Gemin5 proteolysis reveals a novel motif to identify L protease targets

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ABSTRACT

Translation of picornavirus RNA is governed by the internal ribosome entry site (IRES) element, directing the synthesis of a single polyprotein. Processing of the polyprotein is performed by viral proteases that also recognize as substrates host factors. Among these substrates are translation initiation factors and RNA-binding proteins whose cleavage is responsible for inactivation of cellular gene expression. Foot-and-mouth disease virus (FMDV) encodes two proteases, L^pro and 3C^pro. Widespread definition of L^pro targets suffers from the lack of a sufficient number of characterized substrates. Here, we report the proteolysis of the IRES-binding protein Gemin5 in FMDV-infected cells, but not in cells infected by other picornaviruses. Proteolysis was specifically associated with expression of L^pro, yielding two stable products, p85 and p57. *In silico* search of putative L targets within Gemin5 identified two sequences whose potential recognition was in agreement with proteolysis products observed in infected cells. Mutational analysis revealed a novel L^pro target sequence that included the RKAR motif. Confirming this result, the Fas-ligand Daxx, was proteolysed in FMDV-infected and L^pro-expressing cells. This protein carries a RRLR motif whose substitution to EELR abrogated L^pro recognition. Thus, the sequence (R)(R/K)(L/A)(R) defines a novel motif to identify putative targets of L^pro in host factors.

INTRODUCTION

Initiation of translation in a growing number of viral and cellular mRNAs is governed by internal ribosome entry site (IRES) elements (1–6). IRES-driven translation initiation allows for recruitment of the translational machinery to an internal position in the mRNA, and depends on the coordination of RNA structure and RNA–protein interactions (7–12). Picornavirus IRES elements interact with host factors termed IRES transacting factors (ITAFs) that modulate their activity (13,14). Most ITAFs are abundant proteins with RNA-binding motifs that shuttle between the nucleus and the cytoplasm.

In contrast to this mechanism of translation initiation, most cellular mRNAs depend on the m^{7}Gppp (cap) located at the 5′end that, in turn, is recognized by the eIF4F complex (eIF4E, eIF4A and eIF4G). Recruitment of the 43S pre-initiation complex, composed of a 40S ribosomal subunit, eIF3 and eIF2-GTP-met-tRNA$_i$ ternary complex, is accomplished through association between eIF4G and eIF3. Once assembled at the 5′ end of the mRNA, the 43S complex scans in the 5′ to 3′ direction until an AUG triplet in the appropriate context is encountered (15), leading to formation of the 48S pre-initiation complex.

Gemin5 is the RNA-binding factor of the survival of motor neuron (SMN) complex (16) that assembles Sm proteins onto splicesomal snRNAs. This predominantly cytoplasmic protein (17,18) was identified as an RNA-binding protein that interacts with the IRES elements of foot-and-mouth disease virus (FMDV) and hepatitis C virus (HCV) in a riboproteomic approach (19). We have previously shown that Gemin5 down-regulates translation (20) and photocrosslinks to 7-methylguanosine (21). Together, these findings revealed an unanticipated role for Gemin5 as a multifunctional protein acting on translation and splicing.

Various IRES-binding factors, such as eIF4G, polyA-binding protein (PABP), polypyrimidine tract-binding protein (PTB) and poly(rC)-binding protein 2 (PCBP 2), are targets of picornavirus proteases (22–24) supporting a link between picornavirus gene expression and inactivation of nuclear and cytoplasmic RNA-binding proteins. Picornavirus genomes encode proteases that execute proteolytic cleavage of the viral polyprotein. All picornaviruses express 3C^pro; in addition, enteroviruses express 2A^pro while aphthoviruses express L^pro. A relatively large number of host factors have been identified as targets of picornavirus 3C^pro (25–31).

In contrast, the number of host factors which are substrates of 2A^{pro}, and particularly those of L^{pro}, lack behind (32–37). Specifically, the cleavage site sequence of L^{pro} in host factors has been determined only for eIF4GI and eIF4GII (38,39).

Here, we report the specific cleavage of Gemin5 in FMDV-infected cells, but not in other picornavirus infections, SVDV (swine vesicular disease virus) and EMCV (encephalomyocarditis virus). Two active forms of L^{pro} can be expressed in FMDV-infected cells, termed Lab and Lb, depending on the AUG used to initiate translation of the viral RNA (40,41). Transfection of cells with a plasmid expressing Lb demonstrated that Gemin5 proteolysis was specifically associated with expression of L^{pro}, leading to the generation of two stable products, p85 and p57. Mutational analysis of the Gemin5 amino acid sequence that yielded the 85-kDa polypeptide defined a target sequence, RKAR, for this highly specific viral protease. To our knowledge, this is the first time that a substrate sequence of L^{pro} has been identified, other than those of eIF4GI and eIF4GII. Validating our results, definition of this motif facilitated identification of a new host factor, death-domain associated protein (Daxx) (42), which is targeted by the L^{pro} in infected and transfected cells. Furthermore, mutational analysis of the sequence RRLR in Daxx confirmed its recognition as a substrate of L^{pro}

MATERIALS AND METHODS

Constructs

The constructs generated in this work contain the FMDV IRES (43) upstream of the Gemin5 open reading frame and the FLAG epitope fused to the C-terminal end of the protein. The IRES was inserted to confer resistance to the translation shutdown induced by FMDV L protease (27,43).

The vector pGEM-FLAG was generated using primers sflag and asflag (Supplementary Table S1), annealed, extended with Klenow polymerase and inserted into pGEM3 vector (Promega) between SmaI and SalI restriction sites. The region encoding amino acids 776–1138 of Gemin5 was PCR amplified from pcDNA3.1-Gemin5 (21) using the primers sG5.1 and asG5.2, and inserted into the SmaI site of pGEM-FLAG, generating the construct pG5$_{766–1138}$FLAG. Then, the IRES element present in plasmid pBIC (43) was inserted into the SacI site of pG5$_{766–1138}$FLAG yielding pIRESGemin5$_{766–1138}$FLAG.

pIRESGemin5$_{485–838}$FLAG and pIRESGemin5$_{1078–1439}$FLAG were obtained by PCR amplification from pcDNA3.1-Gemin5 using the primers sG5.3 and asG5.4, or sG5.5 and asG5.6, respectively. These products were inserted into the SmaI site of pIRESGemin5$_{766–1138}$FLAG, replacing the G5$_{766–1138}$ fragment.

Point amino acid substitutions in pG5$_{766–1138}$FLAG were generated by overlapping PCR mutagenesis using primers sG5.7 to asG5.14, listed in Supplementary Table S1. The PCR products were inserted into pG5$_{766–1138}$FLAG between SmaI and BglII restriction sites (details are available upon request). The pIRESGemin5 version

of each mutant was obtained by inserting the IRES into the SacI site of each pG5 mutant by SacI.

