Identification and Characterization of a Protein Destruction Signal in the Encephalomyocarditis Virus 3C Protease*

(Received for publication, October 22, 1998, and in revised form, January 14, 1999)

T. Glen Lawson‡, Donna L. Gronros, Paul E. Evans, Michelle C. Bastien, Katherine M. Michalewich, Justin K. Clark, Jay H. Edmonds, Karolina H. Graber, Jonathan A. Werner, Beth A. Lurve, and Julie M. Cate

From the Department of Chemistry, Bates College, Lewiston, Maine 04240

The amino acid sequence LLVRGRTLVV, which is probably located in a strand-turn-strand structure, has been identified as a protein destruction signal in the rapidly degraded encephalomyocarditis virus 3C protease. Mutations within this sequence reduced the susceptibility of the 3C protease toward ubiquitination and degradation in rabbit reticulocyte lysate. This signal is transferable, since poliovirus 3C protease, which is a poor ubiquitin-mediated proteolytic system substrate, was found to be ubiquitinated and degraded when the signal sequence was either generated at an internal location in the protein or fused to the N terminus. An evaluation of the behavior of 3C protease proteins containing mutations in the signal region indicates that considerable variability in the primary structure is tolerated, although the conservation of certain features appears to be required for signal function. Two E3 ubiquitin-protein ligases that recognize the encephalomyocarditis virus 3C protease as a substrate were also partially purified. One of these was identified as the previously described E3a, and this was shown to require the destruction signal sequence to catalyze efficiently the ubiquitination of the 3C protease. The other is a Ubc5-dependent E3 that appears to recognize a different, unidentified feature of the 3C protease.

Evidence accumulated to date has shown that proteins degraded by the ubiquitin (Ub)†-mediated proteolytic system (1, 2) contain structural features that serve as recognition signals for Ub system factors. These signals generally appear to be composed of short primary structure elements. A conserved nine-amino acid sequence, for example, in the A and B cyclins (3, 4), as well as in certain other proteins (5, 6), is required for these molecules to be recognized as substrates for Ub attachment and for degradation. Short regions rich in proline, glutamate, serine, and threonine, or PEST sequences, have been postulated to serve as protein degradation signals (7), and it now appears that some proteins containing these regions are substrates for the Ub-mediated proteolytic system (8–14). Proteins with certain N-terminal amino acids are subject to Ub-mediated proteolysis (15–18). Still other degradation signals have been demonstrated to exist, and in some cases their locations have been at least partially mapped (19–25). Given the small number of destruction signal elements whose locations have been precisely determined, it is difficult to conclude whether it is only the primary structures of these signals that are important for their recognition or whether defined, higher order structural features come into play. The destruction signal of the MATa2 repressor, for example, has been hypothesized to contain an amphipathic α-helix (24).

Very few of the protein destruction signals that have been mapped so far have been shown to be recognized by a particular Ub system factor or factors. It is likely that the recognition of substrate proteins occurs primarily through the binding of a Ub-protein ligase, or E3, to the destruction signal elements (2). Several E3 proteins or protein complexes have been identified to date. As characterized by function, these can be divided into two classes (26). Some, such as SCF complexes composed of SKP1, CDC53, and an F-box protein (27), appear to operate as docking proteins by binding to both the substrate to be ubiquitinated and an E2 Ub-conjugating protein, the latter of which transfers the Ub molecule to the target. Other E3 proteins actually catalyze the attachment of Ub to the target, with a ubiquitinated E2 serving as a Ub donor. These include the mammalian E3α and its yeast homologue Ubr1 (28–31) and the family of hept Ub-protein ligases (32). E3α and Ubr1 are known to ubiquitinate N-end rule substrates (29, 30), and Ubr1 has recently been found to participate in the ubiquitination of the yeast proteins Gα and Cup9 (18, 23), neither of which contains a destabilizing N-terminal amino acid. In the case of Gα, the Ubr1 recognition element has been mapped to a 60-amino acid region near the N terminus (25), and the recognition of Cup9 requires the C-terminal two-thirds of the protein (23). The only other definitively known connection between a protein destruction signal and a Ub-protein ligase is that between the cyclin destruction signal sequence and the cyclosome complex (33, 34).

We have recently identified the 3C proteases of two picornaviruses as substrates for Ub-mediated degradation (35, 36). The 3C proteases are cysteine proteases of approximately 21–24 kDa which exhibit a high degree of substrate specificity (37). These proteins are initially synthesized within large polyproteins encoded by the positive stranded RNA viral genomes. They are responsible for most of the proteolytic processing of the polyproteins, which leads to the generation of individual functional proteins, including free, mature 3C proteases (37, 38). The mature 3C protease of the encephalomyocarditis virus (EMCV) has been shown to be rapidly degraded in vivo and in vitro (39, 40). Indirect evidence indicates that the mature 3C protease of the hepatitis A virus (HAV) is short lived
in vivo (41), and it has been directly proven to be quickly degraded in rabbit reticulocyte lysate (36). The destruction of both of these proteins requires both the synthesis of Ub-3C protease conjugates and the action of the proteasome (35, 36). Neither of these 3C proteases possesses a destabilizing N-terminal amino acid or homology with any reported sequences suspected of being required for recognition by the Ub-mediated proteolytic system.

In order to understand how the short lived 3C proteases are selected for rapid destruction and to facilitate studies of what role this destruction might play in the viral infectious cycles, we have attempted to identify structural features of the EMCV 3C protease that are required for recognition by the Ub-mediated proteolytic system. Because we have recently demonstrated that at least one Ub-conjugating system component in rabbit reticulocyte lysate recognizes both the EMCV and the HAV 3C proteases (36), and because of the difficulties associated with detecting unstable 3C proteases in vivo, we have employed the reticulocyte lysate system in our search. Here we report the identification and characterization of a 10-amino acid destruction signal sequence in the EMCV 3C protease. An analogous sequence is demonstrated to exist in the HAV 3C protease as well. In addition, we have identified two E3 Ub-protein ligases, one being E3o, that recognize the EMCV 3C protease and consequently catalyze the conjugation of Ub to the protein.

