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Human Rhinovirus 2A Proteinase Cleavage Sites in Eukaryotic Initiation Factors (eIF) 4GI and eIF4GII Are Different

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Several picornaviruses shut down host cellular protein synthesis by proteolytic cleavage of the eukaryotic initiation factor (eIF) 4GI and eIF4GII isoforms. Viral RNA translation is maintained by a cap-independent mechanism. Here, we identify the human rhinovirus 2 $2A^{pro}$ cleavage site in eIF4GII in vitro as $PLLNV^{699}$*GSR; this sequence lies seven amino acids C-terminal to the cleavage site previously identified in eIF4GI ($LSTR^{681}$*GPP).

Picornaviruses, which include human rhinovirus (HRV), poliovirus (PV), and coxsackievirus (CV) as well as the animal pathogen foot-and-mouth disease virus, contain a single-stranded RNA of positive polarity. Eukaryotic cellular mRNAs possess at their 5′ ends a cap structure (m^7GpppX, where X is any nucleotide) (27), which is important for ribosomal recruitment mediated by a cap-binding protein complex, eukaryotic initiation factor (eIF) 4F. The genomic viral RNA lacks a 5′-terminal cap structure (9, 22), and virus translation proceeds by a cap-independent mechanism, whereby ribosomes bind directly to an internal ribosome entry site of the viral RNAs (11, 23). The cap-binding protein complex eIF4F is a three-subunit complex. The three subunits are eIF4E, which interacts directly with the cap (19, 21, 29), eIF4A, an ATPase which in conjunction with eIF4B exhibits RNA helicase activity (25), and eIF4G (formerly p220 or eIF4γ [31]), which is a large scaffolding protein that plays a key role in the assembly of the mRNA-ribosome initiation complex. eIF4G binds directly to the ribosome-associated eIF3, thus delivering the small ribosomal subunit to the mRNA (12, 18). We have cloned and characterized a homologue of eIF4G, which we have termed eIF4GII (4), while the original eIF4G (31) was renamed eIF4GI. eIF4GII is 41% identical to eIF4GI, binds eIF4E, eIF3, and eIF4A, and functionally complements eIF4GI (4, 10).

Different picornavirus proteinases can cleave both isoforms of eIF4G, generating in each case their respective N- and C-terminal cleavage products, cp_N and cp_C (reviewed in references 2 and 28). The cp_C of eIF4G retains the capacity to interact with internal ribosome entry sites as well as with eIF3 and eIF4A (15–17, 20, 24) and can therefore support cap-independent translation. Indeed, initiation of translation on HRV and PV RNA is stimulated under these conditions (7, 14, 32). However, as the eIF4G cp_C lacks the eIF4E-binding site, it is unable to support cap-dependent translation of cellular mRNAs (reviewed in reference 8) or does this inefficiently (1).

We have previously shown, using PV1 and HRV14 as model systems (5, 30), that eIF4GII cleavage precisely coincides with the inhibition of host cellular protein synthesis, whereas the cleavage of eIF4GI occurs earlier. However, in HRV2-infected cells, eIF4GI and eIF4GII are cleaved at similar rates, coincident with the shutoff of host cell protein synthesis (26).

To begin to understand the differences in the kinetics of eIF4GI and eIF4GII cleavage, we set out to determine the in vitro HRV2 $2A^{pro}$ cleavage site in human eIF4GII and compare it to that previously determined for HRV2, CVB4, and PV1 $2A^{pro}$ on eIF4GI. First, we used HRV2 $2A^{pro}$ to cleave recombinant eIF4GII (4). After incubation of recombinant eIF4GII (20 μg for 30 h at 30°C) with the purified enzyme in vitro, we examined the status of eIF4GII by using antibodies against N- and C-terminal regions of the protein. The C-terminal fragment of endogenous or purified recombinant eIF4GII generated by in vitro cleavage ran at about 90 kDa, with mobility identical to that found in vivo in HRV16-infected cells (Fig. 1A, compare lanes 2 and 3 to lane 1) or in HRV2-infected cells (data not shown). Five cycles of N-terminal sequencing by automated Edman degradation of the recombinant eIF4GII cp_C (Fig. 1A, lane 3) generated the following amino acids: glycine, serine, arginine, arginine, and serine. These amino acids correspond to the sequence $V^{700}GSRR^{704}$ on eIF4GII, indicating that HRV2 $2A^{pro}$ must cleave eIF4GII at Val_{699}*Gly_{700} (Fig. 2). We attempted to determine the N-terminal sequence of the endogenous eIF4GII cp_C isolated by immunoprecipitation from HRV16-infected cells but were unsuccessful, as the preparation contained a mixture of polypeptides.

