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Evaluation of the Susceptibility of the 3C Proteases of Hepatitis A Virus and Poliovirus to Degradation by the Ubiquitin-Mediated Proteolytic System

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The picornavirus 3C proteases are required for the processing of viral polyproteins during infections of host cells. Here we report that the 3C protease of the hepatitis A virus, like that of the encephalomyocarditis virus, is a substrate for rapid, ubiquitin-mediated degradation *in vitro*. Ubiquitin was shown to stimulate the turnover of the hepatitis virus 3C protease, and labeled protease was found to become incorporated into a mixture of high molecular weight species, which is characteristic of conjugation with polyubiquitin chains. In the presence of methylated ubiquitin, a new 33 kDa species formed, consistent with the generation of a monoubiquitin-3C protease conjugate. The rate of degradation of the 3C protease was reduced by inhibitors of the 26S proteasome. A similar evaluation of the 3C protease of poliovirus revealed that it is stable protein and is not conjugated with ubiquitin. It was also determined that the hepatitis A and encephalomyocarditis virus 3C proteases compete with each other for conjugation with ubiquitin and for degradation. This suggests that the two 3C proteases are both recognized by the same ubiquitin system enzyme, or enzymes, responsible for selecting them as targets for destruction.

Upon infection of a host cell by a member of the virus family *Picornaviridae*, the synthesis of a large polyprotein coded for by the positive stranded viral RNA genome is initiated. Although the specific events which lead to the generation of mature, functional viral proteins from these polyprotein precursors depends upon the virus, all members of the *Picornaviridae* rely upon a protease located in the P3 region of their polyprotein precursor to catalyze the majority of the processing reactions. These proteases, designated the 3C proteases, are 20 to 24 kDa cysteine proteases which appear to be related to the trypsin protease group (1). The 3C proteases act both inter and intra molecularly to catalyze specific cleavages in their polyprotein precursors, which also results in the release of the mature 3C proteases (2,3).

We have shown that the 3C protease produced by one picornavirus, the encephalomyocarditis virus (EMCV[2]), is rapidly degraded by the ubiquitin-mediated proteolytic system (4). Evidence gathered to date suggests that ubiquitin-mediated proteolysis is responsible for the degradation of a number of key cellular regulatory proteins (for reviews see 5-8). Proteins degraded by the ubiquitin system are selectively modified by conjugation, at the ϵ-amino group of one or more lysines, with polyubiquitin chains. This is accomplished by a reaction pathway that requires at least three enzymes, these being a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). Multiple E2 and E3 enzymes exist, and the selection of the target substrate appears to depend upon a specific E3 ligase, or an E3 in conjunction with a particular E2 (5-12). The ubiquitin-substrate protein conjugates are recognized and degraded by the 26S proteasome complex (5-8, 13-15). In the case of the EMCV 3C protease, the ubiquitin systems present in rabbit reticulocytes and in mouse mammary tumor cells have both been shown to be capable of catalyzing the synthesis of ubiquitin-3C protease conjugates and quickly degrading the 3C protease protein (4,16,17).

A number of important questions remain about the proteolytic degradation of the EMCV 3C protease, including what role this turnover might play in the replication of the virus and how the protein is recognized and acted upon by the ubiquitin enzyme system. Con-

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Abbreviations: ATP-γS, adenosine 5′-*O*-(3-thiotriphosphate); DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EMCV, encephalomyocarditis virus; HAV, hepatitis A virus; MG132, carbobenzoxyl-leucinyl-leucinyl-leucinal; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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sidering the relatively small number of natural substrates for ubiquitin-mediated degradation that have been discovered, the emerging evidence for the complexity of the system, and the potential importance of selective degradation of viral proteins in modulating infectious cycles, the value of finding answers to these questions is apparent. These explorations may be facilitated through the identification of other picornavirus 3C proteases that also serve as substrates for ubiquitin-mediated proteolysis. Here we report the results of experiments with two additional 3C proteases. The information we have obtained indicates that the hepatitis A virus (HAV) 3C protease is a substrate for conjugation with ubiquitin and rapid degradation by the proteasome, while the 3C protease of poliovirus is not. In addition, we have obtained evidence suggesting that the rabbit reticulocyte ubiquitin pathway enzyme, or enzymes, which specifically recognizes the EMCV 3C protease also participates in the conjugation of ubiquitin to the HAV 3C protease.