The construct pHIS-Gemin5/845–1508 was generated in two steps using standard molecular biology techniques, with primers sG5.15 to asG5.20 (Supplementary Table S1) (details are available upon request).

Mutagenesis of pcDNA3.1-HisCDaxx construct (generating amino acid substitutions RRLR to EELR or EELE; RRAR to EEAR, or EEAE; and KKRRAR to EERRAR, or EERRAE) was performed using the Quick-change mutagenesis procedure (Agilent Technologies) with primers sD.1 to asD.14 (Supplementary Table S1). All plasmids were sequenced to ensure correctness.

Infection and transfection assays

IBRS-2 and BHK-21 cells were grown in Dulbecco's-modified Eagle's medium supplemented with 10 or 5% foetal bovine serum, respectively. FMDV, SVDV or EMCV were used for infection of IBRS-2 monolayers at a multiplicity of infection (MOI) of 10. After 1 h of adsorption, infection was left to progress to different time post-infection (pi) until the monolayer developed total cythopatic effect.

BHK-21 or IBRS-2 monolayers were transfected with the indicated Gemin5 plasmid ($1 \mu g / 0.5 \times 10^6$) cells and pLb plasmid$_{TM}$ (5 to 20 ng), as described (44) using lipofectamine (Invitrogen). Cells were harvested 20-h post-transfection with lysis buffer (0.5% NP40, 120 mM NaCl, 50 mM Tris–HCl; pH 7.8). Cell debris was eliminated by centrifugation, and the soluble supernatant was kept at −70°C in sample buffer. The concentration of total protein in the sample was determined by the Bradford method.

BHK-21 cells were transfected with pcDNA3.1-HIS6CDaxx (45) or the indicated mutant constructs ($1 \mu g / 0.5 \times 10^6$ cells) using lipofectamine 24 h prior to transfection of the pLb plasmid (10, 20 ng). Cell extracts were prepared 24 h later.

Western blot

Equal amounts of total protein were resolved on SDS–polyacrylamide gels, and transferred to PVDF membranes (Biorad), using a semidry electrotransfer device. Membranes were probed with monoclonal antibodies α-FLAG (Sigma) (1:5000); polyclonal α-Gemin5 (Novus), α-DHX9 (Bethyl), α-eIF4GI-Ct, α-PABP, α-PTB (1:2000) (23); α-Daxx (Novus) (1:1000); α-Xpress (Invitrogen) (1:4000); α-Tubulin (Sigma) (1:5000). The appropriate mouse or rabbit secondary HRP-conjugated antibody (Thermo Scientific) (1:2000) was used, followed by ECL detection (Millipore). The membranes were probed with a second antibody after stripping using restore western blot stripping buffer (Thermo Scientific) according to the instructions of the manufacturer.

RNA synthesis and *in vitro* translation assays

The bicistronic construct containing the FMDV IRES upstream of the luciferase open reading frame was linearized with NotI (44). Plasmid-encoding HIS-Gemin5/845–1508 was linearized with SalI.

Transcription was performed for 2 h at 37°C using 10–50 U of purified T7 RNA polymerase in the presence of 1–3 µg of DNA template, 40 mM Tris–HCl, 50 mM DTT, 0.5 mM rNTPs, as described (9).

Increasing amounts of Gemin5 transcript synthesized *in vitro* (0–50 ng) were translated in 70% rabbit reticulocyte lysate (RRL) (Promega) supplemented with 10 µCi of ^{35}S-methionine 15 min prior to addition of the bicistronic RNA (200 ng) bearing the FMDV IRES, as described (40). Samples were loaded in SDS–PAGE and analysed by autoradiography of dried gels.

RESULTS

Gemin5 is proteolysed in FMDV-infected cells

Gemin5 was recently identified as one of several RNA-binding proteins interacting with the IRES elements of FMDV and HCV (19) and subsequently shown to act as down-regulator of translation (20). Also consistent with the repressor activity of this protein, Gemin5 has the capacity to bind m^{7}Gppp (21). Since various eIFs and ITAFs are proteolysed in picornavirus infected cells by virtue of proteases encoded in the viral genome (46) we sought to analyse the status of Gemin5 during picornavirus infection. To this end, cytoplasmic IBRS-2 cell extracts prepared at different times post-infection with FMDV were used to inspect the integrity of Gemin5. IBRS-2 is a swine cell line that encodes readily detectable Gemin5 protein by western blot using an antibody targeted against a peptide corresponding to residues 1410–1438 (Figure 1A). Two main stable cleavage products, p85 and p57, were observed 2 and 3 hpi with FMDV, concomitant with a decrease of the full-length Gemin5 (p170) intensity (Figure 1B). The same products, p85 and p57, were observed in extracts from IBRS-2 cells transfected with FMDV RNA, and at later times, with the replication-defective RNA ΔSL1 (47). In contrast, no cleavage was observed with a defective RNA bearing a substitution of the L-P1 region with CAT, pT7Rep (27) (Supplementary Figure S1).

To gather information from other picornavirus infections, we chose viruses belonging to the enterovirus and cardiovirus genera that have the capacity to infect IBRS-2 cells. Cytoplasmic lysates from cells infected with swine vesicular disease virus (SVDV), or encephalomyocarditis virus (EMCV), harvested at times post-infection when total cythopathic effect was visible, were immunoblotted using the α-Gemin5 antibody. Gemin5 levels remained unaltered at all times post-infection with SVDV or EMCV, and no cleavage products were observed (Figure 1B). Complete cleavage of eIF4G occurred in both FMDV and SVDV (Figure 1C), as expected. These results demonstrate that Gemin5 cleavage is specific for FMDV infection.

Next, we compared the Gemin5 proteolysis to other host proteins, PABP and PTB, known to be targets of viral proteases (23,24). To this end, IBRS-2-infected extracts were analysed by western blot, probing the same membrane with antibodies against PABP and PTB. Gemin5 cleavage was observed at similar times post-infection as PABP and PTB, but lagged behind that of eIF4G (Figure 1D). However, other IRES-binding factors such as the RNA helicase DHX9 (19) were not proteolysed during infection. We conclude that cleavage of Gemin5 is FMDV-specific and occurs at similar post-infection times as PTB or PABP.