**EXPERIMENTAL PROCEDURES**

**Construction of in vitro Transcription Plasmids Containing 3C Protease Wild Type, Mutated, and Fusion Coding Sequences—** pE3C (36) or pE3C CD+ (35) were employed in the construction of all in vitro transcription plasmids containing mutated EMCV 3C protease coding sequences. pETP3C (36) or pETP3C-142 (42) were used as the starting points for the construction of all plasmids containing mutated poliovirus 3C protease coding sequences. p55P-P3 (43) was used as the source of sequences to construct plasmids containing mutated poliovirus 3C protease coding sequences with one or two lysine to arginine codon changes, the appropriate mutagenic primers and PCR to synthesize a DNA insert in which the codons for the LVV sequence in the N-terminal extension were changed to codons for AAA.

For the construction of pHAV3C pc3a35L and pHAV3C pc3a35L, the appropriate mutagenic primers and flanking primers containing NcoI or EcoRI sites were employed to amplify sequences from pETP3C. The restriction endonuclease-treated inserts were ligated into modified pGEM-3Z.

**Cloning, Expression, and Purification of EMCV 3C Protease and Mutated Protease Proteins—** The preparation of Escherichia coli cells that express the wild type EMCV 3C protease has already been described (35). The mutated 3C coding sequence carried in pE3C pc3a35L was amplified by PCR using primers containing NcoI or BamHI. The restriction endonuclease-treated fragment was ligated into pET-3d. This construct was used to transform E. coli BL21 (pLysS) cells. Expression, refolding, and purification of the protein were carried out as described previously (35).

**Evaluation of the Susceptibility of the Mutated 3C Protease Proteins toward Conjugation with Ub and Degradation—** The in vitro transcription and translations in reticulocyte lysate was analyzed by 12 or 16% SDS-PAGE and autoradiography as described previously (40).

The degradation of the 35S-labeled proteins in the reticulocyte lysate system was monitored in reaction mixtures similar to that described above, except MeUb was replaced with 0.1 mM bovine Ub (Sigma). In some reactions 0.1 μM Ub aldehyde (40), kindly provided by K. Wilkinson, was used in place of Ub. When lactacystin (Boston Biomedical) was included, it was added to give a final concentration of 100 nM. These reaction mixtures were incubated at 30 °C, and 1.5-μl aliquots were removed at the indicated times and analyzed by 12% SDS-PAGE and autoradiography. In some cases the amount of radioactivity present in the 35S-labeled 3C protease proteins was measured by liquid scintillation counting (40).

**Evaluation of the Catalytic Activity of the Mutated EMCV 3C Protease Proteins—** The activity of the in vitro synthesized EMCV 3C protease proteins was tested using 35S-labeled EMCV LVP0 polyprotein as a substrate as described previously (40).

**Protein Purifications and Reactions with E1, E2, and E3 Preparations—** Partially purified rabbit Ube5 and purified rabbit E2–14K and E1 were kindly donated by C. Pickart. Human E3o, purified by affinity chromatography using an E2–14K column, was the generous gift of A. Haas.

Ube5H was purified from expressing E. coli cells kindly provided by P. Howley (see Ref. 47). The Ube5H purification procedure was adapted from procedures developed by L. Mastrandrea and C. Pickart.2 After incubation at 30 °C for 5 h at 0.8 μM, 0.2 mg/ml bovine serum albumin, and 10 μM E3o, the 35S-labeled 3C protease proteins were added to reaction mixtures containing Ube5H as described previously (40).

---

2 L. Mastrandrea and C. Pickart, personal communication.
EMCV 3C Protease Destruction Signal

Identification of a Signal Required for the Conjugation of Ub to the EMCV 3C Protease—In order to determine whether the recognition of the EMCV 3C protease by the Ub-conjugating system occurs because of a discrete, identifiable structural feature within the substrate protein, several fusion and deletion mutants were prepared and evaluated. Portions of the EMCV 3C protease were first fused with portions of the poliovirus 3C protease, which is known to be a poor substrate for the Ub-mediated proteolytic system (36). Although the poliovirus protein does not share extensive sequence homology with the EMCV 3C protease (49, 50), the two do contain the centrally placed common sequence FRD, located at positions 92–94 in the EMCV 3C protease and at positions 83–85 in the poliovirus 3C protease. This shared sequence was utilized to construct in vitro transcription plasmids carrying either the sequence encoding for the N-terminal half of the EMCV 3C protease fused with the C-terminal half of the poliovirus 3C protease or the sequence coding for the N-terminal half of the poliovirus 3C protease fused with the C-terminal half of the EMCV 3C protease (PNEC protein). The ability of proteins encoded by these plasmids (Fig. 1A) to become incorporated into conjugates in reticulocyte lysate in the presence of methylated Ub (MeUb) was evaluated. Although reticulocyte lysate contains endogenous Ub, which can become incorporated into polymeric Ub chains conjugated to substrate proteins, the inclusion of a high concentration of MeUb (45) in the reaction mixtures results in the synthesis of more easily detected and relatively stable mono-Ub conjugates, at least with full-length wild type 3C protease proteins (35, 36). The use of MeUb in this system facilitates the detection of even low levels of ubiquitination, and it allows comparisons to be easily made between the abilities of proteins to serve as substrates for the initial Ub conjugation event. As shown in Fig. 1B, only the ENPC protein serves as a substrate for Ub attachment, demonstrating that the N-terminal 94 amino acids of the EMCV 3C protease include at least some elements required for recognition by the Ub system. This was confirmed by testing the ability of the N- or the C-terminal regions of the EMCV 3C protease alone (Fig. 1A) to serve as substrates for ubiquitination. A polypeptide composed of amino acids 1–101 (E3C(N)) was found to be readily conjugated with at least four MeUb molecules, whereas a polypeptide composed of amino acids 102–205 (E3C(C)) was a poor substrate for conjugate formation (Fig. 1C). A further evaluation of the N-terminal portion of the EMCV 3C protease by deletion mutagenesis revealed that the elimination of the N-terminal 5, 15, or 26 amino acids from the wild type protein had little effect on ubiquitination (Fig. 1A). Elimination of the N-terminal 46 amino acids, however, completely abolished all Ub conjugate formation (Fig. 1D). This suggests that the Ub system recognizes a structural feature located within the region which includes amino acids 27–46.

