Human Rhinovirus 2 2A<sup>pro</sup> Recognition of Eukaryotic Initiation Factor 4GI

INVolvement of an Exosite

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The 2A proteinase (2A<sup>pro</sup>) of human rhinovirus 2 is a cysteine proteinase with a unique chymotrypsin-like fold. During viral replication, 2A<sup>pro</sup> performs self-processing by cleaving between its own N terminus and the C terminus of the preceding protein, VP1. Subsequently, 2A<sup>pro</sup> cleaves the two isoforms of the cellular protein, eukaryotic initiation factor (eIF) 4G. We have previously shown that HRV2 2A<sup>pro</sup> can directly bind to eIF4G isoforms. Here we demonstrate using deletion mutants of eIF4GI that HRV2 2A<sup>pro</sup> requires eIF4GI amino acids 600–674 for binding; however, the amino acids at the cleavage site, Arg<sup>681</sup> -<sup>6</sup>Gly, are not required. The HRV2 2A<sup>pro</sup> binding domain for eIF4GI was identified by site-directed mutagenesis. Specifically, mutations Leu<sup>17</sup> → Arg and Asp<sup>35</sup> → Glu severely impaired HRV2 2A<sup>pro</sup> binding and thus processing of eIF4G in rabbit reticulocyte lysates; self-processing, however, was not affected. Alanine scanning analysis further identified the loop containing residues Tyr<sup>32</sup>, Ser<sup>33</sup>, and Ser<sup>34</sup> as important for eIF4GI binding. Although Asp<sup>35</sup> is part of the catalytic triad, most of the eIF4GI binding domain lies in a unique exosite structure absent from other chymotrypsin-like enzymes and is distinct from the substrate binding cleft. The exosite represents a novel virulence determinant that may allow the development of specific inhibitors for HRV2 2A<sup>pro</sup>.

Many viruses encode proteins that tailor the host cell physiology to their own needs (1). Modification of the cellular machinery of protein synthesis is a favored strategy of RNA viruses, because this can directly increase the efficiency of the expression of their genetic information. Almost all picornaviruses, a family of positive strand RNA viruses, including FMDV,<sup>1</sup> enteroviruses (such as PV), and HRVs, specifically prevent recruitment of capped cellular mRNAs to the 40 S ribosomal subunit (2, 3). This is achieved by virally encoded proteinases, which cleave the two isoforms of the eukaryotic initiation factor (eIF) 4G, eIF4GI, and eIF4GII (4–6). eIF4G isoforms are scaffolding proteins that have binding sites for eIF4E, the protein recognizing the 5′ mRNA cap, and eIF3, a complex of about 10 proteins, which in turn binds to the 40 S ribosomal subunit (7). The virally induced cleavage separates these two binding domains, preventing association between the cellular mRNA and the 40 S ribosomal subunit. Initiation of translation from viral mRNA is unaffected, because it is not cap-dependent but initiates internally from an internal ribosomal entry site (8, 9).

In enteroviruses and HRVs, a cysteine proteinase termed 2A<sup>pro</sup> is responsible for the cleavage of eIF4G isoforms. Viruses possessing certain specific mutations in 2A<sup>pro</sup> have a small plaque phenotype, as exemplified by PV (10) or SVDV (11, 12). In addition, such SVDV mutations show an attenuated phenotype (11, 12). A similar attenuated phenotype is also observed in FMDV when the proteinase responsible for eIF4G cleavage, the Leader proteinase, is deleted (13). Thus, the eIF4G cleavage reaction is an important determination of virulence in many picornaviruses.

The mechanism of cleavage of eIF4G isoforms by the picornaviral proteinases has not been completely resolved. Evidence for direct cleavage by both the 2A<sup>pro</sup> and the L<sup>pro</sup> has been presented (14, 15). However, activities in the PV-infected cell, distinct from the 2A<sup>pro</sup>, have been described that are able to perform cleavage of eIF4G isoforms (3). A further unresolved observation is that the cleavage of eIF4GI and eIF4GII occurs concomitantly in HeLa cells infected either with HRV serotypes 2 or 16 (16, 17). In contrast, in cells infected with PV or HRV14, cleavage of eIF4GI temporally precedes that of eIF4GII (6, 18). Interestingly, HRV2 and HRV16 are members of the genetic A group of HRVs, whereas HRV14 belongs to the B group (19). Further experiments will be required to show whether this difference in cleavage of the eIF4G isoforms extends to other representatives of the two genetic groups.