To further substantiate our results, we introduced mutations at the HRV2 $2A^{pro}$ cleavage site in eIF4GII. Glutathione *S*-transferase (GST)–eIF4GII (amino acids [445 to 744])–FLAG fragments, containing either a single point mutation (G700E)

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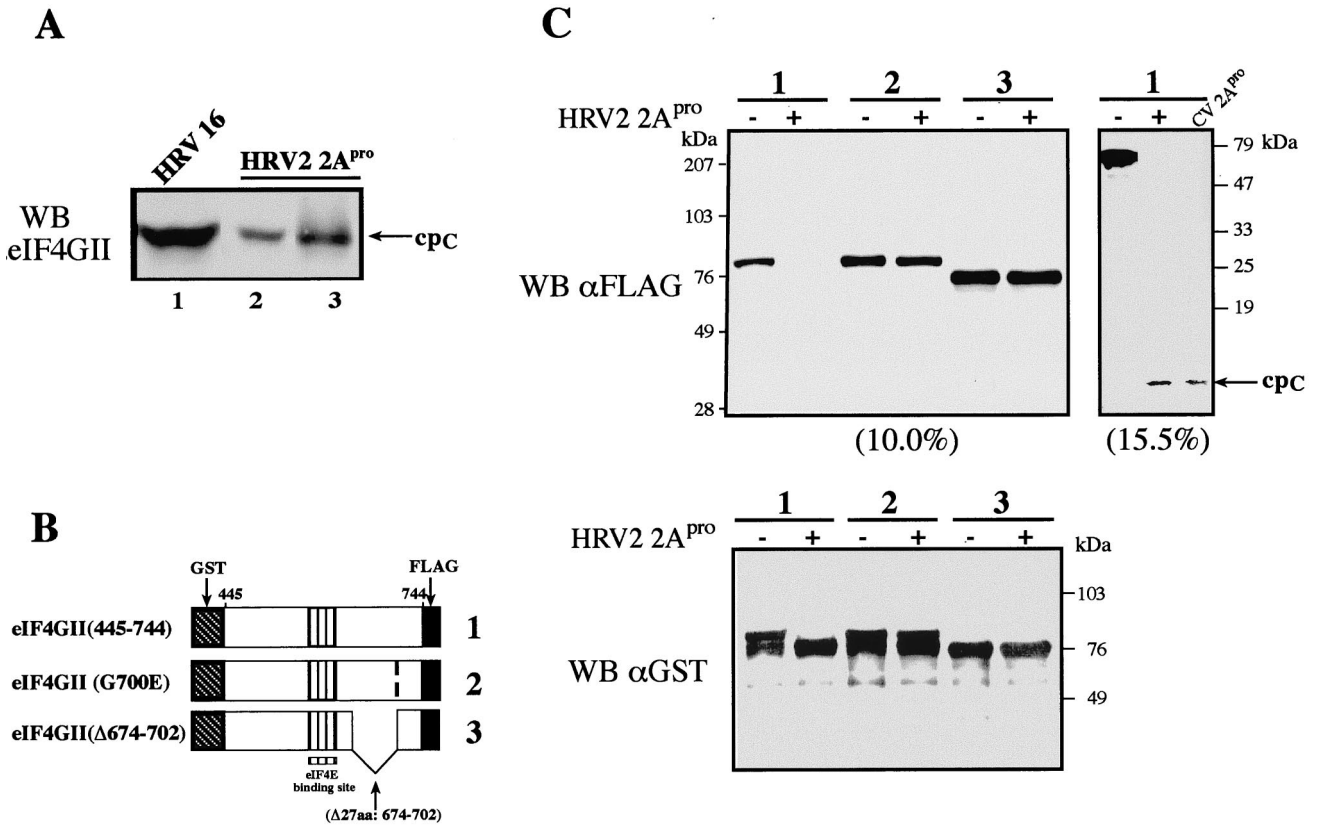