MATERIALS AND METHODS

Plasmid constructions. pHAV3C, which contains the sequence coding for the HAV 3C protease with an N-terminal methionine, was constructed as follows. The DNA coding for the HAV 3C protease was amplified by PCR from pE5P-P3 (18), which was the generous gift of Ellie Eherenfeld, using the primers ATGCAGATCCCATGGAATCTCAGTCAAC and CTGAGTAGGATCCACTTTCATAATTCACTGACTTTCA. Following treatment with Nco I and EcoR I, the sequence was ligated into the previously modified *in vitro* transcription vector pGEM-3Z (4). The *in vivo* expression vector pETHAV3C, was constructed by amplifying the HAV 3C coding-DNA from pE5P-P3 using the first primer from above, along with the primer CACTTTCAGAATTCACTGACTTTC. Following treatment with Nco I and BamH I, the sequence was ligated into pET3d (19). For the construction of pETP3C, which carries the sequence coding for the poliovirus 3C protease with an N-terminal methionine, the sequence coding for the poliovirus 3C protease was amplified by PCR from pT7-1 (20), which was the generous gift of Bert Semler, using the primers CAGCAAAGGCCATGGGACCAGGGTTCGATTACGCAG and TCCACTGGATCCCCTATTGACTCTGAGTGAAGTATGATC. Following treatment with Nco I and BamH I, the sequence was ligated into pET3d. The previously described pE3B′CD* (4) was modified to remove sequences coding for extraneous flanking amino acids, by amplifying the EMCV 3C protease coding region using the primers GTTGGACACCATGGGACCAAACCCTGTG and AATACGACTCACTATAGGGC. Following treatment with Nco I and EcoR I, the sequence was ligated into the modified pGEM-3Z. The resulting plasmid, pE3C, carries the sequence coding for the EMCV 3C protease with an N-terminal methionine. The 3C protease coding sequence in pE3C was mutated by PCR to change the codon for C_{159} to A_{159}, using the mutagenic primers GAAAGGGCTGGGCTGGATCAGCC and GGCTGATCCAGCCCAGCCCTTTC. This modified plasmid was designated pE3C_{A159}. Sequences inserted into vectors were confirmed by sequence analysis with an Applied Biosystems automated DNA sequencer.

In vitro and in vivo synthesis of 3C proteins. RNA transcripts were prepared from linearized *in vitro* transcription plasmids as previously described (4,16). *In vitro* translations in 58% (v/v) nuclease-treated reticulocyte lysate (Promega) in the presence of L-[^{35}S]-methionine were carried out to generate [^{35}S]-labeled 3C protease proteins (4,16). pET vectors carrying 3C protease coding sequences were transformed into *E. coli* BL21 (pLysS), and expression was induced as described (4). Poliovirus and EMCV 3C proteases and EMCV 3C_{A159} protease were refolded from inclusion bodies and purified using the published scheme (4). HAV 3C protease was purified from the soluble cell lysate fraction by filtration through Q-Sepharose (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.9, 1 mM DTT, and 0.1 mM EDTA, followed by passage of the filtrate through a column of Sephadex G-100 (Pharmacia) equilibrated with 50 mM Tris, pH 7.6, 20 mM $KC_2H_3O_2$, 1 mM DTT, 0.1 mM EDTA. The purity of the 3C protease preparations, as monitored by SDS-PAGE (21) was greater than 95%. Correct refolding of the purified poliovirus 3C protease was tested by evaluating catalytic activity using *in vitro* synthesized ^{35}S-labeled poliovirus P1 polyprotein generated by transcription from pT7-1 digested with BstE II and translation of the RNA in reticulocyte lysate (22). Correct refolding of the purified EMCV 3C proteases was tested by evaluating catalytic activity using *in vitro* synthesized ^{35}S-labeled EMCV LVP0 polyprotein as previously described (4,16).

