Mapping the Cleavage Site in Protein Synthesis Initiation Factor eIF-4γ of the 2A Proteases from Human Coxsackievirus and Rhinovirus*

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The rate-limiting step of eukaryotic protein synthesis is the binding of mRNA to the 40 S ribosomal subunit, a step which is catalyzed by initiation factors of the eIF-4 (eukaryotic initiation factor 4) group: eIF-4A, eIF-4B, eIF-4E, and eIF-4A. Infection of cells with picornaviruses of the rhino- and enterovirus groups causes a shut-off in translation of cellular mRNAs but permits viral RNA translation to proceed. This change in translational specificity is thought to be mediated by proteolytic cleavage of eIF-4γ, which is catalyzed, directly or indirectly, by the picornaviral 2A protease. In this report we have used highly purified recombinant 2A protease from either human Coxsackievirus serotype B4 or rhinovirus serotype 2 to cleave eIF-4γ in vitro in the eIF-4 complex purified from rabbit reticulocytes. Neither the rate of cleavage nor fragment sizes were affected by addition of eIF-3. The NH2- and COOH-terminal fragments of eIF-4γ were separated by reverse phase HPLC and identified with specific antibodies, and the NH2-terminal sequence of the COOH-terminal fragment was determined by automated Edman degradation. The cleavage site for both proteases is 478GPPRGGPG148 in rabbit eIF-4γ, corresponding to 478GHTTLSTR 148GPRGGPG148 in human eIF-4γ.

The translation of all cellular and most viral mRNAs in eukaryotic cells requires initiation factors of the eIF-4 group.

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The abbreviations used are: eIF, eukaryotic initiation factor; CVB4, human Coxsackievirus serotype B4; HPLC, high pressure liquid chromatography; HRV2, human rhinovirus serotype 2; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; cp, cleavage product.
linear gradient of Buffer A to 80% Buffer B (0.1% trifluoroacetic acid in 95% acetonitrile), 2 ml of 80% Buffer B, a 2-ml gradient to 100% Buffer B, 2 ml of 100% Buffer B, and a 2-ml gradient back to 0% Buffer B. Cleavage products derived from the COOH terminus were subjected to automated Edman degradation using an Applied Biosystems model 470A sequenator.

Electrophoresis and Western Blotting—SDS-PAGE was performed using the Laemmli buffer system (20) for 1.5 h at 100 V; protein bands were visualized after either staining with silver (21) or Western blotting. For the latter analysis, unstained gels were transferred to polyvinylidene difluoride membranes using a Bio-Rad Mini Trans-Blot cell and electrode buffer (20) as per manufacturer's recommendations. Antibodies 6 antibody was generated against a COOH-terminal eIF-4y peptide and anti-peptide 7, against an NH2-terminal peptide as described previously (18). Detection of immunoreactive species was performed as described previously (18) with development enhanced by inclusion of CoCl2 (22).

RESULTS AND DISCUSSION

In Vitro Cleavage of eIF-4y—Addition of recombinant 2A protease from CVB4 and HRV2 to highly purified eIF-4 resulted in the appearance of a series of faster migrating bands immunologically related to eIF-4y, presumed to be proteolytic fragments (16). The same study described several experiments indicating that eIF-4y is cleaved directly by 2A protease, rather than through activation of an endogenous cellular protease.

To analyze further this proteolytic reaction, eIF-4y purified from rabbit reticulocytes was incubated with recombinant CVB4 2A protease and the reaction time course analyzed (Fig. 1). Commencement of proteolysis was apparent after 5 min as judged by the disappearance of the 200–220-kDa bands of eIF-4y and appearance of the characteristic cleavage products ranging in size from 100 to 130 kDa (cpa and cpa) (18, 24–26). Four major cleavage products were clearly detected in the silver-stained gel shown (Fig. 1A). eIF-4y incubated for 60 min in the absence of protease was unaltered (lane N), showing that the proteolytic activity was not present in the eIF-4y preparation. Comparable results were obtained using HRV2 2A protease (data not shown). The pattern was not changed by overnight incubation (Fig. 1A, 16 hr).

Wyckoff et al. (27) have presented evidence that eIF-3 is required for the cleavage of eIF-4y by poliovirus 2A protease. This initiation factor of 550 kDa contains seven primary polypeptides and several secondary polypeptides derived by proteolysis (1). To determine whether eIF-3 affected either the rate or site of cleavage, we repeated the time course in the presence of this factor at a concentration equimolar to that of eIF-4 (Fig. 1A, +eIF-3). The rate of appearance and mobilities of eIF-4y cleavage products were unchanged. Additionally, no cleavage of eIF-4A, eIF-4E, or the various polypeptides of eIF-3 was observed (Fig. 1A and data not shown). Similar results were obtained when the molar ratio of eIF-3 to eIF-4 was increased to 13 (data not shown). The reason for this apparent discrepancy with the study of Wyckoff et al. (27) is not known, but it may reflect a difference in the purity of components used in the two studies or a difference between poliovirus and Coxsackievirus 2A proteases.