FIG. 1. (A) Identification of the 2A^{pro} cleavage site in eIF4GII. HeLa-I cells were infected with HRV16 (100 50% tissue culture infective doses per cell) as described previously (30). Cell extract (60 μg of protein; lane 1) was prepared 6 h postinfection and loaded on a gel in parallel with 40 μg of HeLa S10 (lane 2) or purified recombinant eIF4GII (1/40 of the reaction mixture, ~0.5 μg; lane 3) that was incubated in the presence of purified HRV2 2A^{pro}. Proteins were resolved by SDS–8% PAGE and blotted onto nitrocellulose. The blot was treated with polyclonal antibodies against the cp$_C$ of eIF4GII. WB, Western blot. (B and C) eIF4GII mutants that are resistant to HRV2 2A^{pro} cleavage. (B) Scheme of wild-type eIF4GII (1) and mutant (2 and 3) fragments. GST-eIF4GII (aa 445 to 744)-FLAG fragments contained a single point mutation (G700E) or a deletion (Δ674-702). (C) eIF4GII proteins were expressed in *E. coli* and purified on a glutathione-Sepharose column (4). Purified proteins (0.5 μg) were incubated in the presence or absence of recombinant 2A^{pro} (2.8 μg) in buffer containing 100 mM potassium acetate, 20 mM Tris-HCl (pH 7.6), 2.5 mM magnesium acetate, and 10% glycerol for 1 h at 30°C. Laemmli buffer was added to stop the reaction. Samples were resolved by SDS-PAGE (10.0, 15.5, or 12.5% [bottom] acrylamide). Proteins were transferred onto a nitrocellulose membrane, which was subsequently incubated with anti-GST or anti-FLAG antibodies as indicated. The small (62-aa) C-terminal cleavage product containing the FLAG epitope was resolved only in a 15.5% gel (cp$_C$). The wild-type eIF4GII fragment was cleaved by HRV2 or CVB4 2A^{pro}, as indicated. Positions of molecular mass standards are shown on the right.

in or a 27-aa deletion (Δ674-702) of the putative hinge region (Fig. 1B) were generated, expressed in *Escherichia coli*, and purified by glutathione-Sepharose affinity chromatography. The purity and integrity of the proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining (data not shown). Equal amounts of the fusion proteins (wild type and two mutants) were incubated with the purified HRV2 2A^{pro} or buffer alone at 30°C for 1 h. Protein samples were resolved by SDS–12.5% PAGE and analyzed by Western blotting using antisera against either the FLAG or GST tag (Fig. 1C). Both mutants were resistant to 2A^{pro} cleavage (lanes 2 and 3), strongly supporting the conclusion that HRV2 2A^{pro} directly cleaves eIF4GII between V_{699} and G_{700}. Identical results were obtained when the eIF4GII mutants were treated with purified recombinant CVB4 2A^{pro} (data not shown), demonstrating that this enzyme also recognizes the same site.

These results identify the cleavage site of HRV2 and CVB4 2A^{pro} in eIF4GII as PLLNV^{699*700}GS, lying 7 aa downstream of the cleavage site identified in eIF4GI as TLSTR^{641*642}GP (13). As the two isoforms are cleaved equally well during infection by HRV2 (26) and HRV16 (data not shown), both eIF4G isoforms must exist in a cleavable form. Moreover, the binding to eIF4E, which is required for efficient cleavage, is not limiting for eIF4G cleavage. In contrast, during HRV14 and PV1 infection, eIF4GI is cleaved more rapidly than eIF4GII, indicating that the respective 2A^{pro} poorly recognizes the eIF4GII cleavage site, compared to recognition of the eIF4GI site. The reason for this discrimination remains, however, unclear. An analysis of the cleavage sites recognized on the viral polyprotein and eIF4G isoforms by HRV2, -14, and -16 2A^{pro} as well as that of PV1 2A^{pro} (Fig. 3) does not indicate any particular pattern which could explain the discrimination. This suggests that the overall conformations of the two isoforms may be important.