Gemin5 is a target of the L protease

Picornavirus genomes encode proteases termed 3C, 2A and L. While 3C^{pro} is expressed by all picornavirus genomes, L^{pro} and 2A^{pro} are encoded by aphthovirus and enterovirus, respectively. The fact that Gemin5 cleavage was specific to FMDV infection, but not SVDV or EMCV, in conjunction with the lack of cleavage in the defective pT7rep RNA construct, prompted us to analyse the possibility that L^{pro} could be responsible for Gemin5 proteolysis. To this end, we co-transfected a FLAG-tagged vector expressing human Gemin5 (21) with increasing amounts of plasmid pLb expressing the FMDV L^{pro} (41,43) in BHK-21 cells. Expression of Gemin5-FLAG protein was readily observed in a western blot with α-FLAG antibody (Figure 2A). As the amount of co-transfected Lb plasmid increased, the intensity of the Gemin5-FLAG product (p170) faded away, and the p85 product appeared immediately below a p90 band derived from the Gemin5-FLAG overexpressed protein. In addition, an unspecific p80 was detected with the FLAG antibody (Figure 2A). However, the p57 product observed in IBRS-2 infected cells with the Gemin5 specific antibody was undetectable.

To test whether the presence of p85 and p57 polypeptides was due to differences between the FMDV infection and the L^{pro} expression system, we performed consecutive immunoblot assays with extracts derived from IBRS-2 cells expressing Gemin5-FLAG prior to FMDV infection. Proteolysis of Gemin5 was first examined with α-FLAG and subsequently, with α-Gemin5 antibodies. As shown in Figure 2B, both antibodies recognized the p85 product but only the α-Gemin5 reacted against p57. This result demonstrated that the p85 cleavage product yielded by L^{pro} expression does not differ from that observed in FMDV-infected cells. Furthermore, it also suggested that another L-cleavage site is present between the C-terminal end of Gemin5 and the epitope recognized by the α-Gemin5 antibody (Figure 2C). No further fragments were detected <55 kDa.

We conclude that Gemin5 is a new target of L^{pro}, yielding at least two relatively stable proteolysis fragments with apparent molecular weights of 85 and 57 kDa, detectable with the α-Gemin5 antibody.

Mapping the L target region in Gemin5

Next, to identify the target region of L^{pro} in Gemin5, three partially overlapping FLAG-tagged polypeptides were designed based on the apparent molecular weights of the cleavage products observed with the full-length Gemin5 protein. Specifically, constructs expressing regions 485–838, 766–1138 and 1078–1439 were generated to allow a wide coverage to map L-induced cleavage products. In addition, the FMDV IRES-dependent translation strategy
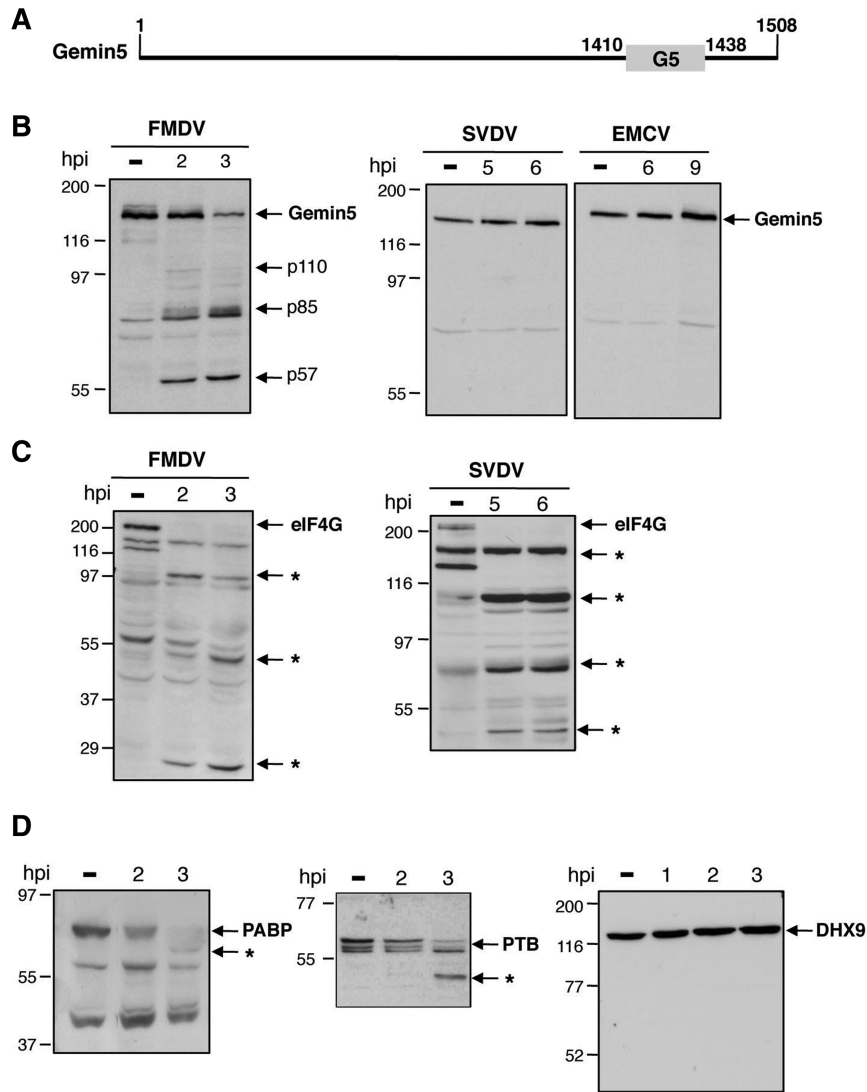
Figure 1. Gemin5 is specifically proteolysed in FMDV-infected cells. (**A**) Schematic of Gemin5 indicating the region used to raise the α-Gemin5 antibody. (**B**) Immunodetection of Gemin5 in IBRS-2 cell extracts prepared at different hours post-infection (hpi) with FMDV, SVDV and EMCV. Samples were fractionated in 8% SDS–PAGE and analysed by western blot using α-Gemin5 antibody. Extracts prepared from mock-infected cells (−) are shown in the first lane of each gel. Arrows depict the position of Gemin5 and the proteolysis products. (**C**) Cleavage of eIF4G in FMDV- and SVDV-infected cells. (**D**) Gemin5 cleavage occurs at similar times than PABP and PTB during FMDV infection. FMDV-infected IBRS-2 cell extracts were used to detect by western blot PABP, PTB and DHX9 using specific antibodies. Asterisks point to the cleavage products. Molecular weight markers are indicated on the left of each gel.

was used to protect the expression of Gemin5 peptides from the cap-dependent inhibition induced by L^{pro} (41,43). Transfection of these three constructs in BHK-21 cells resulted in the expression of the expected FLAG-tagged polypeptides, with apparent molecular masses of 47, 53 and 52 kDa, respectively (Figure 3A).