Although EMCV 3C protease shares little sequence homology with HAV 3C protease (50, 51), the fact that the two proteins are recognized by a common Ub-conjugating system component in rabbit reticulocyte lysate (36) led us to compare amino acid positions 27–46 in the EMCV 3C protease with the similar region in the HAV 3C protease. Fig. 2 shows the sequence of this region of the EMCV 3C protease, aligned with the analogous regions in the HAV and poliovirus 3C proteases (52). Based upon amino acid R group similarity and the location of the conserved catalytic histidine, the LLV sequence at positions 34–36 in the EMCV 3C protease can be aligned with the LGV sequence at positions 35–37 in the HAV 3C protease, and the LVV sequence at positions 41–43 in the EMCV 3C protease can be matched with the LLV sequence at positions 39–41 in the HAV 3C protease. The poliovirus 3C protease contains an LLVG sequence (positions 39–41) in this region as well, but it can be matched with the LLV sequence at positions 35–37, instead of either LVV or LLV. The crystal structures of the HAV, poliovirus, and rhinovirus 3C proteases have been determined (53–55), and all three have very similar secondary and tertiary structural arrangements. Amino acids 32–41 of the HAV protein and amino acids 28–37 of the poliovirus protein are contained within similarly folded structures in which the central four, primarily hydrophilic, amino acids comprise a surface-exposed loop that connects two antiparallel β-sheet strands. There is no reason not to expect that the aligned EMCV 3C protease sequence also exists within a strand-turn-strand structure. Based upon these comparisons, we hypothesized that the 10-amino acid sequence LLVGRGTLVV in the EMCV 3C protease might comprise a protein destruction signal that is recognized by a Ub-mediated proteolytic system component. We also predicted that, if this is the case, then the LGYKDDWLLV segment in the HAV 3C protease could serve this function as well.

In order to test whether the LLVGRGTLVV sequence is involved in the recognition of the EMCV 3C protease for ubiquitination and degradation, the amino acids comprising the LLV and LLYV triplets, at positions 34–36 and 41–43, respectively, were substituted with alanine residues. Whereas the
were degraded (data not shown). This indicates that the effects of the mutations are due to an inhibition of the conjugation of Ub to the 3C protease and not to an increased rate of Ub removal from Ub-3C protease conjugates. It appears, then, that the LLV and LVV triplets in the proposed signal sequence are indeed involved in the attachment of Ub to the EMCV 3C protease and are required for the rapid destruction of the protein.

Further evidence that the LLVRGRTLVV region in the EMCV 3C protease functions as a destruction signal was obtained by mutating the poliovirus 3C protease sequence shown in Fig. 2. Alterations were made in this section of the sequence to increase its homology with the EMCV 3C protease sequence. A mutated poliovirus protein, designated 3C-DS (for destruction signal), containing the sequence LGVHDNVAILP in place of LGVHDNVAILP was prepared. This new sequence is identical to the putative EMCV 3C protease destruction signal, except that Gly-29 and Asp-32 were left unchanged. These residues also occur in the HAV 3C protease sequence (Fig. 2), so we predicted they would allow the signal sequence to function. The poliovirus 3C-DS protein was found to be subject to conjugation with MeUb (Fig. 4A, lanes 1–6). Analysis of the in vitro translation reactions in which the 3C-DS protein was synthesized revealed the presence of poly-Ub-conjugated protein products (Fig. 4A, lanes 7 and 8), which were absent in the non-mutated poliovirus 3C protease synthesis reactions. In addition, although the wild type poliovirus 3C protease was quite stable in the reticulocyte system, the mutated protein was degraded (Fig. 4B). The rate of this degradation was reduced by lacta-
cystin (Fig. 4C; ref. 56), implicating the involvement of the proteasome.

An important question is whether the proposed EMCV 3C protease destruction signal sequence can also function if it is located somewhere else in a substrate protein. In order to answer this, a DNA sequence coding for the EMCV 3C protease destruction signal was fused to the wild type poliovirus 3C protease coding sequence. This allowed for the synthesis of the poliovirus 3C protease with the sequence MGLLVRGRTLVV fused to the N-terminal proline residue of the poliovirus protein (designated the 3C-N-terminal destruction signal or 3C-NDS protein). The presence of the new N-terminal MG results from the construction of the transcription plasmid. The analysis of the in vitro translation product of the RNA coded for by this plasmid revealed the presence of high molecular weight labeled conjugate (Fig. 4D, lanes 1 and 2). The incubation of the poliovirus 3C-NDS protein in the presence of MeUb resulted in the synthesis of a detectable mono-Ub-3C-NDS conjugate (Fig. 4D, lanes 3–5). The 3C-NDS protein was also degraded at a rate similar to that of the poliovirus 3C-DS protein shown in Fig. 4B, lanes 5–8. These results indicate that the fusion of the signal sequence to the N terminus of the poliovirus 3C protease leads to the recognition of the protein as a substrate for ubiquitination. In order to ascertain whether the effects of the N-terminally located destruction sequence are specifically due to the signal itself, and not simply the presence of the extra N-terminal amino acids, the signal sequence was mutated to MGLLVRGRTAA (poliovirus 3C-NDSI). This is analogous to the EMCV 3C protease mutant with the LVV sequence at positions 41–43 replaced with AAA (Fig. 3). The poliovirus 3C-NDSI protein was incorporated into conjugates with MeUb less readily than was the 3C-NDS protein (Fig. 4D, lanes 6–8), indicating that the transfer of susceptibility toward conjugation with Ub results, at least primarily, from the attachment of the destruction signal sequence.