Evaluation of the stabilities of the 3C proteases and susceptibility to conjugation with ubiquitin. RNA transcripts coding for the 3C protease proteins were translated, as described above, in nuclease-treated reticulocyte lysate containing an ATP-synthesis system, and the reactions were terminated by the addition of 0.1 mg/ml cycloheximide. For measurements of protein degradation rates, the mixtures were then incubated at 30°, and aliquots were removed at the indicated times and precipitated with acetone (4,16). In some cases, the reaction mixtures were supplemented with 0.1 mM added ubiquitin (Sigma). Evaluation of the effects of ATP-γ-S (Sigma) was carried out as previously described (16). For tests of the effects of MG132 (Peptide Inst. Inc.), reaction mixtures consisted of a final concentration of 50% (v/v) nuclease-treated reticulocyte lysate (Promega), and included 10% (v/v) of a terminated 3C protease *in vitro* translation reaction mixture, 20 mM HEPES-KOH, pH 7.8, 2 mM ATP, 2 mM $Mg(C_2H_3O_2)_2$, 1 mM DTT, and either 0.05% DMSO or 0.05% DMSO plus 50 μM MG132. The mixtures were incubated at 30° and aliquots were removed at the indicated times and precipitated with acetone. All precipitated aliquots were analyzed by 12% SDS-PAGE, autoradiography, and, in some cases, liquid scintillation counting (4,16). For detection of the formation of monoubiquitin-3C protease conjugates, terminated *in vitro* translation reaction mixtures were incubated at 30° in the presence of 0.1 mM reductively methylated ubiquitin (4,23). Aliquots were removed at the indicated times, precipitated with acetone, and analyzed by 12% SDS-PAGE and autoradiography.

Evaluation of the competitive effects of pure 3C proteases. Reaction mixtures contained a final concentration of 40 % (v/v) nuclease-treated reticulocyte lysate, and included 20% (v/v) of a terminated 3C protease *in vitro* translation reaction mixture, 20 mM HEPES-KOH, pH 7.8, 2 mM ATP, 2 mM $Mg(C_2H_3O_2)_2$, 1 mM DTT, the indicated concentration of added purified 3C protease preparation, and 0.25 mM either added ubiquitin or Me-ubiquitin. Aliquots were removed at the indicated times, precipitated with acetone, and analyzed by 12% SDS-PAGE and autoradiography.

RESULTS

Evaluation of the 3C proteases as substrates for ubiquitin-mediated degradation. The stabilities of the HAV and poliovirus 3C proteases in the rabbit reticulocyte lysate system were determined. The reticulocyte ubiquitin-mediated system is one of the most thoroughly characterized, and it has often been employed in studies of the ubiquitin-mediated turnover of specific proteins (5-8). *In vitro* synthesized ^{35}S-labeled 3C proteases were incubated in reticulocyte lysate in the presence or absence of added ubiquitin, and the relative