Recently, we have shown that the Leader proteinase of FMDV, a cysteine proteinase with a papain-like fold, can bind directly to the isoforms of eIF4G (20); the region recognized on eIF4G1 was mapped to amino acids 640–669 (numbering according to Byrd et al. (21)) but did not include the amino acids at which cleavage occurs (Gly<sup>674</sup> -<sup>6</sup>Arg). The eIF4G binding domain of the L<sup>pro</sup> was shown to comprise the 18 amino acids of the C-terminal extension as well as the residue Cys<sup>133</sup>, which is adjacent to the CTE in the tertiary structure (22). A similar interaction between the 2A<sup>pro</sup> of HRV2 and eIF4G isoforms was also observed (20). However, the binding domain on the HRV2 2A<sup>pro</sup>, which is a cysteine proteinase with a chymotrypsin-like fold (23, 24), was not identified. Furthermore, although the site of interaction on eIF4GI was shown to lie N-terminal to the...
HRV2 2Apro cleavage site (Arg⁶⁶¹ Gly), it was not defined further (20).

Here, we use site-directed mutagenesis to identify certain residues between amino acids 17 and 35 of HRV2 2Apro as being responsible for the binding of eIF4GI. The site recognized on eIF4GI was defined as being within amino acids 600–674.

**EXPERIMENTAL PROCEDURES**

Reagents—pHRV2 VP1–2Apro contains the HRV2 nucleotides 2318–3586, encoding all of VP1 except the first two amino acids, followed by all of 2Apro and two stop codons (25). Mutations in 2Apro were generated by standard PCR mutagenesis and introduced as EcoRI/XhoI fragments into the plasmid pGEX5X (Amersham Biosciences) as required (20). Fragments of eIF4GI for in vitro translation were amplified from plasmid pSKH1 (26), which contains the entire eIF4GI cDNA, and cloned as EcoRI/HincII fragments into pBluescriptKS (Stratagene).

Rabbit polyclonal antiserum #7, raised against the N terminus (kindly provided by R. Rhoads) of eIF4GI, was diluted 1:8000. Secondary goat alkaline phosphatase-conjugated and horseradish peroxidase-conjugated antibodies were diluted 1:5000 (Sigma) and 1:10000 (Bio-Rad), respectively.

**Purification of GST Fusion Proteins**—Escherichia coli BL21(DE3)lysS (Novagen) were transformed with plasmids encoding the GST–2Apro fusion proteins or GST alone. For expression, overnight cultures were diluted 1:10 in 250 ml of medium, and the cells were incubated at 37 °C to an A⁶⁰₀ of 0.8 and induced for 3 h at 18 °C by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.3 mM (20). The fusion protein was purified on glutathione-agarose resin (Amersham Biosciences) using standard techniques.

**GST Pull-down Assays**—Glutathione-Sepharose beads coated with GST fusion proteins were incubated in binding buffer (50 mM Tris-Cl, pH 7.4, 10 mM EDTA, 150 mM NaCl) with either an aliquot (6 μl) of RRL (Promega) or with radiolabeled in vitro translated proteins for 2 h at 4 °C. After three washes with binding buffer, bound proteins were eluted by boiling in SDS-PAGE loading buffer, resolved by SDS-PAGE, and visualized by Western blotting (using enhanced chemiluminescence system of Pierce for detection) or fluorography (20).

**In Vitro Translation**—In vitro translations in RRLs to examine 2Apro self-processing and eIF4GI cleavage were performed using in vitro transcribed RNAs as described (25, 27). In vitro expression of radiolabeled proteins for GST pull-down assays was performed in RRLs (Quick Coupled Transcription/Translation system; Promega) in the presence of [³⁵S]methionine (20 μCi per reaction, Hartmann Analytic) (20). Labeled proteins were resolved by SDS-PAGE and gels were dried and exposed to x-ray films.

**RESULTS**

**Mutations in HRV2 2Apro Specifically Affecting eIF4GI Cleavage but Not Self-processing**—A literature search revealed three mutations in picornaviral 2Apro reported to delay or inhibit eIF4GI cleavage without affecting self-processing (Table I). The poliovirus mutants were identified in an analysis of PV 2Apro by site-directed mutagenesis, whereas the SV DV mutant was determined by the study of virulent and attenuated SV DV strains.