### Table 1

**Effects of single amino acid changes in the destruction signal sequence on the conjugation of Ub to the EMCV 3C protease.**

| EMC virus 3C protease mutant | Destruction signal sequence | Conjugation with Ub inhibited? |
|-----------------------------|----------------------------|-------------------------------|
| 3C                          | LLVRGRTLTVV                | No                            |
| 3C(L34A)                    | ALVRGRTLTVV                | Strongly                      |
| 3C(L34I)                    | MVVRGRTLTVV                | Strongly                      |
| 3C(L34V)                    | VLYRGRTLTVV                | Strongly                      |
| 3C(L35A)                    | LAVRGRTLTVV                | Partially                     |
| 3C(L35N)                    | LNVRGRTLTVV                | Partially                     |
| 3C(V36A)                    | LLARGRTLTVV                | No                            |
| 3C(R37A)                    | LLVAGRTLTVV                | Partially                     |
| 3C(R39A)                    | LLVRCATLTVV                | Partially                     |
| 3C(R39K)                    | LLVRKGTLLVV                | Partially                     |
| 3C(R39D)                    | LLVRRGDTLVV                | Strongly                      |
| 3C(T40A)                    | LLVGRALVV                  | Partially                     |
| 3C(L41A)                    | LLVGRFAVV                  | Partially                     |
| 3C(V42A)                    | LLVGRFTLAV                 | No                            |
| 3C(V42N)                    | LLVGRGTLLNV                | Partially                     |
| 3C(V43A)                    | LLVGRKTLLY                 | No                            |
| 3C(ΔG38)                    | LLVRLTVL                   | Strongly                      |
| 3C(+A38)                    | LLVRCATLTVV                | Strongly                      |

*Indicates the location of mono-Ub conjugates.

**FIG. 3.** Evaluation of the ability of the EMCV 3C(LLV(34–36)AAA) and 3C(LLV(41–43)AAA) proteins to serve as substrates for the reticulocyte Ub-mediated proteolytic system. A, autoradiograph of the SDS-PAGE analysis of aliquots of reaction mixtures in which in vitro synthesized non-mutated and mutated EMCV 3C protease proteins were incubated in the presence of MeUb. Lanes 1–3, non-mutated EMCV 3C protease; lanes 4–6, EMCV 3C(LLV(34–36)AAA) protease; lanes 7–9, EMCV 3C(LLV(41–43)AAA) protease. Aliquots were removed from the reaction mixtures at 0 min (lanes 1, 4, and 7), 15 min (lanes 2, 5, and 8), and 60 min (lanes 3, 6, and 9). * indicates the location of mono-Ub conjugates. B, autoradiograph of the SDS-PAGE analysis of aliquots of reaction mixtures in which in vitro synthesized non-mutated and mutated EMCV 3C protease proteins were incubated in the presence of added Ub. Lanes 1–4, non-mutated EMCV 3C protease; lanes 5–8, EMCV 3C(LLV(34–36)AAA) protease; lanes 9–12, EMCV 3C(LLV(41–43)AAA) protease. Aliquots were removed from the reaction mixtures at 0 min (lanes 1, 5, and 9), 30 min (lanes 2, 6, and 10), 90 min (lanes 3, 7, and 11), and 180 min (lanes 4, 8, and 12).

### Additional Characterization of the Destruction Signal and the Location of Ub Attachment Sites

An effort was made to systematically evaluate which features in the EMCV 3C protease destruction signal are important for recognition by the Ub system. Several mutations, the majority being single alanine substitutions, were generated in the destruction signal sequence. The mutated proteins that were synthesized, and the relative susceptibility of these proteins toward conjugation with Ub in the reticulocyte lysate system, are summarized in Table 1. The effects of the mutations were determined by comparing the fractions of the proteins that were incorporated into mono-Ub conjugates during a 15-min incubation in the presence of MeUb, with the extent of ubiquitination observed with the non-mutated 3C protease. None of the mutated proteins demonstrated a greater susceptibility toward recognition by the Ub system than the non-mutated EMCV 3C protease. The relative rates at which these proteins were degraded correlated with their relative susceptibilities toward conjugation with MeUb. The results indicate few of the single amino acid changes strongly reduced the ability of the destruction signal sequence to function. One exception to this is the first amino acid in the sequence, Leu-34, which is conserved in the analogous positions in the HAV and poliovirus 3C proteases. The substitution of an alanine or methionine residue for this leucine, which would not be expected to induce drastic structural changes, strongly reduced the efficiency of Ub conjugation, as did the substitution of a valine. Whereas the replacement of the arginine at position 39 with an alanine or lysine residue negatively affected the efficiency of ubiquitination of the EMCV 3C protease, the presence of an aspartate in this position resulted in an even more poorly ubiquitinated substrate. This suggests that signal functionality is dependent upon more than simply the presence of polar amino acids in this region. In addition, the simultaneous replacement of both arginines in the central region of the signal with alanine residues virtually eliminated the susceptibility of the 3C protease toward conjugation with ubiquitin. Besides causing an obvious reduction in the hydrophilic character of this part of the signal sequence, these mutations may result in significant higher order structural alterations. The strongly inhibitory effects of eliminating a residue from (ΔGly-38), or adding a residue to (+Ala-38), the sequence indicates that a number of residues in the sequence, or at least in the central region, may also be important. All of the mutated proteins listed in Table 1, as well as the triple alanine substitutions described above (Fig. 3),
were tested for catalytic activity. Except for the 3C[LLV(34–36)AAA] protein, all retained the ability to catalyze the cleavage of the leader (L) protein from the EMCV polyprotein capsid precursor LVP0 (40). For these mutated proteins, at least, the retention of catalytic activity is indicative of minimal alterations in the overall higher order structures.

The severe attenuation of the conjugation of Ub to the EMCV 3C[LLV(34–36)AAA]- and 3C[LLV(41–43)AAA] proteins (Fig. 3A) and the absence of leucines or valines in positions 35 and 36 of the wild type poliovirus 3C protease (Fig. 2) raise the possibility that a high density of leucine and valine residues in the three positions on both ends of the destruction signal sequence are necessary for the signal to function. Support for this hypothesis was obtained by testing the ability of poliovirus 3C protease proteins containing A35L or A35L plus I35L substitutions to serve as substrates for the Ub-mediated proteolytic system. Both the single and double leucine substitutions allowed the protein to become ubiquitinated and to be degraded at rates comparable to that observed for the poliovirus 3C-DS protein, shown in Fig. 4, A and B (data not shown).

