be different from that of HRV2 2A proteinase. The same quantity of untreated extract was therefore added, and the samples were incubated at 30 °C for a further 30 min. The results for elastatinal, antipain, and E64 are shown in Figure 5; inhibition of cleavage of the material added is in the range of 25–50 μM for elastatinal and 50–100 μM for antipain, similar to the IC_{50} values (elastatinal, 55 μM; antipain, 65 μM) observed for the inhibition of HRV2 2A proteinase for peptide cleavage. This was also the case for chymostatin (data not shown). No inhibition was found with E64 at 200 μM. The concentration dependence of the inhibition of cleavage is therefore very similar to that of HRV2 2A (Sommergruber *et al.*, 1992).

Influence of Purified HRV2 2A Proteinase on in Vitro Translation. A system using HeLa cell cytoplasmic extracts was employed to investigate the effect of cleavage of eIF-4γ on *in vitro* translation. Native rabbit globin mRNA was used as capped mRNA; an RNA bearing the HRV2 5′UTR followed by the HRV2 2C coding sequence as reporter gene (transcribed from pHRV2/5′UTR-2C; Figure 1B) served as picornaviral mRNA, the translation of which should be unaffected by the host cell shutoff. This composite RNA was preferred to a subgenomic one comprising the HRV2 5′UTR followed by VP4 and VP2 capsid protein sequences as it is rich in methionine codons.

Figure 6A shows the translation products of the globin mRNA in the presence and absence of 2 μg of HRV2 2A proteinase (compare lanes 5 and 6); translation is almost eliminated after the 5-min preincubation of the HeLa cell

Table I: Purification of Recombinant HRV2 2A Proteinase from 5 g of *E. coli* BL21(DE3) pLysE(pET8c/2A) Cell Paste

step	protein, total amount (mg)	enzyme			
		sp act. (units/mg of protein)	total amount (units)	yield (%)	purification factor (x-fold)
crude cell-free extracts (fraction 1)	602	0.24	144	(100)	1.0
ammonium sulfate precipitation: 20–40 % fraction (fraction 2)	77.5	0.76	59	41	3.2
ion-exchange chromatography: mono-Q: fractions 37–43, 1.35 mL of each, pooled (fraction 3)	9.5	5.20	49	34	21
gel filtration: Superdex: fractions 109–120, 1.5 mL of each, pooled and concentrated (fraction 4)	4.7	8.80	41	28	36

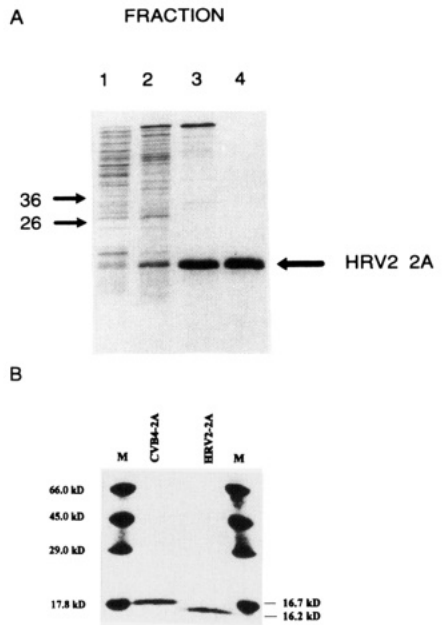

FIGURE 3: (A) SDS–PAGE analysis of the purification of HRV2 2A proteinase. Six micrograms of total protein each from the indicated fractions was analyzed on a gel containing 15% acrylamide; visualization was with Coomassie brilliant blue R250. Positions of marker proteins are indicated. (B) SDS–PAGE analysis of 2A proteinase preparations. Two micrograms each of purified CVB4 and HRV2 2A proteinase was analyzed on a gel containing 15% acrylamide; visualization was as above. Marker proteins are indicated; the eight extra amino acids of the CVB4 enzyme are responsible for the difference in mobility of the two proteins.