In PV 2Apro, Asp³⁸ is the third member of the catalytic triad orienting the catalytic histidine (23, 28). The phenotype of the PV 2Apro D38E mutant (normal self-processing but an inability to process eIF4GI; Table I) implied, however, that this residue had a role in recognizing eIF4GI in addition to its function in the catalytic triad. To investigate whether a similar phenotype could be observed in HRV2 2Apro, we examined self-processing and eIF4GI cleavage activities of a mutant protein bearing the equivalent mutation D35E. For this, we used our previously described assay for measuring HRV2 2Apro self-processing and eIF4GI cleavage in RRLs (25, 27). In this system, an in vitro transcribed mRNA encoding the HRV2 capsid protein VP1 and the subsequent 2Apro was used to program protein synthesis.

As can be seen in Fig. 1A, self-processing can be monitored by examining the status of the newly synthesized radiolabeled protein. The cleavage of eIF4GI, which is abundant in RRLs and migrates as a series of bands with a relative molecular mass of about 220 kDa, is investigated by immunoblotting aliquots of the same samples with an antiserum against the N terminus of eIF4GI (Fig. 1B). When an mRNA encoding the wild-type 2Apro enzyme is translated, 50% of self-processing is observed 20 min after initiation of protein synthesis (Fig. 1A, left panel). Similarly, 50% eIF4GI cleavage is also seen after 20 min (Fig. 1B, left panel). To investigate the behavior of the D35E HRV2 2Apro mutant protein, mRNA was transcribed from an appropriately mutated plasmid and used to program RRLs. As can be seen in Fig. 1A (middle panel), self-processing occurred at wild-type rates. However, processing of eIF4GI was severely delayed in this mutant; a significant degree of cleavage was first observed after 180 min of incubation (Fig. 1B, middle panel). Thus, the replacement of Asp³⁵ by glutamate in the HRV2 2Apro produces a similar phenotype as the equivalent residue in PV 2Apro. Using a bacterial expression system, Sommergruber et al. (29) also investigated the behavior of the HRV2 2AproD35E mutant but found only 16% self-processing activity. Because these experiments required induction at 42 °C for 2 h in bacteria, we believe that our assay system in the RRL gives a more authentic picture of the behavior of the D35E mutant.

Yu and Lloyd (28) identified a second mutation in PV 2Apro, Y89L (Table I), which severely affected eIF4GI cleavage without affecting self-processing. The introduction of the equivalent Y86L mutation into HRV2 2Apro did not essentially affect self-processing (Fig. 1A, right panel). The onset of eIF4GI cleavage was, however, delayed (Fig. 1B, right panel), but cleavage was essentially complete after 90 min. Thus, information derived from one picornaviral proteinase cannot always be directly applied to the corresponding proteinase in another picornavirus.

This notion was further strengthened when a residue in HRV2 2Apro was replaced with one identified as being important in SVDV 2Apro for cleavage of eIF4GI. In this enzyme, the presence of isoleucine at residue 20 is severely detrimental to eIF4GI cleavage (Table I). Replacement of the equivalent residue in HRV2 2Apro, Leu¹⁷, with isoleucine did not affect either self-processing or eIF4GI cleavage in any detectable way in HRV2 2Apro (Fig. 2, left panel). However, the presence of Arg at this position (the virulent wild-type residue in SVDV 2Apro) dramatically inhibited eIF4GI cleavage without affecting self-processing (Fig. 2, right panel). This result is completely the opposite of that observed in SVDV; here the 2Apro from the virulent strain had arginine at the equivalent position, whereas the avirulent strain, in which eIF4GI processing was hampered, possessed Ile (Table I) (11, 12). These results suggest that the recognition of eIF4GI by 2Apro may be different between HRV2 and SVDV.