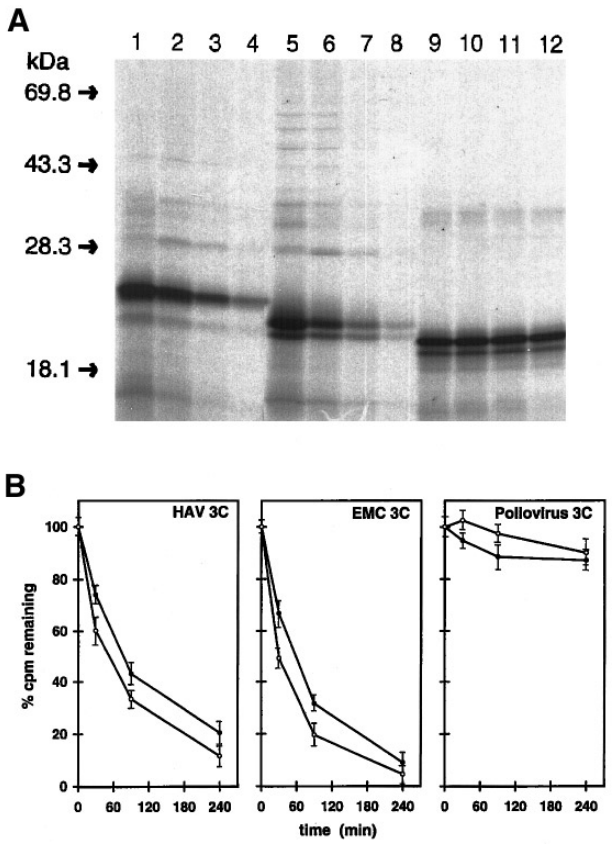

FIG. 1. **A.** SDS-PAGE analysis of reaction mixtures containing the ^{35}S-labeled 3C proteases incubated in reticulocyte lysate in the presence of added ubiquitin. HAV 3C protease samples are in lanes 1 - 4, EMCV 3C protease samples are in lanes 5 - 8, and poliovirus 3C protease samples are in lanes 9 - 12. 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 240 min (lanes 4, 8, and 12). **B.** Measurements of the amounts of the ^{35}S-labeled 3C proteases remaining as a function of time, in the absence (- ● -) and presence (- ○ -) of added ubiquitin. Bars represent the standard deviations for three measurements.

amounts of labeled protein present were measured as a function of time. As Fig. 1A and B show, the HAV 3C protease is quickly degraded, although the rate is less than that of the EMCV 3C protease, data for which is included to allow comparison. The presence of supplemental ubiquitin resulted in an increase in the initial rate at which the proteases were degraded, as well as in an increase in the extent to which degradation occurred. The poliovirus 3C protease, on the other hand, proved to be quite stable, with less than 15 percent disappearing over a four hour period. The presence of additional ubiquitin did not increase the rate at which this degradation occurred. An examination of lanes 1 through 8 of the autoradiogram in Fig. 1A, reveals the presence, at early times in the incubation, of labeled species with incrementally larger masses than either the HAV or EMCV 3C proteases. These species, which are more clearly visible in the EMCV protein samples, disappear with time and are characteristic of the formation of a heterogeneous mixture of polyubiquitin-substrate protein conjugates (4). These apparent conjugates are absent in the poliovirus samples (lanes 9 through 12). The secondary protein bands which migrate immediately below the major 3C protease bands in the gel lanes containing the EMCV and poliovirus protein samples most likely result from translation initiation at nearby downstream in frame start codons which exist within favorable Kozak consensus sequences (24).

Further evidence that the rapid turnover of the HAV 3C protease occurs via ubiquitin-mediated proteolysis was obtained by including methylated ubiquitin in the reaction mixtures. The blocked amine groups allow Me-ubiquitin to become incorporated into monoubiquitin conjugates with other proteins, but the formation of the polyubiquitin chains required for efficient degradation is prevented (23). As shown in Fig. 2, in the presence of Me-ubiquitin a new, easily detected, labeled species with a molecular mass consistent with that of monoubiquitin-HAV 3C protease appeared. It has been shown that a similar species which is synthesized from the EMCV 3C protease contains both the EMCV protein and ubiquitin (4). The results in Fig. 2 also reveal that the poliovirus 3C protease does not become incorporated into monoubiquitin-3C protease conjugates under these conditions.

The data presented thus far support the idea that, like the EMCV 3C protease, the 3C protease of HAV is rapidly degraded by the ubiquitin-mediated system, while the poliovirus 3C protease is not. Since proteins tagged with polyubiquitin chains are recognized and degraded by the proteasome, experiments were carried out to ascertain the involvement of the proteasome in the HAV 3C protease turnover. The effects of two inhibitors of proteasome activity were evaluated. The ATP analog ATP-γS, which supports the conjugation of ubiquitin to substrate proteins but does not support the ATP-dependent functioning of the proteasome, was