Plasmids encoding each of these polypeptides were co-transfected with increasing amounts of pLb and detected by western blot using the α-FLAG antibody. No cleavage product was observed when the Gemin5/485–838-FLAG was analysed (Figure 3A). In contrast, the products p37 and p25 were detected with Gemin5/766–1138-FLAG or Gemin5/1078–1439-FLAG, respectively (Figure 3A). The estimated Mw of these fragments indicated that the target sequence (likely yielding p85)

resided around residue 840 within the peptide 766–1138, while the second target (likely that of p57) was close to residue 1200 within the peptide 1078–1439 (Figure 3B). Also, in support of this conclusion, coexpression of the polypeptide 485–885-FLAG with Lb, revealed a clear decrease in the intensity of the p50 full-length product although we fail to detect the proteolysis fragment, presumably due to its low Mw (Supplementary Figure S2).

Identification of the L target sequence in Gemin5

Based on a study of synthetic substrate specificity (48) as well as amino acid sequences hydrolysed by L^{pro} in the L-VP4 junction, eIF4GI and eIF4GII (38,39), we developed criteria to search for candidate L^{pro} target sites
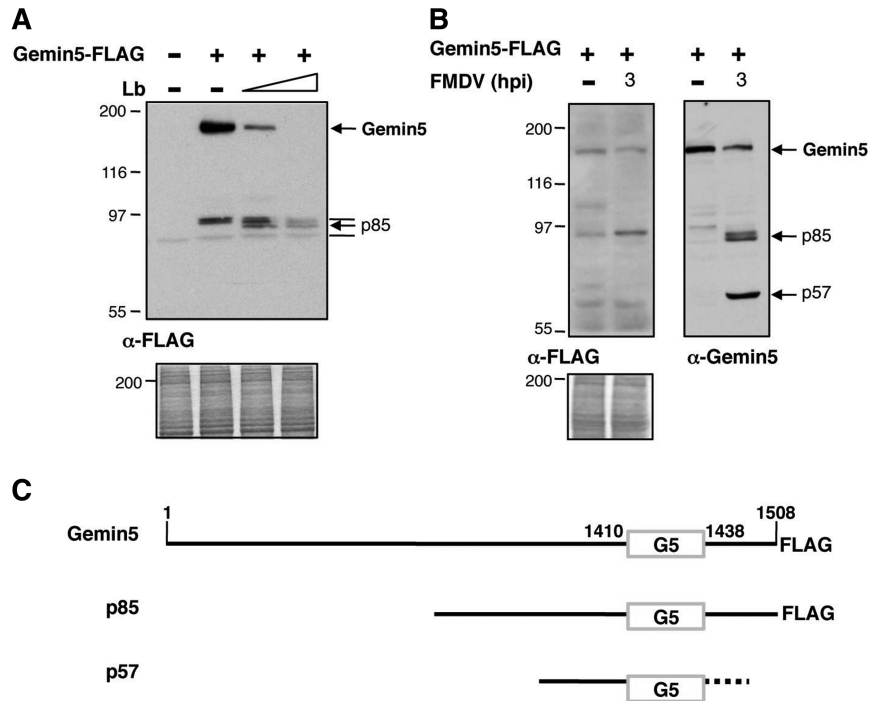
Figure 2. The L protease is responsible for Gemin5 proteolysis. (**A**) The pcDNA3-Gemin5-FLAG construct was transfected 24 hr prior to increasing amounts of the plasmid expressing the FMDV Lb protease in BHK-21 cells. Cell extracts were prepared 20 hpt and analysed by western blot using α-FLAG antibody. Ponceau staining is shown on the bottom panel. Arrows point to the position of the p85 product; thin lines mark the position of a p90 Gemin5-FLAG derived band and a p80 unspecific band; (−), mock-transfected cell extract. (**B**) IBRS-2 cells were transfected with pcDNA3-Gemin5-FLAG, and 24 h later, infected with FMDV (MOI 10). Extracts prepared 3 hpi were analysed by western blot with α-FLAG (left panel) and subsequently, with α-Gemin5 (right panel) antibody. (−), mock-infected cells. Arrows point to the positions of p85 and p57 products. Loading control is shown on the bottom panel. (**C**) Diagram of Gemin5-FLAG with the estimated locations of proteolysis products; a rectangle marks the region recognized by the Gemin5 antibody.

in Gemin5. Basically, targets of 12 residues were first selected if they contain positively charged (K or R) amino acids upstream of polar (three out of eight) residues. Sequences meeting these criteria but having proline or negatively charged residues in the four upstream positions were excluded. From this group, we selected sequences where the third residue was hydrophobic. Thus, *in silico* search of L^{pro} target candidates was carried out dividing Gemin5 polypeptide sequences into all possible overlapping subsets of 12 amino acids. Only four sequences met the criteria mentioned earlier having the peculiarity of being two separate partially overlapping sequences (Figure 4A and B). These potential targets were located within residues 838–854 and 1226–1241, respectively. In support of the functional relevance of our prediction, the size of the cleavage products estimated from the predicted targets matched very closely the observed size of Gemin5 fragments (p85 and p57) in infected cells (Figure 1B), as well as the size of the Lb-mediated proteolysis products observed in the coexpression of truncated polypeptides (p37 and p25 in Figure 3A).

The candidate target sequence in Gemin5/766–1138-FLAG (838-TLIKKRKARSLLPLSTS-854) consists of a stretch of positively charged amino acids that precede polar residues, resembling the L^{pro} target sequence in the viral L-VP4 junction (Figure 4C), but not those of eIF4GI