Indeed, the experiments presented here provide evidence

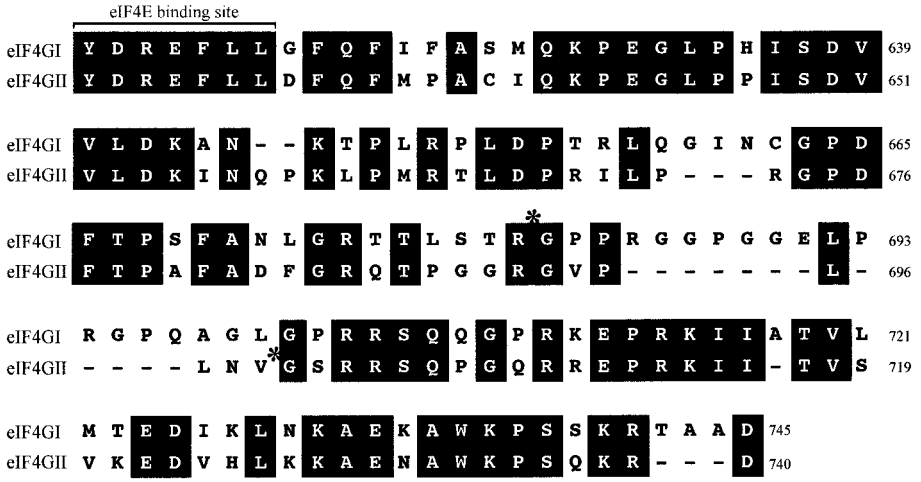

FIG. 2. Sequence alignment of human eIF4GI and eIF4GII proteins (sequence comparison starts from the eIF4E-binding site) (4, 18). Asterisks, positions of $2A^{pro}$ cleavage sites in eIF4GI and eIF4GII. The full-length eIF4GI and eIF4GII sequence accession numbers are AY082886 (3) and AF012072 (4), respectively.

that a significant conformational component in cleavage site recognition is present. Efficient cleavage of the recombinant GST-eIF4GII fragments was achieved in the absence of eIF4E (Fig. 1C). In contrast, the cleavage of eIF4GII, as well as eIF4GI (6), in HeLa extracts could be prevented by the addition of the GST-4E-BP1 fusion protein, which can sequester the eIF4E in the extract and prevent its binding to both eIF4G isoforms (data not shown). Thus, although the cleavage site is present in eIF4GII, it is no longer in a conformation which can be recognized by the enzyme. Interestingly, this is not the case when fragments of eIF4GII are used as in Fig. 2C, as efficient cleavage of recombinant GST-eIF4GII by purified $2A^{pro}$ was observed in the absence of eIF4E. Thus, the remainder of eIF4GII, as well as other factors which may be bound to it, affects the conformation of the cleavage site. Further experiments will be required to examine the nature of these conformational changes and to elucidate in which way they are responsible for the differential cleavage of the eIF4G isoforms during HRV14 and PV infection.

Substrates	Cleavage sites P (5 4 3 2 1)		P′ (1 2 3 4)
HRV16 polyprotein	NLTTV	*	GPSD
HRV2 polyprotein	IITTA	*	**GP**SD
eIF4GI	TLSTR	*	GPPR
eIF4GII	PLLNV	*	GSRR
HRV14 polyprotein	DIKSY	*	GLGP
PV1 polyprotein	DLTTY	*	GFGH

FIG. 3. Comparison of $2A^{pro}$ cleavage sites on the respective polyproteins and eIF4G homologues. The viral polyprotein sequences represent the C terminus of VP1 and the N terminus of $2A^{pro}$, respectively, and are taken from the public database http://www.iah.bbsrc.ac.uk/virus/picornaviridae/sequencedatabase/index.html. The residues shown to be important for HRV2 $2A^{pro}$ substrate recognition by peptide cleavage, mutational analysis, or crystallography are in boldface. P and P′ designate the N-terminal and the C-terminal cleavage products, respectively. Numbers indicate the positions of amino acid residues relative to the cleavage site.

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