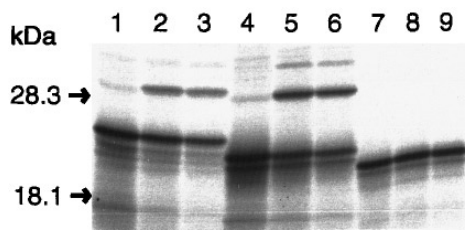

FIG. 2. SDS-PAGE analysis of reaction mixtures containing the ^{35}S-labeled 3C proteases incubated in reticulocyte lysate in the presence of added Me-ubiquitin. HAV 3C protease samples are in lanes 1 - 3, EMCV 3C protease samples are in lanes 4 - 6, and poliovirus 3C protease samples are in lanes 7 - 9. Aliquots were removed from the reaction mixtures at: 0 hr (lanes 1, 4, and 7), 1 hr (lanes 2, 5, and 8), and 4 hr (lanes 3, 6, and 9).

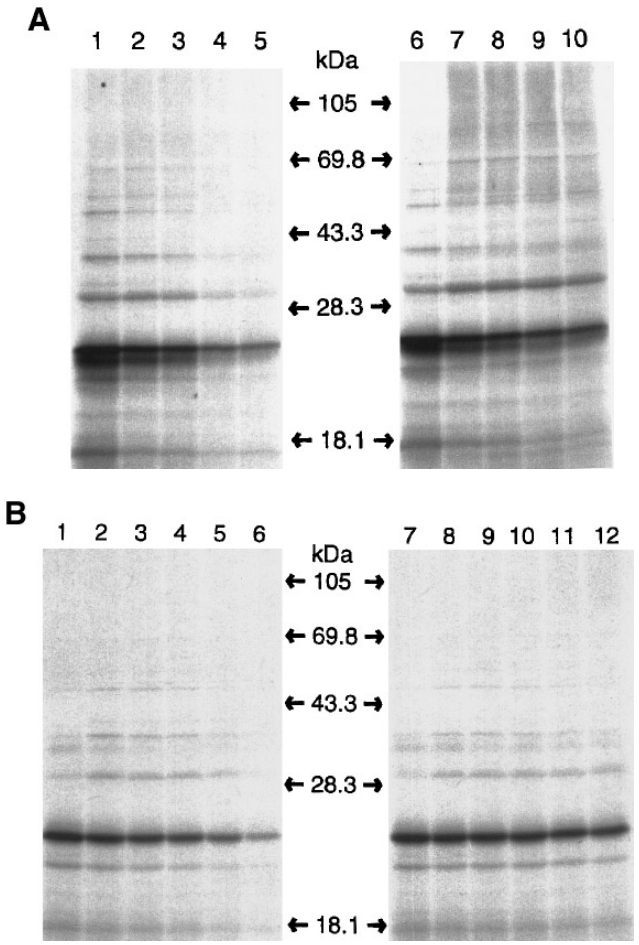

FIG. 3. **A.** SDS-PAGE showing the effects of ATP-γS on the ^{35}S-labeled HAV 3C protease incubated in reticulocyte lysate in the presence of added ubiquitin. Minus ATP-γS samples are in lanes 1 - 5, and plus ATP-γS samples are in lanes 6 - 10. Aliquots were removed from the reaction mixtures at: 0 min (lanes 1 and 6), 30 min (lanes 2 and 7), 60 min (lanes 3 and 8), 120 min (lanes 4 and 9); and 180 min (lanes 5 and 10). **B.** SDS-PAGE showing the effects of MG132 on the ^{35}S-labeled HAV 3C protease incubated in reticulocyte lysate in the presence of added ubiquitin. Minus MG132 samples are in lanes 1 - 6, and plus MG132 samples are in lanes 7 - 12. Aliquots were removed from the reaction mixtures at: 0 min (lanes 1 and 7), 10 min (lanes 2 and 8), 20 min (lanes 3 and 9), 30 min (lanes 4 and 10), 60 min (lanes 5 and 11), and 120 min (lanes 6 and 12).

found to reduce the rate of degradation of the HAV 3C protease (Fig. 3A). Moreover, the presence of this compound resulted in the accumulation of a significant quantity of higher molecular mass labeled material, which very likely represents a heterogeneous mixture of polyubiquitin-3C protease conjugates (4). The peptide analog inhibitor of proteasome proteolytic activity, MG132 (25) was also discovered to reduce the rate of HAV 3C protease degradation (Fig. 3B). In our hands this inhibitor produces a smaller accumulation of polyubiquitin-substrate protein conjugates than does ATP-γS. These results indicate that the actual degradation of the HAV 3C protease is accomplished by the proteasome.