**HRV2 2Apro Mutant Proteins D35E and L17R Fail to Bind eIF4GI**—The ability of the above mutants to perform self-processing at similar levels to the wild-type indicated that the catalytic property of the enzyme had not been reduced by the various mutations. We therefore investigated whether the mutants were defective in their ability to bind eIF4GI, because we have recently shown that a GST-HRV2 2AproC106A fusion protein is an effective eIF4GI recognition site.
protein can bind to the eIF4G isoforms (20). The mutation C106A inactivates the enzyme by replacing the active site cysteine by alanine, preventing any possible cleavage of eIF4GI during the experiment. We constructed GST-HRV2 2AproC106A fusion proteins bearing the respective mutations and expressed them in E. coli (Fig. 3A). The mutant proteins were bound to glutathione-coupled Sepharose and incubated with RRLs as a source of eIF4GI. After washing, proteins bound to the respective HRV2 2AproC106A fusion proteins were resolved by SDS-PAGE and detected by immunoblotting.

eIF4GI is retained by HRV2 2AproC106A and HRV2 2AproC106A-Y86L (Fig. 3B, lanes 2 and 4). In contrast, no binding was observed with the mutant proteins HRV2 2AproC106A-D35E and -L17R (Fig. 3B, lanes 3 and 5), suggesting that the reduction in their cleavage of eIF4GI was due to an impairment of binding.

The Activity of the HRV2 2AproL17R Mutant Protein Can Be Partially Rescued by a Second Site Mutation—To identify further residues involved in the HRV2 2Apro-eIF4GI interaction, we concentrated on residues interacting with Leu17, which were identified using the three-dimensional structure of HRV2 2Apro. Although the mutation D35E was detrimental to eIF4GI cleavage, we were concerned that substitution of its neighbors, with which it forms an extensive hydrogen-bond network (see Fig. 5 in Ref. 24), these residues were therefore not investigated further.

The structure of HRV2 2Apro is shown in Fig. 4. HRV2 2Apro has a chymotrypsin-related fold comprising a unique four-stranded β sheet as the N-terminal domain and a six-stranded β barrel as the C-terminal domain (24). The asymmetric unit contained two HRV2 2Apro molecules (designated A and B). Detailed analysis of the structure revealed that Leu17 (posi-
indicated in purple are shown. Amino acids investigated for their role in eIF4GI binding are proximity. Of the two, Val 30 appeared of greater importance be-

Table II; the equivalent residues in SVDV 2Apro are indicated). In contrast, two other residues, Asn21 and Met24, are positioned

The topology of SGPB (41) and are colored in dark blue except for the loop bearing residue Tyr86 at its

Tyr32 for further investigation because of their consistent prox-

amino acids were selected as they are conserved in 2Apro of HRV2 2Apro Recognition of eIF4GI

To test this hypothesis, we attempted to generate this salt bridge in HRV2 2Apro. We introduced the mutation V30E into HRV2 2AproL17R and measured the ability of this double mutant to cleave eIF4GI compared with HRV2 2AproL17R. As a further control, we also investigated the activity of the single mutant HRV2 2Apro V30E.

Fig. 5 (left panel) shows that self-processing by the mutant protein HRV2 2AproL17R-V30E occurred at wild-type rates; furthermore, the processing of eIF4GI by this double mutant was significantly more efficient than that of the original HRV2 2AproL17R mutant protein. With HRV2 2AproL17R-V30E, 50% cleavage of eIF4GI was observed after 90 min (Fig. 5B, left panel) compared with more than 180 min with the single L17R mutant protein (Fig. 2B, right panel) and 20 min with the wild-type HRV2 2Apro (Fig. 1B, left panel). This suggests that the interaction of Leu17 and Val30 is important in eIF4GI cleavage by HRV2 2Apro.

The positive effect of the presence of glutamate at the residue 30 in the HRV2 2Apro L17R supported the idea of an interaction between residues 17 and 30. This would imply that the single mutant HRV2 2AproV30E would also be handicapped in eIF4GI cleavage. However, examination of the mutant HRV2 2Apro V30E revealed only a minimal effect on self-processing and eIF4GI cleavage (Fig. 5, right panel). This result argues against the importance of interaction between Leu17 and Val30 and stresses, rather, that the occupancy at residue 17 is more critical.

To investigate whether the introduction of the mutation V30E had also restored the ability of the L17R mutant protein to bind to eIF4GI, we expressed the fusion protein GST-HRV2 2AproC106A-L17R-V30E. Fig. 5D (lane 3) shows that the binding of the HRV2 2AproC106A-L17R-V30E to eIF4GI is improved compared with that of the L17R single mutant (Fig. 3B, lane 5). Nevertheless, wild type binding was not restored.