Despite the complexity of products, we propose that the 2A proteases have only a single cleavage site in eIF-4y. The migration of eIF-4y on SDS-PAGE is heterogeneous, with three major, closely spaced forms apparent (19, 24, 29), but the cause of this is unknown. Cleavage by poliovirus in vivo produces three major fragments detected with an antibody directed against NH2-terminal peptide sequences (collectively referred to as cpa) but only a single fragment (cpb) detected with an antibody against COOH-terminal sequences (18). These results suggest that the heterogeneity in the intact eIF-4y polypeptide is localized to the NH2-terminal segment. To determine if Coxsackievirus 2A protease behaved similarly in vitro, we analyzed the polypeptides in Fig. 1A with the COOH-terminal antibody (Fig. 1B). A single COOH-terminal product was detected, appearing simultaneously with the NH2-terminal products. The kinetics of appearance of cpa, were not affected by eIF-3, and no further cleavage occurred even after overnight incubation (Fig. 1B, 16 hr). Interestingly, we have consistently observed with purified rabbit eIF-4y preparations that the antibody reacted much more strongly with cpa than with intact eIF-4y, suggesting that the epitope may be obscured in the intact protein. As a further argument that 2A protease has a single cleavage site, all cleavage products appeared simultaneously (Fig. 1A), and the pattern did not change in complexity during incubation, as would be expected if there were multiple cleavages and intermediate products.

It has been shown previously that cleavage products of eIF-4y, produced by poliovirus infection of HeLa cells, co-migrate electrophoretically with products formed after infection of HeLa cells by either human rhinovirus (28) or Coxsackievirus (10). In contrast, in vitro cleavage of eIF-4y by extracts containing poliovirus 2A protease yields products that co-migrate with cleavage products generated in vivo during poliovirus infection (10, 27). These results support the view that all three viral proteases cleave at the same site and also that in vitro cleavage of eIF-4y reflects the in vivo situation. The ability to cleave eIF-4y in vitro in a system containing only purified components enabled us to define the cleavage products biochemically.

Purification and Identification of the Carboxyl-terminal Fragment of eIF-4y—Purified eIF-4y was incubated with either CVB4 or HRV2 2A protease under the reaction conditions described under “Experimental Procedures.” The reaction was terminated when less than ~2% of the intact eIF-4y remained. Reaction products were resolved by reverse phase HPLC on a
C4 column, subjected to SDS-PAGE, and detected by silver staining. The results obtained with CVB4 2A protease are shown in Fig. 2; similar results were obtained with HRV2 2A protease (data not shown). The three slowest migrating fragments of eIF-4γ eluted together in fraction 30, whereas the fastest migrating fragment eluted in fractions 42–44. Interestingly, although the latter fragment had the weakest silver-staining characteristics of the eIF-4γ fragments, it possessed the greatest absorbance at 280 nm. eIF-4A eluted in fraction 40. As the polyacrylamide concentration of 6.5% was employed to resolve the various fragments of eIF-4γ, polypeptides below 30 kDa were not resolved. Identification of the other peaks in the chromatogram was made from additional HPLC runs with individual components of the reaction mixture (see figure legend).

HPLC fractions containing eIF-4γ cleavage products were tested with antibodies raised against synthetic peptides representing NH₂- and COOH-terminal sequences of eIF-4γ (18). Anti-NH₂- and COOH-terminal antibodies recognized different subsets of fragments (Fig. 3). The fragments co-eluting in fraction 30 were all recognized by the anti-NH₂-terminal antibody and not by the anti-COOH-terminal antibody, indicating they were derived from the NH₂ terminus. Conversely, the fragment eluting at fraction 43 was specifically recognized by the anti-COOH-terminal antibody and was not by the anti-NH₂-terminal antibody. Fragments generated by HRV2 2A protease gave identical results (data not shown). Therefore, reverse phase HPLC under these conditions readily resolves cpb and cpbB. A minor product of ~80 kDa, which was also recognized by antibodies against the COOH terminus, appeared only after reverse phase HPLC (Fig. 3C, lane 43), but was not apparent in the original digest (Fig. 1B). It is possible that it resulted from a cleavage by CVB4 2A protease at a second site. Alternatively, as it was detectable only after HPLC, it may have resulted from breakdown of cpb under the acidic conditions used (pH 2).

**Amino-terminal Sequence Analysis**—HPLC fractions containing cpb generated upon cleavage by CVB4 or HRV2 2A protease were subjected to automated Edman degradation for determination of NH₂-terminal sequence (Table I). The results indicate that cleavage (↓) of rabbit eIF-4γ by both proteases

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**Fig. 2. Separation of eIF-4γ cleavage products by reverse phase HPLC.** eIF-4γ (40 μg/ml) cleaved in a total reaction volume of 2 ml with CVB4 2A protease (60 μg/ml) was loaded directly on a C4 column. Panel A shows SDS-PAGE analysis on 6.5% gels followed by silver staining of fractions obtained. U, C, and FT represent lanes containing unreacted eIF-4γ, CVB4-cleaved eIF-4γ, and flow-through (pooled fractions 6–8), respectively. Panel B represents the eluting proteins as monitored by absorbance at 280 nm. Peaks not identified by SDS-PAGE in panel A were identified in separate runs as buffer components (fractions 41, 46, and 55), 2A protease (fraction 29), and eIF-4E (fraction 39).