Evaluation of the 3C proteases as competitive inhibitors of degradation and ubiquitin conjugate formation. Given that both the HAV and EMCV 3C proteases are degraded by the ubiquitin-mediated system, the question arises as to whether the two proteins share features that allow them to be recognized by the same ubiquitin conjugating pathway factor or factors. In order to test this possibility, HAV, poliovirus, and EMCV 3C proteases were purified to near homogeneity from expressing *E. coli* cells. These proteins were then evaluated for their ability to inhibit the degradation, and incorporation into ubiquitin conjugates, of labeled *in vitro* synthesized 3C proteases. While performing these experiments, it was discovered that the EMCV 3C protease catalyzes the hydrolysis of the HAV 3C protease at one or more undetermined sites. The result is an HAV 3C protease reduced in mass by about 1 kDa (data not shown). A mutant of the EMCV 3C protease was therefore prepared, in which the catalytic cysteine at position 159 (26) was changed to alanine. This protein was employed in the experiments involving labeled HAV 3C protease.

As shown in Fig. 4A, purified HAV 3C protease was found to inhibit the degradation of the labeled versions of both itself and the EMCV 3C protease in reticulocyte lysate. Purified EMCV 3C protease proved to be an even more potent inhibitor of the turnover of itself and of the HAV 3C protease. This result is consistent with the idea that these two proteins compete with each other for degradation. Purified poliovirus 3C protease had no effect on the degradation of either the HAV or EMCV 3C proteins. That the purified EMCV $3C_{A159}$ protein lacks catalytic activity was confirmed by demonstrating its inability to cleave the EMCV polyprotein capsid precursor LVP0 (Fig. 4B; ref. 16). This mutant protein is, however, as effective as the wild type protein in inhibiting the degradation of the labeled *in vitro* synthesized EMCV 3C protease (Fig. 4C). In an attempt to determine if the observed reductions in degradation rates could be caused, at least in part, by competition for a limiting component of the ubiquitin conjugating pathway, the effects of the purified 3C proteases on the synthesis of labeled Me-ubiquitin-3C protease conjugates were tested. The EMCV 3C protease was observed to strongly inhibit the attachment of Me-ubiquitin to both labeled HAV and EMCV 3C proteases (Fig. 5). Neither the purified HAV or poliovirus 3C proteins measurably inhibited the incorporation of either labeled protein into conjugates with Me-ubiquitin. While these results suggest that both the HAV and EMCV 3C proteases are conjugated to ubiquitin in pathways that share at least one substrate-recognizing component, the failure of the purified HAV protein to act as an inhibitor of conjugate synthesis may indicate