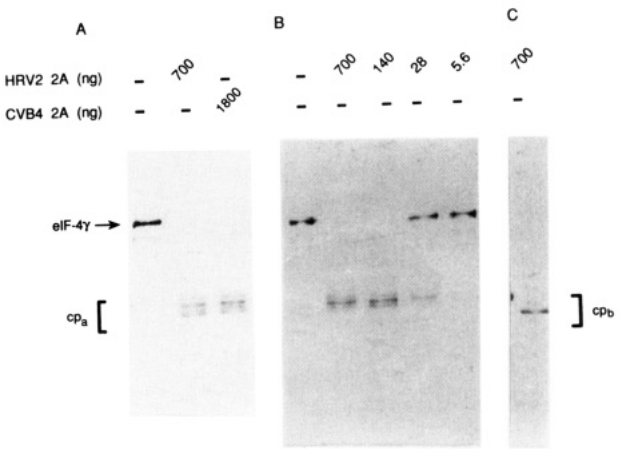

FIGURE 4: In vitro cleavage of rabbit eIF-4γ by 2A proteinases. (A) 0.1 μg of purified rabbit eIF-4 was incubated for 30 min at 30 °C with the indicated amounts of HRV2 or CVB4 2A proteinases. Samples were analyzed by SDS–PAGE on a gel containing 6% acrylamide; the gel was blotted onto nitrocellulose and probed with the rabbit anti-eIF-4γ peptide 7 antiserum. (B) 0.1 μg of purified rabbit eIF-4 was incubated with decreasing amounts of HRV2 2A proteinase as indicated. Samples were analyzed as in panel A. (C) 0.1 μg of purified rabbit eIF-4 was incubated with 700 ng of HRV2 2A proteinase; the sample was analyzed as in panel A, except that the anti-eIF-4γ antiserum 6 was used. Key: cp$_a$, N-terminal cleavage products; cp$_b$, C-terminal cleavage product.

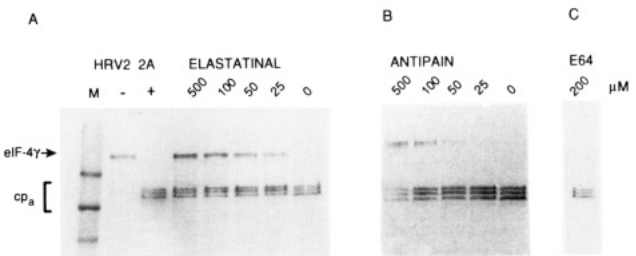

FIGURE 5: Inhibition of HRV2 2A proteinase cleavage of eIF-4γ in HeLa cell cytoplasmic extracts. HRV2 2A (8 μg) was incubated with 42 μg of HeLa cell cytoplasmic extract for 30 min at 30 °C. Aliquots were removed, and inhibitor was added. After 10 min on ice, a further 6 μg of HeLa cell cytoplasmic extract was added, and the samples were incubated for a further 30 min at 30 °C. Samples were analyzed by immunoblotting as in Figure 4, panel A. Inhibitors were (A) elastatinal, (B) antipain, and (C) E64. Concentrations are shown above the lanes. The state of the initial digest before (–) and after (+) the incubation with HRV2 2A is shown in panel A. Key: cp$_a$, N-terminal cleavage products.

cytoplasmic extract with HRV2 2A proteinase. In contrast, translation of the RNA transcribed from the plasmid pHRV2/5′UTR-2C was stimulated about 2–3-fold by preincubation with HRV2 2A proteinase (compare lanes 1 and 2). That the ionic strength or the composition of the buffer in which the 2A proteinase was stored was not responsible for this effect is shown in lanes 4 and 8; translation of both mRNAs is not significantly different from that in lanes 1 and 5, respectively, in which the extracts were preincubated without any addition whatsoever. Preincubation of the HRV2 2A proteinase with the inhibitor elastatinal at 0.5 mM before addition prevented the negative effect on globin mRNA translation (lane 7) and the positive effect on the translation of pHRV2/5′UTR-2C RNA (lane 3).

The state of the eIF-4γ protein from the translation reactions analyzed in Figure 6A was then examined; aliquots were analyzed by SDS–PAGE, blotted onto nitrocellulose, and probed with the anti-eIF-4γ peptide 7 antiserum (Figure 6B). eIF-4γ is only cleaved in reactions to which active 2A proteinase had been added (lanes 2 and 6); furthermore, the specific proteolysis appeared to be complete. This demonstrates that translation of pHRV2/5′UTR-2C was indeed occurring in the absence of intact eIF-4γ. An overexposure of the gel in Figure 6A showed that some residual translation of the rabbit globin mRNA was also visible, although no residual intact eIF-4γ could be detected (see Figure 6A,B, lanes 6).