Given that the EMCV 3C protease undergoes conjugation with Ub primarily at a single lysine (35), an attempt was made to determine if a specific lysine in the EMCV 3C protease, perhaps because of its location relative to that of the destruction signal, is exclusively utilized as the site for the initial attachment of Ub. For this purpose, the ability of poliovirus 3C protease conjugates in the reticulocyte lysate system was only slightly less than that observed for the non-mutated protein (data not shown). Additional mutagenesis was performed to prepare in vitro translation reaction vectors that code for the 3C protease with lysine to arginine substitutions at positions 10, 14, 68, 74, and 78 (3C[K1–5R] protease), positions 151, 156, 171, and 172 (3C[K6–12R] protease), and at all 12 lysine positions (3C[K1–12R] protease). The results of incubating these proteins in reticulocyte lysate in the presence of MeUb are shown in Fig. 5A. Only the 3C[K1–12R] protease protein was found to be completely resistant to ubiquitination, although the other mutated proteins were less susceptible to conjugation with MeUb than was the non-mutated 3C protease. A comparison of the stabilities of these proteins is shown in Fig. 5B. Whereas the 3C[K1–5R], 3C[K8–12R], and 3C[K6–12R] proteins were degraded less rapidly than was the non-mutated EMCV 3C protease, presumably a reflection of their reduced susceptibility toward ubiquitination, the 3C[K1–12R] protease was found to be degraded quite slowly. This no lysine version of the 3C protease was degraded at a rate about one-fourth that measured for the non-mutated protein, and this degradation was not inhibited by lactacystin (Fig. 5C). The ubiquitin and proteasome-independent destruction of the EMCV 3C protease is likely due to one or more other proteases present in the reticulocyte lysate. We have previously observed, for example, that other EMCV proteins, including the LVP0 polyprotein and the 3D polymerase are degraded in the reticulocyte system, although at rates that are considerably slower than that of the mature 3C protease (40). This turnover does not appear to require the ubiquitination of these proteins (35). All of the lysine to arginine mutants retained levels of catalytic activity comparable to that of the non-mutated protein, and this degradation was not inhibited by lactacystin (Fig. 5C).

The ubiquitin and proteasome-independent destruction of the EMCV 3C protease is likely due to one or more other proteases present in the reticulocyte lysate. We have previously observed, for example, that other EMCV proteins, including the LVP0 polyprotein and the 3D polymerase are degraded in the reticulocyte system, although at rates that are considerably slower than that of the mature 3C protease (40). This turnover does not appear to require the ubiquitination of these proteins (35). All of the lysine to arginine mutants retained levels of catalytic activity comparable to that of the non-mutated protein, and this degradation was not inhibited by lactacystin (Fig. 5C).

The ubiquitin and proteasome-independent destruction of the EMCV 3C protease is likely due to one or more other proteases present in the reticulocyte lysate. We have previously observed, for example, that other EMCV proteins, including the LVP0 polyprotein and the 3D polymerase are degraded in the reticulocyte system, although at rates that are considerably slower than that of the mature 3C protease (40). This turnover does not appear to require the ubiquitination of these proteins (35). All of the lysine to arginine mutants retained levels of catalytic activity comparable to that of the non-mutated protein, and this degradation was not inhibited by lactacystin (Fig. 5C).

Confirmation That the HAV 3C Protease Also Contains a
EMCV 3C Protease Destruction Signal

FIG. 5. Evaluation of the ability of lysine to arginine mutants of the EMCV 3C protease to serve as substrates for the reticulocyte Ub-mediated proteolytic system. A, autoradiograph of the SDS-PAGE analysis of aliquots of reaction mixtures in which in vitro synthesized EMCV 3C protease proteins were incubated in the presence of MeUb. Lane 1, non-mutated EMCV 3C protease; lane 2, non-mutated EMCV 3C protease, 0 min; lane 3, EMCV 3C[K1–5R] protease, 0 min; lane 4, EMCV 3C[K1–5R] protease, 60 min; lane 5, EMCV 3C[K8–12R] protease, 0 min; lane 6, EMCV 3C[K8–12R] protease, 60 min; lane 7, EMCV 3C[K6–12R] protease, 0 min; lane 8, EMCV 3C[K6–12R] protease, 60 min; lane 9, EMCV 3C[K1–12R] protease, 0 min; and lane 10, EMCV 3C[K1–12R] protease, 60 min. * indicates the location of mono-Ub conjugates. B, autoradiograph of the SDS-PAGE analysis of aliquots of reaction mixtures in which in vitro synthesized non-mutated and mutated HAV 3C protease proteins were incubated in the presence of MeUb. Lanes 1–4, non-mutated HAV 3C protease; lanes 4–6, HAV 3C[LGV(32–34)AGA] protease; lanes 7–9, HAV 3C[LGV(39–41)AAA] protease. Aliquots were removed from the reaction mixtures at 0 min (lanes 1, 4, and 7), 15 min (lanes 2, 5, and 8), and 60 min (lanes 3, 6, and 9). * indicates the location of mono-Ub conjugates. C, autoradiograph of the SDS-PAGE analysis of aliquots of reaction mixtures in which in vitro synthesized non-mutated and mutated HAV 3C protease proteins were incubated in the presence of added Ub. Lanes 1–4, non-mutated HAV 3C protease; lanes 5–8, HAV 3C[LGV(32–34)AGA] protease; lanes 9–12, HAV 3C[LGV(39–41)AAA] protease. Aliquots were removed from the reaction mixtures at 0 min (lanes 1, 5, and 9), 30 min (lanes 2, 6, and 10), 90 min (lanes 3, 7, and 11), and 180 min (lanes 4, 8, and 12).

Destruction Signal—As discussed above, we predicted that the HAV 3C protease contains a destruction signal sequence, presumably the 10-amino acid sequence that is aligned with the EMCV 3C protease signal sequence in Fig. 2, i.e. LGVKDDWLLV. This prediction was tested by evaluating the ability of HAV 3C protease with mutations in the LGV and LLV triplets to serve as substrates for conjugation with Ub. The results of incubating the in vitro synthesized proteins containing either L32A plus V34A or L39A, L40A, and V41A mutations in reticulocyte lysate in the presence of MeUb is shown in Fig. 6A. The ability of both mutants to become incorporated into mono-Ub conjugates was sharply attenuated. The rates at which both mutated proteins are degraded was also reduced (Fig. 6B). These results confirm that the HAV 3C protease LGVKDDWLLV sequence comprises at least part of a destruction signal sequence.