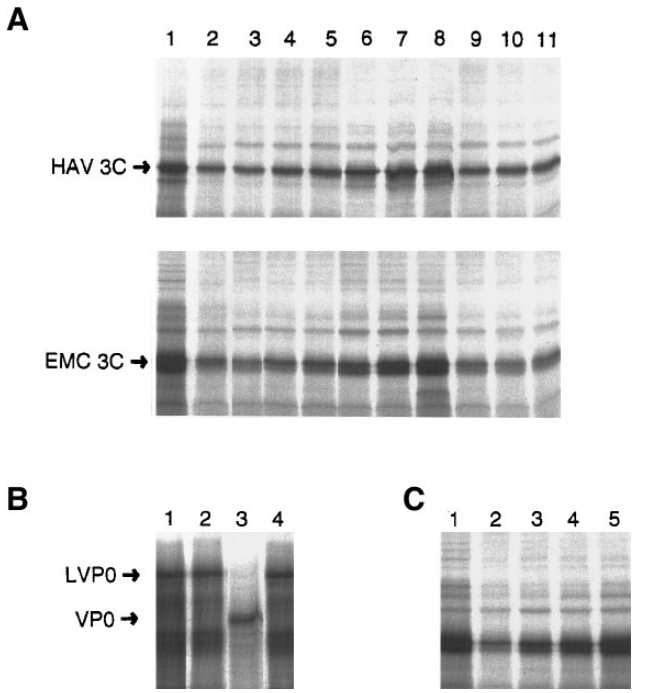

FIG. 4. **A.** SDS-PAGE showing the effects of added purified 3C proteases on the degradation of the ^{35}S-labeled HAV and EMCV 3C proteases in reticulocyte lysate. Aliquots were removed from the reaction mixtures at 2 hr, except for the samples in lane 1, which were removed at 0 hr. The samples analyzed are: no added protease (lanes 1 and 2); 2, 4, and 8 μM added HAV 3C protease (lanes 3, 4, and 5, respectively), 2, 4, and 8 μM added EMCV $3C_{A159}$ protease (lanes 6, 7, and 8, respectively, top panel only), 2, 4, and 8 μM added EMCV 3C protease (lanes 6, 7, and 8, respectively, bottom panel only), and 2, 4, and 8 μM added poliovirus 3C protease (lanes 9, 10, and 11, respectively). **B.** Activity assay of the EMCV 3C and $3C_{A159}$ proteases. ^{35}S-labeled EMCV LVP0 polyprotein was incubated with EMCV 3C or $3C_{A159}$ proteases as described in Materials and Methods. Aliquots were removed from the reaction mixtures at 2 hr, except for the sample in lane 1, which was removed at 0 hr. The samples analyzed are: no added protease (lanes 1 and 2), added 3C protease (lane 3), and added $3C_{A159}$ protease (lane 4). **C.** SDS-PAGE showing the effects of added purified EMCV $3C_{A159}$ protease on the degradation of ^{35}S-labeled EMCV 3C protease in reticulocyte lysate. Aliquots were removed from the reaction mixtures at 2 hr, except for the sample in lane 1, which was removed at 0 hr. The reactions analyzed are: no added protease (lanes 1 and 2) and 2, 4, and 8 μM added EMCV $3C_{A159}$ protease (lanes 3, 4, and 5, respectively).

differences exist in the kinetic details of the two processes.

DISCUSSION

Previous reports have suggested, based upon indirect evidence, that the 3C protease of HAV might be unstable in reticulocyte lysate (27) and in infected cells (28). The results of this study confirm that the HAV 3C protease is a rapidly degraded protein, and that it, like the EMCV 3C protease (4), is a substrate for the ubiquitin-mediated proteolytic system. *In vitro* synthesized ^{35}S-labeled HAV 3C protease was shown to be degraded in the rabbit reticulocyte lysate system with a half-life of approximately 60 min, which is about twice the value for the EMCV protein (4). Evidence that this turnover involves the conjugation of ubiquitin to the HAV 3C protease includes the stimulation of the degradation by increased ubiquitin concentration and the synthesis, in the presence of Me-ubiquitin, of a product with the expected mass of about 33 kDa for a monoubiquitin-3C conjugate. ATP-γS and MG132 were both found to markedly reduce the rate at which the HAV 3C protease is degraded, implicating the 26S proteasome as the proteolytic agent (4,25).

In contrast to the results with the HAV 3C protease, the 3C protease of poliovirus was found to be quite stable in the reticulocyte lysate system. No evidence for the conjugation of ubiquitin with this protein was observed, and the addition of ubiquitin did not diminish the stability of the protein. It is possible that reticulocytes lack a ubiquitin conjugating system enzyme which is required for the recognition of the poliovirus 3C protease. Such an enzyme may be present in the virus's natural human host cells, but preliminary experiments in which concentrated cytoplasmic extracts of cultured human lung fibroblasts were added to labeled poliovirus 3C protease in reticulocyte lysate did not induce either the synthesis of ubiquitin-3C protease conjugates or the degradation of the protein (data not shown).