or eIF4GII. To determine the relevance of R and K residues for L^{pro} hydrolysis in Gemin5/766–1138-FLAG, we generated single, double and triple substitution mutants to E (Figure 5A). For this, we took advantage of previous data that substitution of K and R by E in synthetic peptides resembling the L-VP4 sequence prevented the hydrolysis of the peptide by L^{pro} *in vitro* (48). Western blot of BHK-21 cell extracts transfected with each of the mutant constructs and increasing amounts of pLb were performed using the α-FLAG antibody. The p37 product resulting from the hydrolysis of the wild-type polypeptide (WT) was also detected in extracts prepared from the single substitution R846E (Figure 5B), indicating that L-induced hydrolysis was not impaired. In contrast, no p37 cleavage product was identified in extracts prepared from the double substitution (RKAR/EEAR) and the triple substitution mutants (RKAR/EEAE), indicating that residues RK are critical for L^{pro} recognition of this target (Figure 5B). However, the double mutant IKKR/IEER, carrying substitutions of the positively charged residues not conserved with the L-VP4 junction was a substrate of L^{pro}. The negative effect of the most upstream KK residues was only observed when assisted by a third substitution (KKRKAR/EERKAE). These results demonstrated that the Gemin5 candidate sequence accurately matched the L^{pro} target.
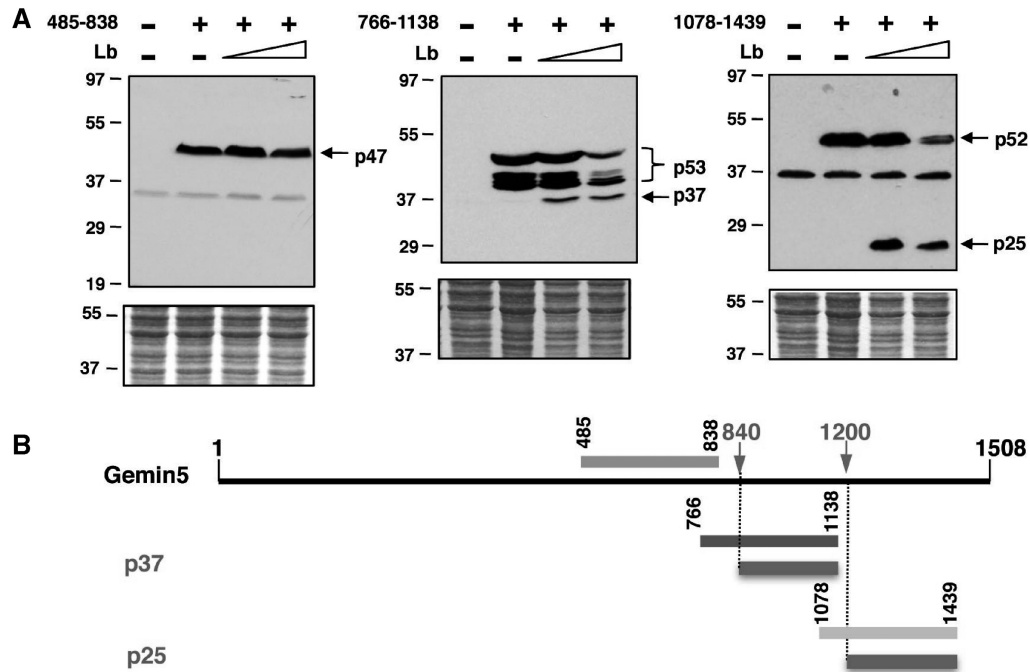
Figure 3. Mapping the Gemin5 products induced by the L protease. (**A**) IRES-Gemin5-FLAG constructs were cotransfected with increasing amounts of Lb plasmid in BHK-21 cells. Translation of Gemin5-FLAG polypeptides p47, p53 and p52, were analysed in 12% SDS–PAGE by α-FLAG western blot. Arrows point to the positions of Lb-induced products, p37 in Gemin5/766–1138, and p25 in Gemin5/1078–1439. Ponceau staining is shown in the bottom panel. (**B**) Schematic representation of the location of L cleavage sites in Gemin5 (depicted by gray arrows) estimated from the apparent molecular weight of proteolysis products, p37 and p25, arising from the peptides 766–1138 and 1078–1439, respectively, overexpressed in BHK-21 cells.
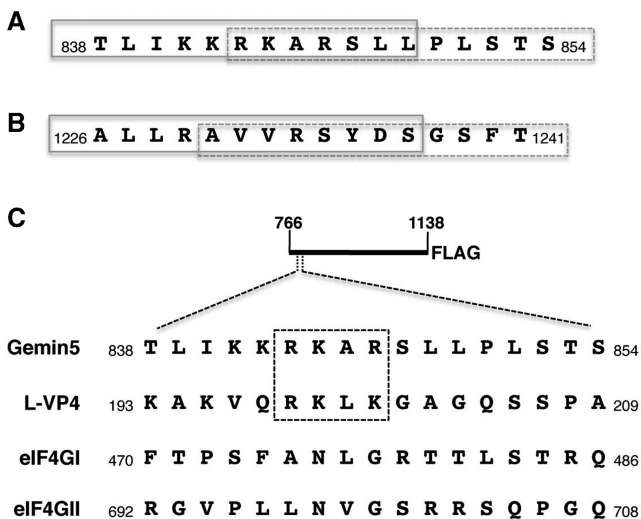

Figure 4. Gemin5 amino acid sequences retrieved in the search *in silico* of putative substrates of L protease. The Gemin5 open reading frame (1508 amino acids) was subdivided into overlapping subsets of 12 amino acids. Putative targets were selected if they contain K or R amino acids upstream of polar (three out of eight) residues. Sequences meeting these criteria but having proline or E, D residues in the four upstream positions were excluded. Two sequences where the third residue was hydrophobic were selected, 838–854 (**A**) and 1226–1241 (**B**). (**C**) Comparison of the Gemin5 amino acid sequence 838–854 with that of the L-VP4 junction in the viral polyprotein, eIF4GII and eIF4GI. A broken line rectangle depicts the motif of higher resemblance between Gemin5 and L-VP4 junction. Subscript symbols indicate amino acid positions.

We conclude that the sequence TLIKKRK ARSLLPLSTS, located within polypeptide 766–1138 of Gemin5, yields a fragment corresponding to the 85-kDa product observed during FMDV infection. Furthermore, based on our mutational analysis, the RKAR sequence defines a new motif to identify putative targets of the L protease.

Relevance of the Gemin5 proteolysis products to translation control

We have previously shown that Gemin5 binds directly to the IRES element and acts as a translation repressor using either cell free extracts or shRNA-depleted cells (20). Thus, it was of interest to determine whether the Gemin5 proteolysis products retained their capacity to repress translation. To this end, expression of the polypeptide 845–1508 was used to monitor the effect on IRES-dependent translation by measuring the efficiency of translation of bicistronic constructs bearing the FMDV IRES between CAT and luciferase reporter genes in rabbit reticulocyte lysates. The results indicated that the Gemin5/845–1508 amino acid product exerted a moderate dose-dependent repressor effect on IRES-driven translation of luciferase, as well as 5′ end-dependent translation of CAT (Figure 6A), although the repressor effect was somewhat more effective on the first cistron than that of IRES-dependent translation (Figure 6B), and in both cases smaller that that exerted by the uncleaved Gemin5
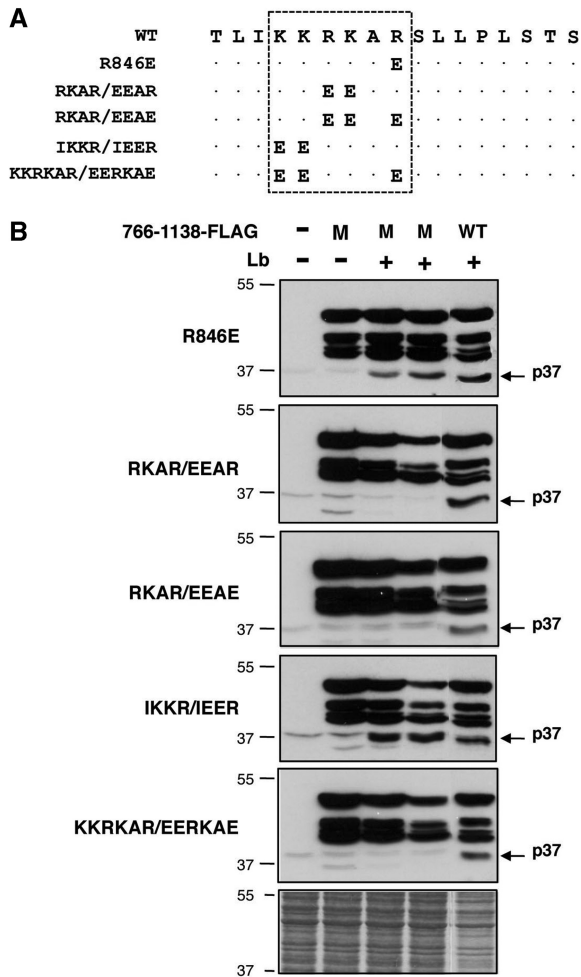
Figure 5. Mutational analysis of the sequence recognized as substrate of L[pro] in Gemin5/766–1138. (**A**) Diagram of the single, double and triple amino acid substitutions introduced in the wild-type Gemin5/766–1138-FLAG. In all cases, K or R residues were replaced by E. A dot in the sequence alignment is used to indicate no change with respect to the wild-type sequence. (**B**) Plasmids expressing the Gemin5/766–1138-FLAG (WT) or the substitution mutants (M, carrying substitutions indicated on the left of each panel) in an IRES-dependent manner were cotransfected with increasing amounts of a plasmid expressing the L protease in BHK-21 cells. Cell extracts were analysed by 12% SDS–PAGE and western blot using α-FLAG antibody. Arrows indicate the pattern of L-induced products (p37), which was always detected in the WT sequence loaded in parallel. The bottom panel shows a ponceau staining as loading control.