We also investigated the role of a third residue interacting with Leu17, methionine 24 (Table II). The equivalent residue in SVDV 2Apro is Trp27 and may be in a position to cover and thus stabilize the putative salt-bridge between Arg20 and Glu33. Accordingly, the triple mutant HRV2 2AproC106A-L17R-M24W was constructed; however, it showed no increase in activity on eIF4GI when compared with the double mutant (data not shown).

Alanine Scanning Reveals the Involvement of Residues Tyr32, Ser33, and Ser34 in eIF4GI Recognition—To investigate the importance of other residues bordering on strand e2 (Fig. 4) in eIF4GI binding, we carried out alanine scanning analysis of amino acids lying close to Leu17. This method allows the removal of side-chain interactions without inducing extreme perturbations into the overall fold of the molecule. Two sets of three amino acids, Phe30-Asn31-Ser32 and Tyr32-Ser33-Ser34 were chosen for replacement with alanine. These groups of amino acids were selected as they are conserved in 2Apro of

iso leucine replaces Arg20. Interestingly, the equivalent residues at these positions in several coxsackieviruses (24) are also arginine and glutamate, strengthening the idea of an important ionic interaction.

The distances (Å) of residues neighbouring Leu17 in HRV2 2Apro are

| Residue | HRV2 2Apro | SVDV 2Apro |
|---------|------------|------------|
| Leu17(Cα) | A          | A          |
| Met24(S8) | 4, 11      | 8, 31      |
| Val20(Cβ) | 4, 06      | 3, 63      |
| Tyr22(Cζ1) | 4, 89     | 4, 93      |
| Asn21(Cβ) | 4, 03      | 10, 06     |
| Glu33(Cε) | 4, 11      | 8, 31      |
| Trp27      |            |            |
| Gly35      |            |            |
| Thr34      |            |            |

| Residue | Distance (Å) |
|---------|--------------|
| Met24(S8) | 4, 11      |
| Val20(Cβ) | 4, 06      |
| Tyr22(Cζ1) | 4, 89     |
| Asn21(Cβ) | 4, 03      |
| Glu33(Cε) | 4, 11      |
| Trp27      | 4, 06      |
| Gly35      | 4, 11      |
| Thr34      | 4, 06      |

Fig. 3. GST-2AproC106A-D35E and GST-2AproC106A-L17R do not bind eIF4GI. A, Coomassie Brilliant Blue staining of purified GST (lane 1) and modified GST-2AproC106A fusion proteins (lanes 2–5). B, 8 µl of RRL (input lane 0, corresponding to 2 µl of RRL) was incubated with glutathione-Sepharose beads coated with either GST (lane 1) or the indicated GST-2AproC106A fusion proteins (lanes 2–5); bound eIF4GI was detected by immunoblotting using an anti-eIF4GI antiserum.

Fig. 4. The structure of HRV2 2Apro. Molscript (39, 40) diagram in stereoview of the 2Apro molecule A (24). α-helices are colored in green. The β strands in the N-terminal region are labeled according to the topology of SGPB (41) and are colored in cyan. The C-terminal β strands are colored in dark blue, except for the loop bearing residue Tyr86 at its top, which is shown in gray. The side chain of Tyr86 is omitted for clarity. Members of the catalytic triad (His34, blue; Asp35, purple; Cys36, light yellow), the zinc ion (blue sphere), and the zinc binding site are shown. Amino acids investigated for their role in eIF4GI binding are indicated in purple (Met24, Val20, Tyr22, Leu17, and Asn31) as well as the loop containing amino acids 32–34. The Protein Data Bank identifier for HRV2 2Apro is 2HRV.