The apparent slow turnover rate of the poliovirus 3C protease suggests that rapid degradation of the 3C

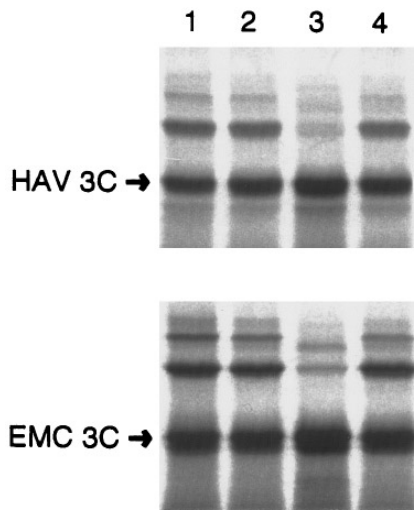

FIG. 5. SDS-PAGE showing the effects of added purified 3C proteases on the incorporation of the ^{35}S-labeled HAV and EMCV 3C proteases into monoubiquitin conjugates during incubation in reticulocyte lysate in the presence of Me-ubiquitin. Aliquots were removed from the reaction mixtures at 40 min. The reactions analyzed are: no added protease (lane 1), 8 μM added HAV 3C protease (lane 2), 8 μM added EMCV 3C protease (lane 3), and 8 μM added poliovirus 3C protease (lane 4).

proteases will not prove to be a necessary event for the successful replication of all picornaviruses. It remains to be seen what role, if any, the destruction of 3C proteases plays in the infectious cycles of HAV and EMCV. The poliovirus, HAV, and EMCV proteases all have different substrate specificities (2,3,18), and it is probable that if the HAV and EMCV proteases recognize any cellular proteins as substrates during their infectious cycles, these proteins are different than those known to be cleaved by the poliovirus 3C protease (29-31). Stability of the poliovirus 3C protease may therefore be vital for efficient poliovirus replication, but the preservation of mature HAV and EMCV 3C protease function in infected cells may interfere with HAV and EMCV reproduction. The evolution of the HAV and EMCV 3C proteases, which share little sequence homology (32-33), into substrates for the same highly selective cellular proteolytic system, lends support to the possibility that the rapid degradation of these proteins is an important event in the replication of HAV and EMCV.

In spite of the lack of similarity between the HAV and EMCV 3C protease sequences, data was obtained which suggests that both proteins are recognized by the same ubiquitin system enzyme, or enzymes, which selects them for degradation. The HAV and EMCV 3C proteases were shown to compete with each other for access to the reticulocyte proteolytic machinery, as indicated by the ability of each to reduce the rate at which the other is degraded. The EMCV protein was found to be the stronger competitor in these experiments. The poliovirus 3C protease was determined not to serve as an inhibitor of the degradation of either of the other two other 3C proteases, which is consistent with the evidence that it is not a substrate for the ubiquitin system. While at least some of the competition between the HAV and EMCV 3C proteases for degradation may occur during the binding of the polyubiquitin-3C protease conjugates to the 26S proteasome complex, competition between the two 3C proteins definitely occurs during the ubiquitin conjugation stage. This is indicated by the observation that the purified EMCV 3C protease strongly inhibits the formation of conjugates between Me-ubiquitin and the labeled *in vitro* synthesized HAV 3C protease. The most straightforward interpretation of this result is that the EMCV 3C protease binds to an enzyme of the ubiquitin conjugation pathway that is required for the recognition of both the EMCV and HAV 3C proteases. The most likely candidate for this unidentified protein is an E3 ubiquitin-protein ligase, since the members of this class of enzymes appear to be responsible for protein substrate recognition by the ubiquitin pathway (5-12). The purified HAV 3C protease did not act as an inhibitor of ubiquitin-EMCV or HAV 3C protease conjugate synthesis, at least under the conditions employed here. One explanation for this is that the HAV 3C protease binds to the recognizing-enzyme with an affinity which is considerably lower than that of the EMCV 3C protease. The data may also indicate that the attachment of ubiquitin is a rate-limiting step in the degradation of the EMCV 3C protease, but not in the destruction of the HAV 3C protein. In any case, the identification of two ubiquitin system substrates which share recognition elements will facilitate the search for what these elements are. This in turn will assist in the understanding of how substrate proteins are recognized by the ubiquitin conjugation pathway.

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