Purified eIF-4 Restores Translation in HRV2 2A-Treated Translation Extracts. To investigate whether the reduction in translation was due to the cleavage of eIF-4γ, HeLa cell cytoplasmic extracts were treated with an excess of HRV2 2A to ensure a rapid inhibition of translation; possible side-effects of the preincubation could therefore be excluded. eIF-4 was added after HRV2 2A proteinase inhibition with elasta-

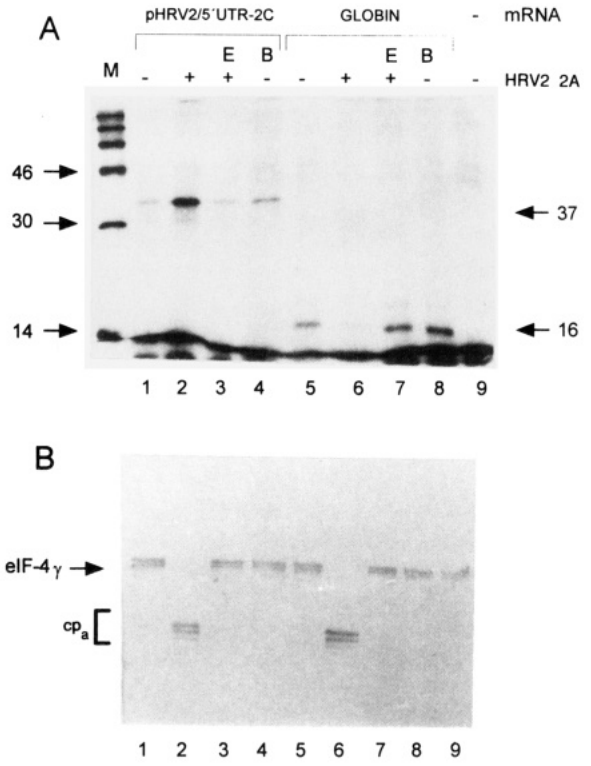

FIGURE 6: Effect of purified HRV2 2A proteinase on *in vitro* translation in HeLa cell cytoplasmic extracts and fate of eIF-4γ. *In vitro* translation with HeLa cell cytoplasmic extracts was performed as described under Materials and Methods. (a) Seven-microliter aliquots were analyzed for globin synthesis by SDS–PAGE on a gel containing 15% acrylamide; the translation products were visualized by subsequent fluorography. Those of the pHRV2/5'UTR-2C and rabbit globin RNAs are indicated (37 and 16 kDa, respectively). (B) Seven-microliter aliquots were analyzed for the state of eIF-4γ as in Figure 4, panel A. Lanes 1–4, 1 μg of RNA transcribed from pHRV2/5'UTR-2C; lanes 5–8, 1 μg of rabbit globin RNA; lane 9, no RNA. In lanes 2 and 6, 2 μg (18 milliunits) of HRV2 2A proteinase was added to the translation extract prior to the addition of RNA. Lanes 1 and 5 were controls with no addition. In lanes 3 and 7, 2 μg of HRV2 2A proteinase pretreated (5 min, 30 °C) with 0.5 mM elastatinal was added. In lanes 4 and 8, the same volume of buffer A was added as in lanes 2 and 6. Key: M, marker proteins; B, buffer A; E, elastatinal; cp_a, N-terminal cleavage products.

tinal and the translational capability of the extracts examined (Figure 7). In panel A, translation is again reduced by the addition of 2A proteinase (compare lanes 1 and 2 with lanes 3 and 4). However, translation is restored almost to the previous level by the addition of 90 ng (compare lanes 1, 3, and 5) and 150 ng (compare lanes 2, 4, and 6) of eIF-4. The increased KCl concentration is the reason for the higher absolute level of translation in lanes 2, 4, and 6 than in lanes 1, 3, and 5.

Panel B shows the state of the eIF-4γ. The protein is intact in lanes 1 and 2 [the faint band running in the region of the cleavage products is occasionally produced during micrococcal nuclease treatment, presumably through a calcium-dependent proteinase (Wyckoff *et al.*, 1990b)]. In lanes 3 and 4, the cleavage products constitute the predominant species; however, the amount of intact material present in lanes 5 and 6 is higher, due to the addition of purified eIF-4. Although the original concentration of uncleaved eIF-4γ was not reached, it was sufficient to restore translation to previous levels.

DISCUSSION

Koenig and Rosenwirth (1988) described the purification to homogeneity of small amounts (30 μg) of poliovirus 2A