(p170) (20). Thus, we conclude that the p85 product only partially retains the translation repression capacity.

Definition of a general motif to identify targets of L[pro] in host proteins

The precise target sequence of L[pro] in host factors is limited to eIF4GI and II (49). Thus, the results described earlier for Gemin5 prompted us to search for novel targets of L[pro] in host factors. To this end, we conducted a search *in silico* of proteins carrying sequences highly similar to KKRKAR and variations of this motif. Among the proteins retrieved in this search (Supplementary Table S2) was Daxx harboring the sequence K/QKRRAR
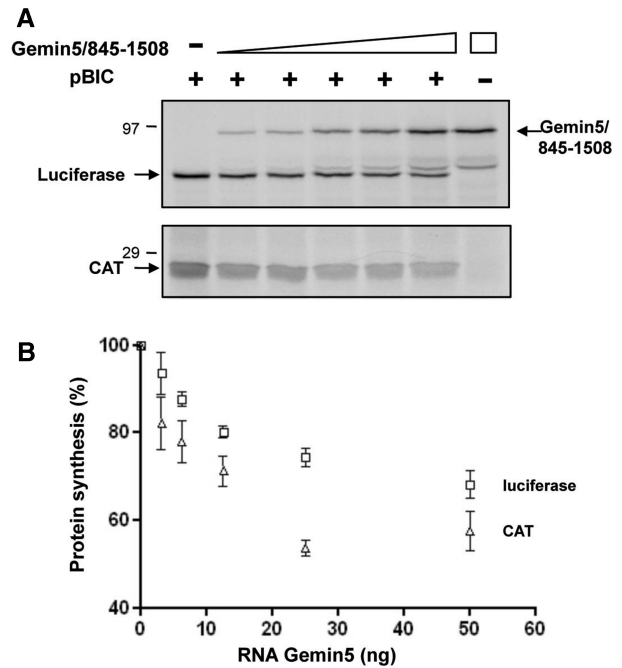

Figure 6. Gemin5/p85 represses translation in a cell-free system. (**A**) Equal amounts of the bicistronic pBIC RNA, encoding CAT as the first cistron and luciferase as the IRES-dependent cistron, were added to RRL previously incubated during 15 min with increasing amounts of Gemin5/845–1508 RNA. Samples were analysed by SDS–PAGE 12% followed by autoradiography. (**B**) The intensity of luciferase and CAT proteins observed in four independent assays, normalized to the value obtained with the pBIC RNA alone, is represented against the amount of Gemin5 RNA added to the translation assay.

(depending on the organism Homo sapiens, Mus musculus, or Bos taurus). Daxx is a multifunctional protein with critical roles in transcription control, apoptosis and innate immune antiviral response (42,50,51).

Comparison of the Daxx sequence to Gemin5/p85 (Figure 7A) showed two closely located motifs, RRLR and RRAR, embedded in a larger sequence that fits all the criteria mentioned before (Figure 4A). To test the hypothesis that proteins retrieved in the search might be authentic targets of L[pro], we analysed extracts prepared from either BHK-21 or IBRS-2 cells infected with FMDV by western blot using α-Daxx antibody (Figure 7B). Remarkably, Daxx protein was degraded during FMDV infection although no stable proteolysis products were detected (two unspecific bands that appeared depending on the cell line analysed, were undetected in HeLa cell extracts included for antibody specificity (Figure 7C)). Confirming the proteolysis of Daxx during FMDV infection, we found that overexpression of N-terminal Xpress-tagged Daxx (45) prior to L[pro] expression allowed us to observe a p60 band, coincident with the molecular weight of the expected cleavage products (Figure 7C). Similar results were observed with α-Daxx antibody overexpressing Daxx in transfected cells (Supplementary Figure S3). By contrast, we have not been able to detect cleavage products in EMCV or
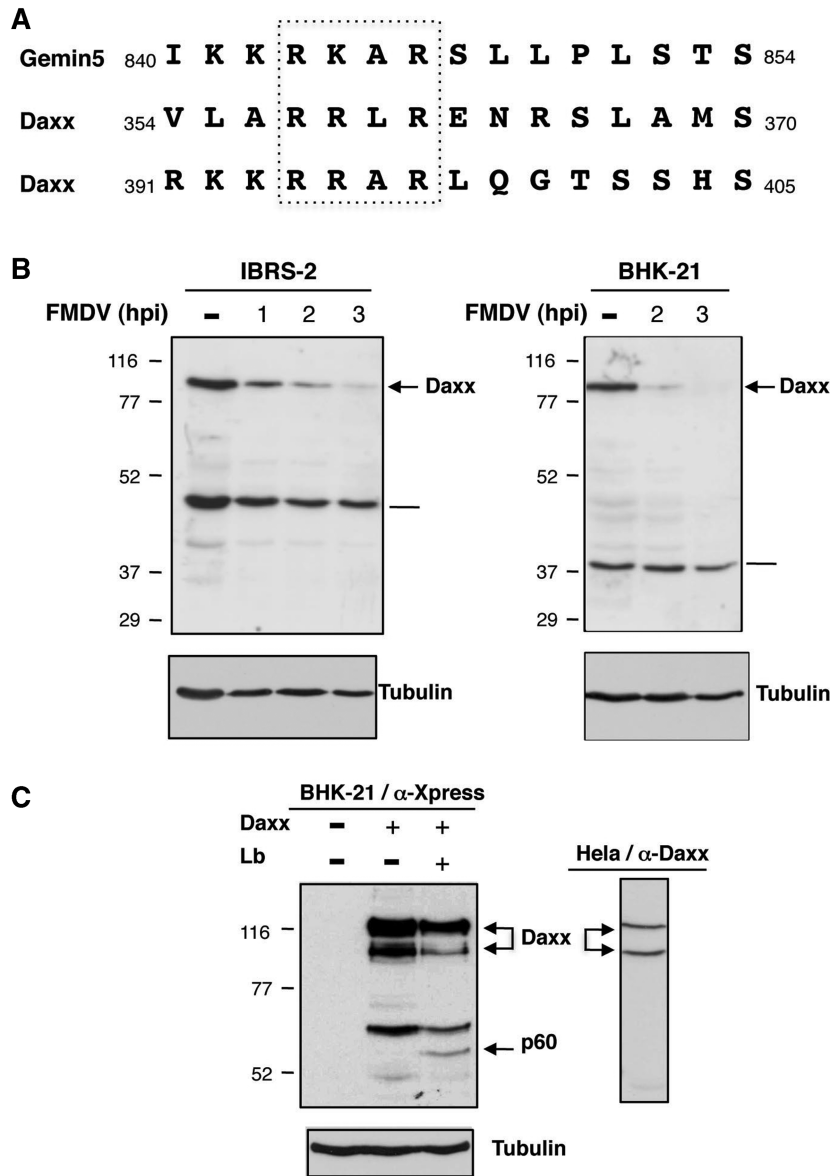
A