Partial Purification and Identification of an E3 Ub-Protein Ligase That Recognizes the EMCV 3C Protease Destruction Signal—By having identified a sequence in the EMCV 3C protease signal sequence that is recognized by the E3 Ub-protein ligase, we were able to identify partial purification of this enzyme from HeLa cells. A summary of this work is shown in Fig. 7A. The purification was carried out on immobilized RNase A-Sepharose and the enzyme was assayed on polyvinylpolypyrrolidone-Sepharose and agarose (Fig. 7A). The enzyme activity was shown to be associated with the polypeptide fraction of the protein (Fig. 7B). The enzyme was stably expressed in Escherichia coli and this material was used as a source of the enzyme for further studies. Utilization of the enzyme in the analysis of ubiquitinated proteins is shown in Fig. 7C. The enzyme was able to detect ubiquitinated proteins as well as those containing the 3C protease destruction signal sequence.
protease required for the attachment of Ub to occur, we attempted to at least partially purify the E3 Ub-protein ligase that interacts with this signal. Reconstitution experiments with rabbit reticulocyte lysate fraction II (48) and a crude rabbit Ubc5 E2 preparation revealed that Ubc5 can support the ubiquitination of the EMCV 3C protease, presumably by functioning in a pathway with the E1-activating enzyme and an E3 present in fraction II. A screening of (NH4)2SO4 precipitate fractions revealed that this E3 activity resides in the 0–30% (NH4)2SO4 fraction. This material was used as a starting point for a small scale purification of the E3 activity. The progress of the purification was monitored using a reconstitution mixture composed of purified wild type EMCV 3C protease, Ub, purified rabbit E1, human UbcH5 purified from expressing E. coli cells (47), and E3 ligase-containing fractions.

The details of the purification scheme are described under “Experimental Procedures.” The 0–30% (NH4)2SO4 precipitate material was further fractionated by anion exchange chromatography on a column of Q-Sepharose, size exclusion chromatography on a column of Sephadex S-200, and hydrophobic interaction chromatography on a column of methyl-HIC resin. The composition of the most active fraction recovered from the methyl-HIC resin is shown in Fig. 7A. Six major proteins are visible, along with several minor ones. A demonstration of the catalytic activity of this E3 fraction is shown in the Western blot, probed with anti-3C protease antibodies, in Fig. 7B, lanes 1–4. The formation of mono- and poly-Ub conjugates of the EMCV 3C protease is apparent, and the synthesis of these products requires the E3 preparation.

Several other E2 enzymes were tested for their ability to function with the E3 preparation, in place of UbcH5. As Fig. 7B, lane 6, shows, E2–14K from rabbit reticulocytes supported the formation of Ub-EMCV 3C protease conjugates in the presence of the E3 preparation. These results were something of a surprise, since E2–14K appears to work exclusively with E3α (57). The E3 preparation contains a protein that migrates in SDS-PAGE gels with a mass consistent with that of a subunit of E3α, which was about 180 kDa (Ref. 28; Fig. 7A). These observations support the notion that the preparation contains E3α, which is capable of recognizing the EMCV 3C protease as a substrate for ubiquitination. In order to confirm this, affinity purified human E3α was tested in the reconstituted system.

This E3, in combination with E2–14K, was indeed found to catalyze the synthesis of Ub-EMCV 3C protease conjugates (Fig. 7C).

These results demonstrate that the Ub-protein ligase E3α recognizes the EMCV 3C protease as a substrate for ubiquitination. A second, Ubc5-dependent E3 protein also appears to be capable of participating in the synthesis of Ub-3C protease conjugates. The two E3 activities apparently co-precipitated in the same (NH4)2SO4 fraction and at least partially co-eluted from each of the chromatography matrices employed in the purification procedure. In order to determine directly whether the recognition of the EMCV 3C protease by one, or both, of these E3 proteins requires the unidentified destruction signal, experiments were carried out using a purified 3C protease with a mutated destruction signal sequence. The EMCV 3C[LVV(41–43)AAA] protease, MeUb, E1, E2–14K, and E3α. Lanes 5–8 show the analysis of reaction mixtures containing 1 μM 3C protease, MeUb, E1, E2–14K, and E3α. Lanes 5–8 show the analysis of reaction mixtures containing 1 μM 3C[LV(41–43)AAA] protease, MeUb, E1, E2–14K, and E3α. Aliquots were removed at 0 min (lanes 1 and 5), 10 min (lanes 2 and 6), 20 min (lanes 3 and 7), and 60 min (lanes 4 and 8). B, demonstration of the activity of the partially purified Ubc5-dependent E3 in catalyzing the synthesis of Ub-3C protease conjugates. Lanes 1–4 show the analysis of reaction mixtures containing 1 μM 3C protease, MeUb, E1, E2–14K, and E3a. Lanes 5–8 show the analysis of reaction mixtures containing 1 μM 3C[LV(41–43)AAA] protease, MeUb, E1, E2–14K, and E3α. Aliquots were removed at 0 min (lanes 1 and 5), 10 min (lanes 2 and 6), 20 min (lanes 3 and 7), and 60 min (lanes 4 and 8). The results shown were generated using Western blotting and development with ECL.

**DISCUSSION**

Although considerable understanding of how the Ub-mediated proteolytic system functions to selectively degrade proteins has been gained in recent years, the mechanism by which proteins are recognized as substrates for this system has...
largely remained undefined. We have identified a previously unknown protein destruction signal in the rapidly degraded EMCV 3C protease, a protein that is crucial for viral replication. The sequence LLVRGRTLVV, located at positions 34–43, has been demonstrated here to contribute, at least in vitro, to the recognition of the EMCV 3C protease for ubiquitination and to be necessary for the rapid destruction of the protein. Evidence was also obtained which suggests that the HAV 3C protease contains a destruction signal sequence (LGVKD-DWLLV) in a location homologous with that of the EMCV 3C protease.