tioned in the N-terminal domain) interacts closely with Val30 and Tyr32 in both molecules in the crystal structure (Fig. 4 and Table II; the equivalent residues in SVDV 2Apro are indicated). In contrast, two other residues, Asn21 and Met24, are positioned about 4 Å from Leu17 in molecule A, but are much more distant in molecule B (Table II). It is worth noting that the loop containing residues 17–30 shows the greatest conformational difference between the two molecules (24). We selected Val30 and Tyr32 for further investigation because of their consistent proximity. Of the two, Val30 appeared of greater importance because it was closer and also faces Leu17 in both molecules. In addition, in the SVDV 2Apro of the virulent strains, the equivalent residues to Leu17 and Val30 are Arg20 and Glu33; thus, in this SVDV strain, Arg20 and Glu33 could form a salt bridge, providing that they are oriented in a similar fashion. This positive salt bridge would be lost in the avirulent strain when
other group A human rhinoviruses (24). Furthermore, Tyr32 was of interest because of its proximity to Leu17 (Fig. 4 and Table II). Following construction of the respective plasmids, RNAs were prepared in vitro and used to program RRLs. Self-processing of HRV2 2Apro was essentially unaffected by the substitution of alanine residues at either of the two positions (Fig. 6A). In contrast, an effect on eIF4GI cleavage was observed with both mutant proteins (Fig. 6B, left panel). The Tyr32→Ser33→Ser34 alanine-scanning mutant was, however, more seriously handicapped in eIF4GI cleavage, with 50% cleavage not occurring during the time frame observed (Fig. 6B, right panel).

Examination of the eIF4GI binding by the corresponding GST fusion proteins (Fig. 6D) once again revealed that the significant delay in eIF4GI cleavage correlated with the binding of eIF4GI. Thus, the Phe20→Asn21→Ser22 alanine-scanning mutant was still able to bind eIF4GI (Fig. 6D, lane 3), whereas almost no binding was observed with the Tyr32→Ser33→Ser34 (Fig. 6D, lane 4) alanine-scanning mutant. Thus, this experiment further defines the loop region Tyr32→Ser33→Ser34 as part of the HRV2 2Apro binding domain, while pointing to only a minor role for Phe20→Asn21→Ser22.

**Definition of the Amino Acids Recognized on eIF4GI by HRV2 2Apro**—We showed previously (20) that HRV2 2Apro binds to the N-terminal cleavage fragment of eIF4GI generated by the Lpro (residues 1–674). Furthermore, HRV2 2Apro also bound a fragment of eIF4GI comprising amino acids 220–674 but did not bind one comprising amino acids 220–669. The N-terminal boundary was, however, not defined. We therefore prepared suitable eIF4GI fragments (Fig. 7A), expressed the encoded proteins in RRLs using a coupled transcription/translation system, and investigated whether the GST-HRV2 2AproC106A fusion protein could recognize them in a binding assay. Bound proteins were resolved by SDS-PAGE, and the gels were subjected to fluorography.

As a control, Fig. 7B shows the binding of the GST-2AproC106A fusion protein to the in vitro translated eIF4GI fragment spanning amino acids 260–674. In addition, the eIF4GI fragment 600–739 was also recognized (Fig. 7C); the fragment was extended relative to the 260–674 fragment to
make it more easily detectable on PAGE. Extension of the deletion analysis was complicated by the presence of the binding site for eIF4E (eIF4GI amino acids 609–623 (30)). The presence of eIF4E greatly accelerates the cleavage of eIF4G isomers by HRV2 2Apro (31), suggesting that its presence may also be required for HRV2 2Apro binding. Thus, the fragment comprising eIF4GI amino acids 640–820 was only poorly bound, if at all (Fig. 7D). Although the interpretation of this last experiment is complicated by some binding to the GST protein alone (Fig. 7D, lane 2), it suggests that the presence of eIF4E bound to eIF4G significantly increases the binding by HRV2 2Apro and that the eIF4E binding site defines the N-terminal boundary of the HRV2 2Apro binding domain.

**DISCUSSION**

The interaction of human entero- and rhinoviral 2Apro with the eIF4G isoforms has, despite a great deal of research, remained controversial. Mutations that affect this reaction result in a reduction of virulence in these viruses; thus, a thorough understanding of the mechanism of this interaction is of fundamental importance.

The above experiments define the regions of the HRV2 2Apro and eIF4GI required for their interaction. In HRV2 2Apro, this domain involves some of the residues 17–35, which form a loop-strand-loop structure at the edge of the N-terminal domain (Fig. 4 (24)). Of these residues, Leu17, Asp35, and together Tyr32, Ser33, and Ser34 were shown to be crucial for binding eIF4G. The role of Asp35 is especially noteworthy, because this residue forms part of the active site. Because none of the mutations examined here, not even the D35E mutation, had a significant effect on self-processing, this indicates that the interaction with eIF4GI is much more sensitive to perturbation.