Gemin5	840	I	K	K	R	K	A	R	S	L	L	P	L	S	T	S 854
Daxx	354	V	L	A	R	R	L	R	E	N	R	S	L	A	M	S 370
Daxx	391	R	K	K	R	R	A	R	L	Q	G	T	S	S	H	S 405

B

C

Figure 7. Search of putative substrates of L protease by homology to the motif identified in Gemin5. (**A**) Alignment of amino acid sequences present in Gemin5 and Daxx around the RKAR motif. Subscript symbols indicate amino acid positions. (**B**) Proteolysis of Daxx in FMDV-infected cells. Extracts of FMDV-infected cells (IBRS-2 and BHK-21 cells) immunoblotted with α-Daxx; the unspecific band detected in each of this cell line (depicted by thin lines) was not observed in Hela cell extract. (**C**) Extracts of BHK-21 cells transfected with pcDNA3.1-HIS6CDaxx (expressing the human Daxx protein) 24 h prior to transfection of Lb plasmid, immunoblotted with α-Xpress (left panel); two polypeptides of ∼116 and 100 kDa are detected by immunodetection of the endogenous human Daxx in HeLa cells (right panel). Arrows depict the Daxx protein and the p60 proteolysis product; tubulin was used as loading control.

SVDV infected cells (Supplementary Figure S4). These results were in agreement with the hypothesis that proteins carrying a motif similar to RKAR are candidate substrates of the L protease.

To validate this hypothesis, we generated multiple mutants in both motifs (RRLR and RRAR) substituting basic amino acids, R or K, by E (Figure 8A and B). Transfection of Daxx constructs expressing these substitution mutants with the L protease construct indicated that substitution of the RRLR motif by EELR or EELE fully abrogated the recognition of the protein by L^{pro}

(Figure 8A). Substitution of the second site RRAR to EEAR did not affect proteolysis, whereas substitution to EEAE diminished the intensity of the proteolysis products (Figure 8B). Similarly, and in agreement with the results obtained with Gemin5/p85, the triple mutant EERRAE interfered with the recognition of the protein by L^{pro} while the double mutant EERRAR was not affected (Figure 8B). Therefore, we conclude that the sequence 354-VLARRLRENRSLAMS-370 of Daxx protein constitutes the preferential recognition motif for the L protease.
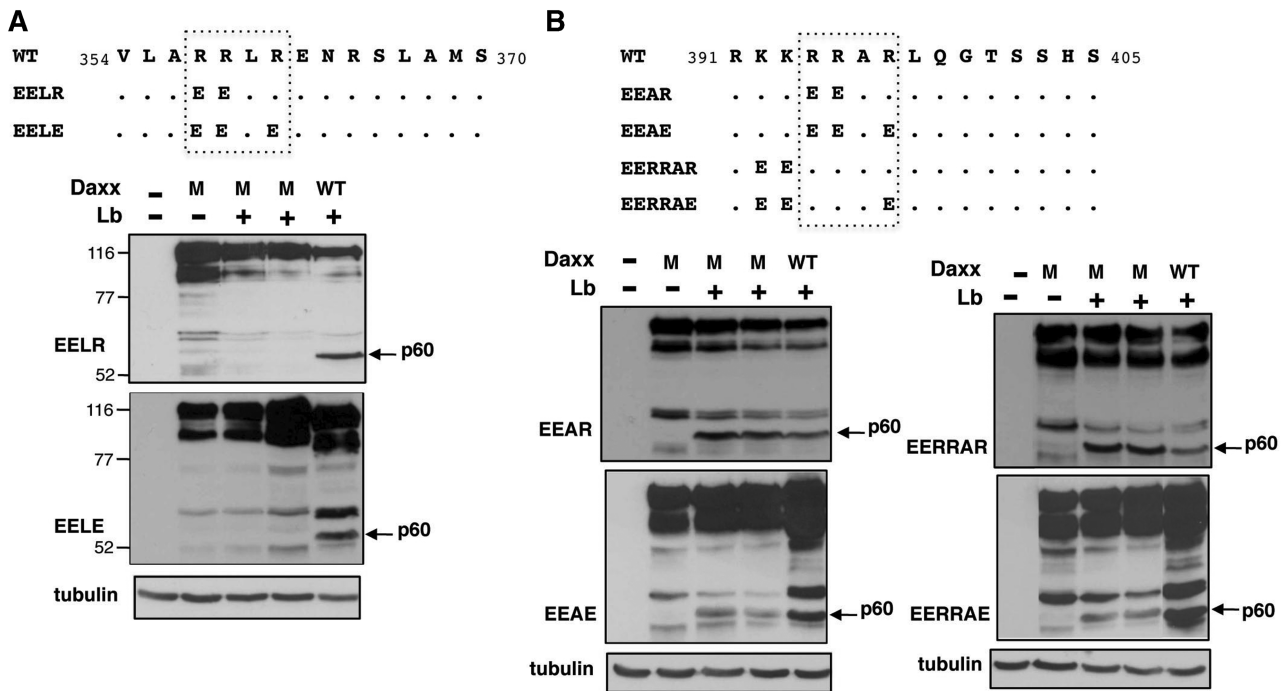
Figure 8. Mutational analysis of the sequence recognized as substrate of L^{pro} in Daxx protein. (**A**) Diagram of the double and triple amino acid substitutions introduced in the RRLR motif of the wild-type Daxx. A dot in the sequence alignment is used to indicate no change with respect to the wild-type sequence. Plasmids expressing the Daxx (WT) or the substitution mutants (M, carrying substitutions indicated on the left of each panel) were transfected 24 h prior to transfection of Lb plasmid in BHK-21 cells. Cell extracts were analysed by 10% SDS–PAGE and western blot using α-Xpress antibody. Arrows indicate the position of L-induced products (p60), which was always detected in the WT sequence loaded in parallel. The bottom panel shows a tubulin immunoblot as loading control. (**B**) Diagram of the amino acid substitutions introduced in the KKRRAR motif of the wild-type Daxx. Transfections and immunoblots were carried out as in (A).