**Fig. 3. Identification of HPLC fractions by immunoblotting.** Selected HPLC fractions (see Fig. 2) were subjected to electrophoresis on 6.5% gels. Gels were stained with silver (panel A) or transferred to polyvinylidene difluoride membrane and probed with anti-NH₂- and COOH-terminal antibodies. Lanes U and C contain unreacted and CVB4 2A protease-cleaved eIF-4γ, respectively. Lanes 30 and 43 contain aliquots from HPLC fractions 30 and 45, respectively.

**Table I**

| Source of sequence | Sequence |
|--------------------|----------|
| Rabbit eIF-4γ cpb | GPPRGPGP |
| CVB4 2A digestion | XPPRGGPGXELPRG |
| Rabbit eIF-4γ cpb | XPPRGGPGXELPRG |
| HRV2 2A digestion | XPPRGGPGXELPRG |
| Rabbit eIF-4γ cDNA | 47GRPALSSR GPYGHQSG |
| Human eIF-4γ cDNA | 47GRPALSSR GPYGHQSG |
| HRV2 polypeptide | 47GRPALSSR GPYGHQSG |
| CVB4 polypeptide | 47GRPALSSR GPYGHQSG |

*See footnote 2.
Ref. 18.
Ref. 31.
Ref. 32.
X indicates that an unambiguous assignment could not be made.
occurs at GPPRGGPG. As there is considerable similarity in the amino acid sequences of rabbit and human eIF-4y (18), it is likely that the site determined for the rabbit protein is valid for human eIF-4y and corresponds to the sequence GPPRGGPG. From the amino acid sequence of eIF-4y and the cleavage site determined above, it can be calculated that cp, would be 102 kDa, consistent with the mobility of the ~100-kDa cp, fragment on SDS-PAGE. Furthermore, this indicates that the portion of the eIF-4y molecule that causes the intact 154-kDa protein to migrate abnormally is located somewhere in the NH2-terminal one-third.

Relationship of the eIF-4y Cleavage Site to Substrate Determinants for HRV2 2A Protease—Several studies have suggested that 2A protease cleaves eIF-4y in vivo by activation of an endogenous protease (13–15). However, Liebig et al. (16) have argued for a direct cleavage. An essential precondition for direct cleavage is the ability of 2A protease to recognize the cleavage site assigned on rabbit and human eIF-4y. Knowledge of the cleavage site now permitted comparison with previously determined substrate requirements (23, 30). For HRV2 2A protease, essential residues in substrate recognition have been determined by systematic replacement of amino acids in the viral polyprotein (30) or in synthetic peptides (23). In both cases, the occupancy of P2 and P1 was critical for cleavage; at P2, of 16 substituted peptides tested, only Ser, Asn, or Arg could replace the wild type Thr. The P1' residue was even more restrictive, with only the wild type Gly being accepted (23).3 In contrast, several amino acids could be tolerated at the P1 site (23, 30).

Table I shows that the 2A protease cleavage sites on rabbit and human eIF-4y satisfy these requirements, both containing Gly at P1', Ser or Thr at P2, and the well tolerated Arg residue at P1. Moreover, similarity is evident with the cleavage sites of both proteases at P7 (Arg) and P2' (Pro), positions that are also important in determining cleavage (23). Further similarity exists between both eIF-4y cleavage sites and the cleavage site of CVB4 polyprotein at P4 (Leu) and between the rabbit eIF-4y site and the HRV2 cleavage site at P6 (Pro; see Table I). Interestingly, although the P' regions of human and rabbit eIF-4y are identical from P1 to P1', there is little similarity with the viral cleavage sites. This is consistent with the fact that P residues play only a minor role in influencing cleavage efficiency on peptide substrates (23).

Knowledge of the cleavage site of eIF-4y shown in Table I resolves the difficulty raised by the poor cleavage of HRV2 2A protease on peptides derived from the cleavage sites of other rhino- and enterovirus serotypes (23, 30). The inference of these studies was that a common sequence on the cellular target of 2A proteases could not be defined. Given that most 2A cleavage sites have a negatively charged residue at P7 or P6; Thr, Asn, or Ser at P2; and Gly at P1', it is probable that most 2A proteases will cleave eIF-4y at the position determined here.

Furthermore, the unusual nature of the P' region (containing 5 Gly and 3 Pro residues in a space of nine residues) may induce a conformation suitable for 2A protease cleavage.

Our finding that the γ subunit of eIF-4 purified from rabbit reticulocytes could be cleaved in vitro by 2A protease provides additional support for the direct cleavage of eIF-4y in vivo. However, the possibility could be raised that a second protease activity is present as a contaminant in the eIF-4, or is an inherent activity of eIF-4 itself. In experiments to be published elsewhere,3 a 16-mer peptide matching the human eIF-4y cleavage sequence was synthesized and shown to be cleaved by both viral proteases. This argues against participation of an endogenous cellular protease, since the proteases were from bacterial sources and the peptide was chemically synthesized.

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