Eukaryotic Initiation Factor 4GII (eIF4GII), but Not eIF4GI, Cleavage Correlates with Inhibition of Host Cell Protein Synthesis after Human Rhinovirus Infection

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For many members of the Picornaviridae family, infection of cells results in a shutoff of host protein synthesis. For rhinoviruses and enteroviruses, the shutoff has been explained in part by the cleavage of eukaryotic initiation factor 4G (eIF4G), a component of the cap-binding protein complex eIF4F. The cleavage of eIF4G occurs rapidly, i.e., within an average of 2 h postinfection, and coincides with the shutoff of host cell protein synthesis after poliovirus infection (Gradi et al., Proc. Natl. Acad. Sci. USA 95:11089-11094, 1998). We wished to determine whether eIF4GII cleavage kinetics could also explain the lack of correlation between the kinetics of eIF4G and eIF4GII cleavage and the shutoff of host protein synthesis after rhinovirus infection. In this study, we examined the correlation between human rhinovirus-induced shutoff of host protein synthesis and cleavage of eIF4GI and eIF4GII. In HRV-14-infected HeLa cells, almost no intact eIF4GI could be detected by 4 h postinfection, while only 4% of eIF4GII was cleaved at this time. By 6 h, however, 67% of eIF4GII was cleaved, and this cleavage coincided with a significant (60%) decline of host translation. These results suggest that cleavage of both eIF4GI and eIF4GII is required for HRV-mediated inhibition of host cell protein synthesis and that the cleavage of eIF4GII is the rate-limiting step in the shutoff of host cell protein synthesis after rhinovirus infection.

The genome of human rhinoviruses (HRV), similar to that of other picornaviruses, contains a single long open reading frame which encodes a large polyprotein precursor. The polyprotein is processed, partially in the nascent state, by two virus-specific proteases. Protease 2Apro performs the initial cleavage of the nascent polyprotein that liberates the capsid protein complex (2). Poliovirus 2Apro mutants defective in eIF4G cleavage are also defective in inducing the shutoff of host translation (3, 32). 2Apro of human rhinovirus 2 (HRV-2) and coxsackievirus B4 cleave eIF4G directly (24, 25). Poliovirus 2Apro is also capable of directly cleaving eIF4G (7, 17); however, it was concluded that the major eIF4G-specific cleavage activity in poliovirus-infected HeLa cells is not associated with a 2Apro (8). The C-terminal cleavage product of eIF4G interacts with eIF4A and eIF3 and supports cap-independent translation (6, 31, 37). However, it lacks the eIF4E recognition domain and is unable to support cap-dependent translation. In addition, cap-independent viral protein synthesis is stimulated by the C-terminal cleavage product of eIF4G of several (6, 31, 47, 48), but not all (5, 41), picornaviruses.

Although eIF4G proteolysis provides an attractive explanation for the shutoff phenomenon, several earlier findings obtained with poliovirus are inconsistent with such a model. For example, there is no good temporal correlation between the proteolysis of eIF4G and the shutoff of host translation in poliovirus-infected HeLa cells (13). While the cleavage of eIF4G occurs rapidly, i.e., within an average of 2 h postinfection, the shutoff is observed at 2.5 h or even later. Moreover, a complete cleavage of eIF4G occurs in the presence of guanidine-HCl and other inhibitors of poliovirus replication, which strongly mitigate the inhibition of host cell protein synthesis (4, 21, 36). It was suggested, therefore, that complete inhibition of host cell protein synthesis following poliovirus infection requires a second event in addition to the cleavage of eIF4G (4). An alternative interpretation of these data is that eIF4G cleavage is irrelevant to the shutoff of host translation (21). These apparent discrepancies may now be explained by recent find-
ings. We have recently identified a functional homolog of eIF4G, termed eIF4GII (16). eIF4GII is 46% identical to the original eIF4G (46), which was renamed eIF4GI. eIF4GII is cleaved both in vivo and in vitro by poliovirus 2Apro (17). However, after poliovirus infection, the cleavage of eIF4GII is slower than that of eIF4GI and, in contrast to cleavage of eIF4GI, it coincides with the shutoff of host protein synthesis (17).