Comparison of the eIF4GI binding site on HRV2 2Apro with that on the FMDV Lpro, which we determined earlier (20), reveals no similarity in the binding sites. In the FMDV Lpro, the eIF4GI binding domain does not involve the residues from the catalytic triad. Instead, it is located more than 25 Å away from the active site and comprises part of the flexible CTE. Thus, the enzymes have evolved different structural domains to recognize the same cellular target.

Given the different structure of the binding domains, it is not surprising that the two enzymes bind to somewhat different fragments of eIF4G (600–674 for HRV2 2Apro and 640–669 for FMDV Lpro). Nevertheless, two similarities can be found. First, binding by both enzymes is enhanced by the presence of...
the eIF4E binding site on eIF4GI, with the HRV2 2Apro enzyme being more sensitive to the presence of eIF4E. It seems likely that the enzymes bind to the conformation generated when eIF4E binds to eIF4GI (32). Second, the C-terminal boundaries of the binding sites are separated by at least five amino acids, a similar number to the seven found between the cleavage sites of the two proteinases on eIF4GI.

Is the HRV2 2Apro binding domain for eIF4GI likely to be present in 2Apro encoded by other human rhino- or enteroviruses? Analysis of amino acid sequences shows that the equivalent residues to HRV2 2Apro 17–35 are highly conserved in A group human rhinoviruses. As mentioned in the results, the residues 20–22 and 32–34 were chosen for alanine scanning analysis partly for this reason (24). However, little conservation between residues 17–35 of the HRV2 2Apro and HRV14 2Apro (the only representative of the B group human rhinoviruses sequenced to date) and between the HRV2 2Apro and those of enteroviruses is evident. Furthermore, little identity between the 2Apro of enteroviruses themselves is found in this region. Indeed, it was not possible to replace residues Leu17 and Val30 of HRV2 2Apro with those (arginine and glutamate) found in SVDV and most coxsackieviruses (Fig. 5, HRV2 2AproL17R-V30E). This illustrates the complexity of this part of the picornaviral 2Apro and indicates that the binding abilities of other rhino- and enterovirus 2Apro will have to be determined experimentally. Possibly, differences in binding behavior may explain why the eIF4G isoforms are cleaved simultaneously in cells infected by HRV2 and HRV16 and at different times during infection by HRV14 and poliovirus (6, 16, 18).

Residues 19–29 of HRV2 2Apro forming the loop between the catalytic His18 and strand eI2 (Fig. 4), previously attracted attention during the structure determination (24). Indeed, this loop is found in two almost unrelated conformations in the two molecules in the asymmetric unit of the HRV2 2Apro crystal. A further indication of the singularity of the loop region 19–29 can be seen on comparison with the Streptomyces griseus B proteinase and the 3Cpro of PV (24, 33). These proteinases both possess a minimal chymotrypsin-like fold and are structurally the closest relatives of 2Apro. Despite their minimal fold, however, they both possess two β-strands (dI and eI1) between the catalytic histidine and β-strand eI2. Strands dI and eI1 are lacking in HRV2 2Apro. Furthermore, the comparisons show that the β-strand eI2 of HRV2 2Apro is substantially truncated compared with that in S. griseus B proteinase and PV 3Cpro. Thus, in these two enzymes, an equivalent structure to that adopted by residues 19–29 of HRV2 2Apro is not possible.

The ability of the HRV2 2Apro to recognize a substrate using a domain outside the active site is, however, not unique among chymotrypsin-like enzymes. At least two members of this family, thrombin and factor VII, can interact with substrates or inhibitors at sites away from the classic substrate binding site (34). Indeed, thrombin has two exosites, which mediate intermolecular interactions. Both are positively charged; exosite I recognizes fibrinogen, exosite II recognizes heparin (35, 36). Interestingly, small two-domain protein inhibitors of thrombin have been shown to occupy both the active site and exosite I (e.g. hirudin (37)) or the active site and exosite II (e.g. hemadin (38)). These examples suggest that potent specific inhibitors of
HRV2 2Apro might be designed that bind both to the active site and the eIF4GI binding exosite.

In summary, the N-terminal domain of HRV2 2Apro lacks 4 beta-strands found in almost all other proteinases bearing a chymotrypsin fold. Nevertheless, we show here that the N-terminal domain has evolved to perform a vital interaction in the function of the enzyme and to make a significant contribution to determining virulence.

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