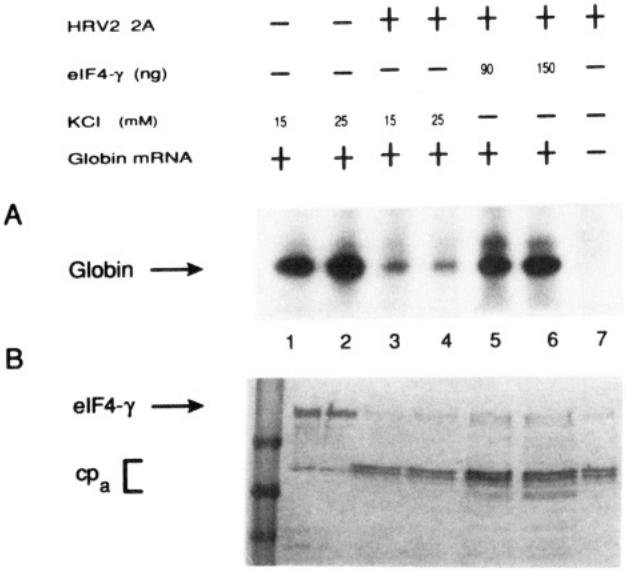

FIGURE 7: Reduction of translation of a capped mRNA induced by HRV2 2A proteinase can be overcome by addition of purified eIF-4. *In vitro* translation with HeLa cell cytoplasmic extracts was performed as described under Materials and Methods. (A) Seven-microliter aliquots were analyzed for globin synthesis as in Figure 6A. (B) Seven-microliter aliquots were analyzed for the state of eIF-4γ as in Figure 4, panel A. Lanes 1–6, 1 μg of rabbit globin RNA. Lane 7, no RNA. Eight micrograms of HRV2 2A proteinase was added to lanes 3–7; an equivalent volume of buffer A was added to lanes 1 and 2. All samples were incubated for 1 min at 30 °C. Elastatinal (final concentration 500 μM) was then added to all samples, followed by incubation for 10 min on ice; 90 and 150 ng of eIF-4 was subsequently added to lanes 5 and 6, respectively. The ionic strength of lanes 1 and 3 was adjusted to that of lane 5 and those of lanes 2 and 4 to that of lane 6 by increasing the KCl concentration by the amounts shown. Key: cp_a, N-terminal cleavage products.

proteinase from HeLa cells infected with poliovirus. However, the properties of this poliovirus preparation differ greatly from those of the HRV2 and CVB4 2A proteinases. In contrast to the two recombinant proteins, the poliovirus enzyme was extremely heat-labile and could only be isolated from infected cells by solubilization with detergent. The low protein concentrations of the poliovirus 2A proteinase preparations may have adversely affected the stability. Association of the protein with intracellular membranes may explain the need for detergent.

The HRV2 and CVB4 2A proteinases have a similar K_m on their cognate peptides; however, one major difference in the two enzymes is noteworthy, namely, the elution from the gel exclusion column. HRV2 2A proteinase appears to be behaving as a dimer, whereas the CVB4 2A proteinase runs as a monomer. Further evidence for the HRV2 2A dimer was obtained upon changing the buffer conditions; under conditions of high salt (500 mM) and low pH (under pH 5.9), HRV2 2A elutes as a monomer with an apparent molecular mass of 17 kDa (H.-D. Liebig, unpublished observations). Molecular mass determinations of picornaviral 2A proteinases by gel filtration must therefore be approached with caution.

The mechanism by which human rhinoviruses and enteroviruses impair the ability of the host cell to translate capped mRNAs has been the subject of much investigation. The most favored hypothesis before the work presented here was that the 2A proteinase activated a host cell proteinase which cleaved and inactivated the host cell protein eIF-4γ, a component of the eukaryotic initiation factor eIF-4 (Wyckoff *et al.*, 1992). The activation of the host cell proteinase appeared to be eIF-3-dependent (Wyckhoff *et al.*, 1990a). Concrete evidence for this hypothesis is, however, not available;

neither has the putative proteinase of molecular mass 55–60 kDa activated by rhinoviral and enteroviral 2As been identified (Lloyd *et al.*, 1986; Wyckhoff *et al.*, 1990a, 1992) nor has the exact contribution of the cleavage of eIF-4γ to host cell shutoff been elucidated (Etchison *et al.*, 1982; Bonneau & Sonenberg, 1987; Urzainqui & Carrasco, 1989). Recently, even the role of the 2A proteinase in initiating host cell shutoff has been placed in doubt (Perez & Carrasco, 1992), despite genetic (Bernstein *et al.*, 1985; O'Neill & Racaniello, 1989) and biochemical experiments (Lloyd *et al.*, 1986; Kraeusslich *et al.*, 1987) implicating 2A proteinase activity in this phenomenon.

The availability of purified reagents made it possible to examine these open questions. The results presented in Figures 4 and 5 suggest that HRV2 and CVB4 2A proteinases can cleave eIF-4γ directly; if a second proteinase is involved, it must copurify with eIF-4 and have a very similar inhibition profile to that of HRV2 2A.