An examination of the crystal structures of the picornavirus 3C proteases that have been determined to date reveal that they are conserved (53–55). The putative HAV 3C protease destruction signal sequence coincides with segments of two antiparallel β-sheet strands connected by a surface-exposed turn. The internal sequence changes made in the poliovirus 3C protease, which converted it to a substrate for the Ub system, are located in an identically placed structure. It is likely that the EMCV 3C protease signal sequence is placed in a similar location as well. This would mean that the central four, mostly hydrophilic, amino acids in the EMCV 3C protease signal exist in a surface-exposed loop which connects two strands of an antiparallel β-sheet. The leucine/valine-rich hydrophobic triplets would occupy the strand portions of the signal. The comparable strand sequences in the HAV and poliovirus 3C protease strand segments are not exposed on the surfaces of the proteins (53, 55), but it is possible to imagine them becoming accessible though a partial unfolding of the protein structure. It may be that the secondary structure of the signal region in the native protein contributes to its identity as a recognition element for the Ub-mediated proteolytic system. That the EMCV 3C protease signal sequence linked to the N terminus of the poliovirus 3C protease was observed to bring about the ubiquitination of the poliovirus protein may, however, indicate that the strand-turn-strand motif is not an absolute requirement for the signal to function. The folding of the signal sequence in this context may be different than that which occurs in the internal location in the native EMCV 3C protease.

An analysis of the effects of mutations in the EMCV 3C protease signal sequence indicates that the signal can tolerate considerable sequence variability and remain functional, although certain definable characteristics appear to contribute to its ability to function. The data, along with the comparisons between the EMCV, HAV, and poliovirus 3C protease sequences, suggest a leucine must be present in the first position of the signal sequence and that the three positions on either end of the signal sequence must contain a high density of amino acids with branched aliphatic R groups (leucines and valines). The absence of these residues in the C-terminal triplet of the poliovirus 3C protease sequence which aligns with the EMCV 3C and putative HAV 3C protease destruction signals was shown to be a major contributing factor to the stability of the poliovirus protein. These results are also consistent with observations made in studies with artificial substrates (58, 59), which indicate that short sequences of bulky hydrophobic amino acids can cause proteins to be recognized for Ub-mediated degradation. Given the strand-turn-strand structure in which the signal appears to reside, the four amino acids that occupy the center of the signal may also comprise an another important feature, since these residues would contribute to the formation of the hydrophilic loop. Although the data obtained shows that sequence in this region can be highly variable, any combination of polar amino acids does not support the generation of a functional destruction signal. Other experiments with EMCV 3C protease proteins containing lysine to arginine substitutions revealed that the location of the destruction signal does not restrict the initial attachment of Ub to a specific lysine residue. The absence of unique ubiquitination sites has been reported to exist for other substrates of the Ub-mediated proteolytic system (19, 60, 61).

Two E3 Ub-protein ligases were shown to be involved in the ubiquitination of the EMCV 3C protease. One of these was identified as E3α, and this was demonstrated by reconstitution experiments to require the identified destruction signal sequence to participate in the attachment of Ub to the 3C protease protein. Although the most straightforward interpretation of these results is that E3α binds to the EMCV 3C protease signal during initial substrate recognition, it is possible that a necessary interaction between E3α and the signal occurs subsequently to this step. Preliminary experiments have demonstrated that E3α can also participate in the ubiquitination of the HAV 3C protease, presumably by interacting with the destruction signal identified in that protein. E3α has been thought to recognize only proteins with certain N-terminal amino acids (29, 30). Its yeast homologue, Ubr1, has, however, recently been found to participate in the ubiquitination of yeast proteins Gs and Cup9, both of which have internally located destruction signals (18, 23, 25). A search of the reported signal regions for both of these proteins did not reveal the presence of a sequence that mimics the EMCV or HAV 3C protease destruction signal sequences. This may indicate that mammalian E3α and yeast Ubr1 recognize dissimilar internally located signal elements or that these E3 proteins are capable of interacting with more than one type of internal signal feature.

The second E3 shown to be capable of catalyzing the ubiquitination of the EMCV 3C protease functions with the E2 Ubc5. This may, therefore, be an E3 that belongs to the hect family of Ub-protein ligases (32). This E3 was found not to require the LLVRGRTLVV destruction signal sequence to recognize the 3C protease. It is unclear what structural features are recognized by the Ubc5-dependent E3. The fact that no ubiquitination was observed to occur in reticulocyte lysate with proteins containing only the C-terminal 113 amino acids or with N-terminal deletion mutants lacking the first 46 amino acids suggests the N-terminal region contains a recognition element necessary for this E3 to interact with the 3C protease. It appears that, in reticulocyte lysate, at least, the Ubc5-dependent E3 is responsible for a much smaller fraction of the Ub-3C protease conjugate synthesis than is E3α. This is indicated by the sharply attenuated rate of ubiquitination and degradation that was observed for some of the EMCV 3C protease proteins containing mutations in the E3α destruction signal sequence.

It is likely that the protein destruction signal identified here also exists in one or more cellular proteins, since the ability of the ubiquitin system to recognize this signal would be expected to have evolved to meet the needs of cells not infected with picornaviruses. Because of the apparent degeneracy of the signal sequence, and because the secondary structure of the signal region may contribute to the efficiency with which it functions, meaningful searches of protein data bases for other examples of the signal are difficult. A search of the Swiss-Prot database, however, revealed the existence of at least 17 eukaryotic cellular and 5 (in addition to the EMCV and HAV 3C proteases) mammalian viral proteins with candidate destruction signal sequences. These proteins all contain the sequence Leu-(Gly, Leu, or Val)-(Leu or Val)-X2-(Leu or Val)n, where X represents amino acids frequently found in reverse turns.

3 T. G. Lawson, D. L. Gronros, P. E. Evans, M. C. Bastien, K. M. Michalewicz, J. K. Clark, J. H. Edmonds, K. H. Graber, J. A. Werner, B. A. Lurvey, and J. M. Cate, unpublished data.
It remains to be seen if the identified destruction signal functions in vivo the same way it functions in vitro. Similarities between the kinetics of the EMCV 3C protease degradation in vivo and in vitro (35, 39, 40) suggest similar mechanisms of destruction occur in both systems. Detailed studies of the behavior of destruction signal mutants of the 3C protein in vivo will be required to answer this question. It is also not yet clear what function, if any, the rapid degradation of the EMCV and HAV 3C proteases serves during the replication of these viruses. In the case of EMCV, at least, the amount of 3C protease present reaches a maximum 2–3 h into the infectious cycle and then rapidly declines to near undetectable levels by the time the cells lyse (39). It may be that the presence of 3C protease activity during the later phases of the infectious cycle is detrimental to virus reproduction. It is also unclear why the 3C protease of poliovirus has evolved to be relatively stable. The ability to now generate catalytically active, relatively stable versions of the EMCV and HAV 3C proteases will allow future studies into whether the rapid and selective degradation of these proteins is biologically important.