In human rhinovirus 14 (HRV-14)-infected HeLa cells, as in poliovirus-infected cells, there is a discrepancy between the time course of eIF4GI cleavage and the shutoff of host protein synthesis (12). It is possible that this discrepancy can be explained for rhinovirus, as was shown for poliovirus, by the slower kinetics of cleavage of eIF4GII. Here, we addressed this possibility.

HeLa-I cells (Ohio strain) (1) were grown in monolayers in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS). HRV-14 was kindly provided by R. Rueckert. Virus stock was generated by passages in HeLa cells at 34°C in DMEM containing 2% FBS. Virus titers were determined in four replicates in a 50% tissue culture infective dose (TCID50) assay on HeLa cells, using 10-fold virus dilutions in 96-well microtiter plates. Virus dilutions were mixed with 10^4 cells in DMEM containing 2% FBS in a total volume of 0.3 ml. The plates were incubated at 34°C for 4 days. Following incubation, the cytopathic effect was determined, and the titers were estimated according to the method of Reed and Muench (40). For a one-step infection, HeLa cells were grown to ~80% confluency in 10-cm petri dishes. The cells were infected in serum-free medium at a multiplicity of infection of 50 TCID50 per cell. Following virus adsorption at room temperature for 30 min, the cells were washed with phosphate-buffered saline (PBS) and further incubated in 5 ml of methionine-free DMEM without serum at 34°C. At the times indicated in the figure legends, the cells were incubated for 30 min with [35S]methionine (10 μCi/ml; 1 Ci = 37 GBq), washed with PBS, and scraped on ice into 0.2 ml of cold buffer containing 20 mM HEPES-KOH (pH 7.6), 150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and a protease inhibitor cocktail (Boehringer Mannheim). Cells were lysed by three cycles of freezing and thawing. Cell debris was pelleted by centrifugation, and protein concentration in the supernatant was measured by the Bio-Rad assay. The protein concentration was adjusted to 4 mg/ml by diluting with PBS. Proteins were denatured with an equal volume of 2× Laemml sample buffer and boiled for 2 min. Aliquots were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide) for protein analysis. The gels were processed for fluorography with En3 Hance (Dupont). Antibodies and the protocol for Western blotting have been described previously (16, 17). Briefly, 40 μg of HeLa cell protein extract was subjected to SDS-PAGE (6% acrylamide). The proteins were transferred onto 0.45-μm pore-size nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked overnight with 5% skim milk in PBS at 4°C and probed with the indicated antibodies for 3 to 4 h at 4°C in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Tween 20, and 5% skim milk. After washing, the blots were incubated in the same buffer, but without skim milk, with donkey anti-rabbit immunoglobulin-horseradish peroxidase (Amersham) at a 1:5,000 dilution for 45 min at room temperature. Following extensive washing, the blots were developed with the Renaissance ECL system (Amersham). The intensities of the bands were quantified by using a BioImage densitometer (Milligen/Biosearch).

HRV-14 infection results in rapid cleavage of eIF4GI between 1 and 2 h after infection (12, 29). However, the shutoff of host protein synthesis lags considerably behind the cleavage of eIF4GI, as significant inhibition of host protein synthesis occurs only after 3 h of infection (12). To determine whether eIF4GII is more resistant to proteolysis than eIF4GI, we examined the integrity of eIF4GII following HRV-14 infection. The state of eIF4GI and the pattern of protein synthesis in
HRV-14-infected HeLa cells were analyzed in parallel. Samples of mock- or virus-infected HeLa cells (50 TCID$_{50}$ per cell) were grown in monolayers in 10-cm petri dishes and pulse-labeled for 30 min with $[^{35}S]$methionine at different times after infection. Aliquots of cytoplasmic extracts were processed for SDS-PAGE followed by autoradiography or Western blotting using polyclonal anti-eIF4GI or anti-eIF4GII antibody as specified in the figure legends.