Clearly, this conclusion is in contrast to the data with poliovirus 2A proteinase implying that the cleavage of eIF-4γ is mediated by a second proteinase (Lloyd *et al.*, 1986; Kraeusslich *et al.*, 1987; Wyckoff *et al.*, 1990a, 1992). Kraeusslich *et al.* (1987) showed that cleavage by the poliovirus 2A proteinase in infected cell extracts on an *in vitro* synthesized precursor bearing the VP1/2A cleavage site could be inhibited by an anti-2A proteinase antiserum; however, this was not the case for eIF-4γ cleavage. To explain the discrepancy, one must assume that eIF-4γ cleavage can still occur in the presence of the anti-2A proteinase antiserum.

Wyckoff and co-workers (Wyckoff et al., 1990a, 1992) have isolated an eIF-4γ cleavage activity from poliovirus-infected HeLa cells. The activity has an apparent molecular mass of 55–60 kDa on size exclusion columns; no poliovirus 2A proteinase could be detected by an antiserum in this activity. Inhibition of the cleavage activity was possible with alkylating agents and certain metal ions, but not by E64, a characteristic inhibitor of papain-like cysteine proteinases. However, as noted by Wyckoff *et al.* (1992), this profile is reminiscent of those of poliovirus 2A proteinase (Koenig & Rosenwirth, 1988) and HRV2 2A proteinase (Sommergruber *et al.*, 1992). As only one concentration of inhibitor was tested for the eIF-4γ cleavage activity, it is difficult to compare the profiles directly; however, the experiments shown in Figure 5 indicate that the inhibition of eIF-4γ cleavage *in vitro* has the same inhibitor dependence profile as HRV2 2A proteinase.

One difference in the profiles is, however, apparent; antipain inhibits HRV2 2A proteinase in both peptide (IC_{50} 55 μM) and eIF-4γ cleavage assays, whereas the cleavage of eIF-4γ induced by extracts of poliovirus-infected HeLa cells was not influenced by antipain at 100 μM. This may reflect differences in the poliovirus and HRV2 2A proteinases.

The reported molecular mass of 55–60 kDa of the eIF-4γ cleavage activity from poliovirus-infected HeLa cells may have two origins, if it is assumed that the amount of poliovirus 2A proteinase present in this activity lies under the detection limit of the antiserum. First, the poliovirus 2A proteinase may have an aberrant mobility on size exclusion columns as shown by HRV2 2A proteinase (Figure 2). Second, the proteinase could be associated with other proteins which elute with the reported molecular mass. Nevertheless, the reported requirement of eIF-3 cannot be explained, unless the partially purified eIF-4γ used as substrate by Wyckoff et al. (1992) requires a factor which the purified eIF-4 used here does not. Finally, however, it cannot be ruled out that the polio- and rhinoviruses utilize a different mechanism for eIF-4γ cleavage.

Sommergruber *et al.* (1992) proposed, on the basis of peptide cleavages with HRV2 2A, that the common target of rhinoviral and enteroviral 2A proteinases would be Thr-X-Gly, with cleavage taking place between X and Gly. For direct cleavage of eIF-4γ to be valid, this sequence must be found in the eIF-4γ amino acid sequence; in fact, it appears 5 times, at positions 15, 484, 745, 971, and 1226. However, only the middle three could give cleavage products which might correspond to those actually observed. Sequencing of the cleavage products will be required to determine which, if any, of these sequences is actually recognized.

These experiments show clearly that purified HRV2 2A proteinase can inhibit the translation of a capped mRNA *in vitro* and strongly support the view that rhinoviral and enteroviral 2A proteinases are indeed responsible for this phenomenon. Furthermore, the reduction of translation by 2A proteinases was mediated by the cleavage of the eIF-4γ component of the cap-binding complex, eIF-4. This factor has long been known to restore cap-binding activity to extracts of poliovirus-infected cells (Tahara *et al.*, 1981); the experiments in Figure 7 demonstrate clearly that the HRV2 2A proteinase is responsible for its inactivation. Nevertheless, despite the apparent completion of the specific cleavage of eIF-4γ in Figure 6, a residual level of translation was still present. Further work will be necessary to show whether other events are required for a complete shutoff as has been suggested (Bonneau & Sonenberg, 1987; O'Neill & Racaniello, 1989).

The biochemical evidence presented here that HRV2 2A can stimulate translation driven from the HRV2 2A 5′UTR correlates well with the very recent genetic evidence of Hambidge and Sarnow (1992) showing that the expression of poliovirus 2A proteinase enhanced translation driven from the virally-encoded 5′UTR. The system characterized here, based on the use of purified components, is currently being used to investigate whether the cleavage products of eIF-4γ are responsible, as has been proposed (Hambidge & Sarnow, 1992), and to identify regions of the 5′UTR necessary for stimulation to occur.

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