DISCUSSION

Gemin5 is a 170-kDa RNA-binding protein that contains 13 WD repeats at the N-terminal end and a coiled-coil motif at the C-terminal end (52). Previous work identified the 5th WD motif as responsible for both cap-binding and snRNA-binding (16,21). Additionally, Gemin5 binds directly to the FMDV and HCV IRES and exerts a down-regulatory role on translation (20). Here, we have determined the status of Gemin5, in parallel to other IRES-binding factors, following the expression of viral proteases in naïve and infected cells. Immunoblots carried out with soluble cell extracts indicated that Gemin5 is hydrolysed in some picornavirus infections (FMDV) but not in others (SVDV, EMCV). Based on the different protease-coding capacity of these three picornaviruses, we predicted that the protease responsible for Gemin5 cleavage would be L^{pro}.

Degradation of specific host factors in picornavirus-infected cells reveals the interference of cellular pathways by viral infection. Host factors eIF4GI and II, eIF3a, PTB, PABP, each involved in translation control, are proteolysed during picornavirus infection (23,24). Other host proteins, such as Gemin3 (the RNA helicase that forms part of the SMN complex), NF-κB (the nuclear factor kappa B, a transcription factor that controls inflammatory response), RIG-I (the retinoic acid-inducible gene 1, a cytoplasmic RNA helicase that senses viral infection), MAVS and TRIF (the innate immune adaptor molecules

mitochondrial antiviral signaling and the Tol/IL1 receptor domain-containing adaptor inducing interferon-beta proteins) or the stress granules protein G3BP (35,53–57) are also substrates of picornavirus proteases. As reported by Pacheco *et al.* (19,20), Gemin5 interacts with the FMDV and HCV IRES and exerts a down-regulatory role on translation initiation. Thus, we investigated whether this protein might be a host factor recognized by picornavirus proteases.

L^{pro} is a papain-like cysteine protease (58) for which two substrates have been characterized in depth, eIF4GI and II, in addition to the viral polyprotein (38,39,49,59). Given our results that specific cleavage of Gemin5 was unequivocally observed in cells expressing L^{pro}, we sought to investigate the putative L^{pro} target sequences. Taking into account biochemical analysis of substrate specificity of this protease carried out with synthetic peptides (48), a close inspection of the amino acid composition of Gemin5 revealed a sequence partially homologous to the L-VP4 junction. Interestingly, the sizes of the proteolysis products identified in infected cells matched very closely the estimated molecular weight of peptides arising from the putative target site for L^{pro}. Furthermore, mutational analysis of the RKAR motif indicated that replacement of the positively charged R or K amino acids by the negatively charged E residues was sufficient to inhibit L-induced cleavage (Figure 5B). A critical feature of this motif is the presence of R and K upstream of 8 residues of which at least three are polar amino acids (Figure 4A).

Characterization of the motif recognized in Gemin5 helped in the identification of novel targets of this protease in mammalian cells. Interestingly, the list of proteins retrieved from databases with this motif includes RNA-binding proteins, DNA-binding proteins and several factors involved in cell cycle arrest, differentiation and tumorigenesis. One of these factors is Daxx, a fas-ligand and multifunctional adaptor that play important roles in transcriptional control, apoptosis and the innate immune antiviral response (42,50,51). Validating our criteria of L^{pro} target definition, we found that Daxx was also proteolysed in FMDV-infected cells (Figure 7B) and that a cleavage product of the expected size was detected in cells overexpressing Xpress-tagged Daxx with L (Figure 7C). Mutational analysis of the Daxx amino acid sequence revealed that the RRLR motif plays a crucial role in the recognition of this protein by the L protease (Figure 8A), with a second cleavage site in the nearby RRAR motif (Figure 8B). Another potential target of L^{pro} is Neuroguidin, an eIF4E-binding and CPEB-binding protein that is important in neuronal development (60) and harbors the AKRRALS sequence. Furthermore, PABP1, a target of several viral proteases (61) that carries the RRSL motif was previously shown to be substrate of the L^{pro} in transfected cells (23) and in FMDV-infected cells as well (Figure 1D).

Alignment of the target sequence of L^{pro} within the viral polyprotein, L-VP4 junction, with Gemin5/p85 (Figure 4C) shows homology in certain positions centered on the RKAR motif, but also extends beyond these six amino acids. It is worth noting that the target sequence identified by mutational analysis yielding p85 shows higher resemblance with the L-VP4 cleavage site than to those of eIF4GI or eIF4GII (38,39) (Figure 4C). Remarkably, the RRLR and RRAR motifs found in Daxx are highly similar to that of Gemin5 (Figure 7A). Recognition of the first one, RRLR, as substrate for L^{pro} was abolished by mutation to EELR or EELE (Figure 8A). By contrast inactivation of the second requires a triple substitution to EEAE or EERRAE (Figure 8B).

We show here that Gemin5 is proteolysed during infection at similar times as the RNA-binding proteins PABP and PTB, previously implicated as ITAFs in internal initiation. Our discovery of Gemin5 proteolysis opens new avenues to understand its functional relevance in FMDV-infected cells. The fact that several enteroviruses have been reported to induce Gemin3 cleavage (35) suggests that inactivation of the SMN complex may be a general feature of picornavirus infections. As the SMN complex is responsible for the cytoplasmic deposition of Sm cores onto snRNAs, a critical step in the pathway of snRNP biogenesis, its deficiency would be expected to induce widespread defects in mRNA splicing and perhaps contribute to shutdown of gene expression in infected cells. Additionally, since a significant fraction of Gemin5 is present in the cell cytoplasm unassociated with the SMN complex (18) and has the capacity to down-regulate translation (20), Gemin5 may possess functions unrelated to splicesomal biogenesis, such as regulation of cellular mRNA translation, which may be modified by picornavirus infection.

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