HRV-14 infection resulted in a feeble inhibition of host cell
protein synthesis (Fig. 1; for quantitation see Fig. 2C). The partial inhibition of host translation (∼60% at 5.5 to 6 h postinfection) occurred concomitantly with the synthesis of the bulk of virus proteins. Inhibition of [35S]methionine incorporation into cellular proteins has been assessed by quantification of the amount of radioactivity in an arbitrarily chosen representative cellular polypeptide (p50). Western blot analysis of infected cell extract revealed that while both eIF4GI and eIF4GII were cleaved during infection, the cleavage kinetics of the two isoforms varied dramatically. Cleavage of eIF4GI was detectable at 1.0 to 1.5 h after infection and was almost complete by 4 h (Fig. 2A). The disappearance of the full-size eIF4GI was accompanied by accumulation of the characteristic cleavage products that have been described for both rhinovirus and poliovirus (12, 13). Cleavage of eIF4GII occurred much later than that of eIF4GI (Fig. 2B). Strikingly, only a minute fraction of eIF4GII was cleaved at 4 h postinfection as judged by the first appearance of the characteristic cleavage products. Quantification of intact eIF4GII showed that about one-third of eIF4GII remained intact even at 6 h after infection (Fig. 2C). The low rate of eIF4GII cleavage is consistent with the inefficient inhibition of host mRNA translation after HRV-14 infection (Fig. 1 and 2C).

Our results show a coincidence between the cleavage of eIF4GII and the inhibition of host protein synthesis in HRV-14-infected cells. eIF4GI is cleaved before eIF4GII, and its cleavage is most probably required for the inhibition of host protein synthesis. Thus, the decrease in translation during HRV-14 infection correlates with eIF4GII cleavage, because this is the last event to occur. Several factors could account for the differential cleavage of eIF4GI and eIF4GII by HRV-14, as is the case for poliovirus infection (17). First, eIF4GII might be a poorer substrate for HRV-14 2Apro than eIF4GI. Second, the fraction of eIF4GI and eIF4GII in the cell that is associated with eIF4E might be different. The susceptibility of eIF4GI and eIF4GII to HRV-2 2Apro proteolysis is dramatically increased when these factors are bound to eIF4E (16, 18, 30). It would, therefore, be important to determine what fraction of eIF4GI and eIF4GII is present as a complex with eIF4E. It is also possible that eIF4GI-eIF4E and eIF4GII-eIF4E complexes differ in their sensitivities to cleavage by 2Apro.

An important issue in addressing the correlation between eIF4GII cleavage and the inhibition of host translation concerns the relative abundance of eIF4GI and eIF4GII in cells. We determined the concentration of eIF4GI and eIF4GII in HeLa cell extracts by quantitative Western immunoblotting (35) using a range of eIF4GI and eIF4GII concentrations. Purified recombinant eIF4GI and eIF4GII proteins expressed by the baculovirus system (16) were used as standards. The eIF4Gs possess an Xpress epitope (Invitrogen) within their N-terminal portions. The concentrations of the eIF4G isoforms were estimated after SDS-PAGE and Coomassie blue staining. The intensities of the recombinant eIF4GI and eIF4GII standards were used to generate a calibration curve. The dashed line indicates the signal of the HeLa cell S10 fraction.

**FIG. 3.** Quantification of eIF4GI and eIF4GII in HeLa S10 cell extracts. (A) HeLa cell protein (40 μg) was analyzed side by side with known amounts of recombinant eIF4GI by SDS-8% PAGE and Western immunoblotting using anti-eIF4GI antibodies (insert). The signals of the eIF4GI standards were used to generate a calibration curve. The dashed line indicates the signal of the HeLa cell S10 fraction. (B) HeLa cell protein (80 μg) was analyzed together with known amounts of recombinant eIF4GII as described above, with anti-eIF4GII antibodies (insert). The signals of the eIF4GII standards were used to generate a calibration curve. The dashed line indicates the signal of the HeLa cell S10 fraction.
any 4E-BP1 dephosphorylation in the course of HRV-14 or
(9), cannot be excluded. We, however, were unable to detect
Finally, it should be noted that although the cleavage of eIF4G
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