Acknowledgments—We are deeply indebted to Cecile Pickart and Art Haas for valuable discussions, advice, and the provision of enzyme preparations.

REFERENCES

1. Varshavsky, A. (1997) Trends Biochem. Sci. 22, 383–387
2. Scheffner, M., Smith, S., and Jentsch, S. (1998) in Ubiquitin and the Biology of the Cell (Peters, J.-M., Harris, J. R., and Finley, D., eds) pp. 65–98, Plenum Publishing Corp., New York
3. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) Nature 349, 132–138
4. Kletzschüchter, A., Stewart, E., Harrison, D., and Hunt, T. (1996) EMBO J. 15, 3053–3064
5. Kaplun, T., and Jacquet, M. (1995) EMBO J. 14, 5023–5029
6. Funabiki, H., Yamano, H., Nagao, K., Tanaka, H., Yasuda, H., Hunt, T., and Tanaka, H. (1997) EMBO J. 16, 5977–5987
7. Rogers, S., Wells, R., and Rechsteiner, M. (1998) Science 234, 364–368
8. Konnitzer, D., Raboy, B., Kula, R. G., and Fink, G. R. (1994) EMBO J. 13, 6021–6030
9. Salama, S. R., Hendriks, K. B., and Thorner, J. (1994) Mol. Cell. Biol. 14, 7953–7966
10. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) Science 267, 1485–1488
11. Tsurumi, C., Ishida, N., Tamura, T., Kakizuka, A., Nishida, E., Okumura, E., Kishimori, T., Inagaki, M., Okazaki, R., Sagata, N., Ichihara, I., and Tanaka, K. (1995) Mol. Cell. Biol. 15, 5682–5687
12. Yaglom, J., Linskens, M. H., Sudis, S., Rubin, D. M., Furcht, B., and Finley, D. (1995) Mol. Cell. Biol. 15, 731–741
13. Krapapp, D., Wulczyn, F. G., and Scheideer, C. (1996) EMBO J. 15, 6716–6726
14. Roth, A. F., Sullivan, D. M., and Davis, N. G. (1998) J. Cell Biol. 142, 949–961
15. Bachmair, A., Finley, D., and Varshavsky, A. (1986) Science 234, 179–186
16. Johnson, S., Gonda, D. K., and Varshavsky, A. (1990) Nature 346, 287–291
17. Nishizawa, M., Furuno, N., Okazaki, K., Tanaka, H., Ogawa, Y., and Sagata, N. (1998) EMBO J. 17, 4021–4027
18. Madura, K., and Varshavsky, A. (1994) Science 265, 1454–1458
19. Treier, M., Staatzewski, L., and Bohm, D. (1994) Cell 78, 787–798
20. Hateboer, G., Kerkhoven, R. M., Stivarts, A., Bernards, R., and Boijersbergen, R. L. (1996) Genes Dev. 10, 2969–2979
21. Huijbregste, J. M., Scheffner, M., Beaudenon, S. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3656–3661
22. Nielsen, K. H., Papapageorgiou, A. V., Vass, W. C., Willumsen, B. M., and Lowy, D. (1997) Mol. Cell. Biol. 17, 7132–7138
23. Byrd, C., Turner, G. C., and Varshavsky, A. (1998) EMBO J. 17, 269–277
24. Johnson, P. R., Swanson, R., Rakhlina, L., and Hochstrasser, M. (1998) Cell 94, 217–227
25. Schauber, C., Hofmann, K., Goebel, M., Harper, J. W., and Elledge, S. J. (1996) Cell 86, 263–274
26. Hershko, A., Heller, H., Eytan, E., and Reiss, Y. (1986) J. Biol. Chem. 261, 11992–11999
27. Reiss, Y., Heller, H., and Hershko, A. (1989) J. Biol. Chem. 264, 10378–10383
28. Bartel, B., Winning, I., and Varshavsky, A. (1988) EMBO J. 7, 3179–3189
29. Reiss, Y., Reiss, V., Fried, V. A., Hershko, A., Yoon, J. K., Gonda, D. K., Sangan, P., Copeland, N. G., Jenkins, N. A., and Varshavsky, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7998–7993
30. Huijbregste, J. M., Scheffner, M., Beaudenon, S., and Howley, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2563–2567
31. King, R. W., Peters, J.-M., Tugeuondsch, S., Rolfe, M., Hieter, P., and Kirschner, M. W. (1995) Cell 81, 279–288
32. Sudakin, V., Ganz, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. (1995) Mol. Cell Biol. 6, 185–198
33. Lawson, T. G., Gronros, D. L., Werner, J. A., Aye, C. M., DiGeorge, A. M., Lockhart, J. L., Wilson, J. W., and Wintride, P. L. (1994) J. Biol. Chem. 269, 28429–28435
34. Gladding, R. L., Haas, A. L., Gronros, D. L., and Lawson, T. G. (1997) Biochem. Biophys. Res. Commun. 235, 119–125
35. Knaussich, H.-G., Ninkin, M. J. H., Lee, C.-K., and Wimmer, E. (1988) Biochemie (Pari) 70, 119–130
36. Palmenberg, A. C. (1990) Annu. Rev. Microbiol. 44, 603–623
37. Lawson, T. G., Smith, L. L., Palmenberg, A. C., and Thach, R. E. (1989) J. Virol. 63, 5013–5022
38. Oberst, M. D., Golland, T. J., Gupta, M., Peura, S. R., Zyldeleski, J. D., Sudarsanam, P., and Lawson, T. G. (1993) Virology 193, 28–40
39. Klotzbucher, A., Stewart, E., and Hunt, T. (1996) EMBO J. 15, 731–741
40. Oberst, M. D., Golland, T. J., Gupta, M., Peura, S. R., Zyldeleski, J. D., Sudarsanam, P., and Lawson, T. G. (1993) Virology 193, 28–40
41. Klotzbucher, A., Stewart, E., and Hunt, T. (1996) EMBO J. 15, 731–741