A thermosensitive mutant of HRV2 2A proteinase: evidence for direct cleavage of eIF4GI and eIF4GII

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Abstract Infection of mammalian cells with picornaviruses like entero-, rhino-, and aphthoviruses leads to an inhibition of cap-dependent cellular protein synthesis by the cleavage of both translation initiation factors, eIF4GI and eIF4GII. In entero- and rhinovirus infection this cleavage process is mediated by the viral 2A proteinase ($2A^{pro}$). In order to discriminate between a direct mode of eIF4G cleavage and an indirect cleavage via activation of a cellular proteinase, a thermosensitive $2A^{pro}$ mutant (ts-$2A^{pro}$) of human rhinovirus 2 was employed. Temperature shift experiments of cytoplasmic HeLa cell extracts incubated with ts-$2A^{pro}$ strongly support a direct mode of cleavage of eIF4GI and eIF4GII by the viral $2A^{pro}$.

Key words: Human rhinovirus; Host cell shut-off; Viral proteinase; eIF4G cleavage

1. Introduction

Many viruses have developed intricate strategies to manipulate their host cells in order to promote their own replication. Picornaviruses, single-stranded RNA viruses of positive polarity, encode proteinases which are essential for the viral life cycle [1]. In entero- and rhinoviruses, the 2A proteinase ($2A^{pro}$) and the 3C proteinase are necessary to process the viral polypeptide precursor into functional proteins [2]. In addition, these proteinases cleave a limited number of cellular proteins in order to subvert the cellular metabolism for optimal viral replication [3].

In the early stage of the infection the main cellular targets of $2A^{pro}$ are the eukaryotic initiation factors (eIFs) eIF4GI and eIF4GII. Each of the eIF4G homologues forms a complex with eIF4E, which recognises the cap structure of cellular mRNAs, and eIF4A, which functions as a helicase. This complex is termed eIF4F. eIF4F is essential for bringing together the capped mRNAs and the ribosomes (for reviews, see [4–9]). eIF4GI and/or eIF4GII serve as scaffolding proteins by binding eIF4E at the N-terminal domain, and eIF4A and eIF3, a protein complex bound to the small ribosomal subunit, at the C-terminal two-thirds of the molecule [10]. Cleavage of eIF4GI and eIF4GII by $2A^{pro}$ abolishes translation initiation of capped mRNAs, because it segregates the N-terminal domain comprising the cap binding function from the rest of the factor complex. As a consequence, cellular translation initiation is shut down [11,12]. The translation of most picornaviral RNA, which is not capped, remains unaffected or is even stimulated by the cleavage of eIF4GI and eIF4GII as initiation occurs internally at an internal ribosomal entry site (IRES; for reviews, see [5,13–15]).

Numerous experiments have demonstrated that cleavage of eIF4GI and/or eIF4GII is mediated by the $2A^{pro}$ of entero- and rhinoviruses [11,12,16]. However, the mode of this cleavage process is still being debated. In some reports it has been concluded that the major eIF4G cleavage is not directly caused by $2A^{pro}$ but occurs instead via a yet unknown cellular proteinase activated by $2A^{pro}$ [17–20]. Recently, two cellular eIF4GI cleavage activities (eIF4Gase-α and -β) have been identified in poliovirus-infected HeLa cells [21]. These activities are also observed at low level in uninfected cells, in particular upon induction of apoptosis. In contrast, other experiments have provided strong support for a direct cleavage of eIF4G by the $2A^{pro}$ [22–27]. However, in these experiments no discrimination has been made between cleavage of eIF4GI and of eIF4GII. In the present study a different experimental approach has been taken to investigate the mode of eIF4GI and eIF4GII cleavage by using a temperature-sensitive variant of $2A^{pro}$ (ts-$2A^{pro}$) of human rhinovirus serotype 2 (HRV2). The data obtained are consistent with a mechanism of direct cleavage of eIF4GI and eIF4GII by the viral proteinase.

2. Materials and methods

2.1. Peptide cleavage

The TRPIITTA-*para*-nitroanilide (pNA) peptide was a gift from W. Sommergruber (Boehringer Ingelheim, Austria). This chromogenic peptide was found to be cleaved efficiently by $2A^{pro}$ [28–30].

2.2. Proteinase activity assay

Recombinant HRV2 $2A^{pro}$ (wt and ts) was purified as described previously [23,31]. To examine the cleavage activity of $2A^{pro}$, 50 nmol pNA peptide was incubated with 30 nmol $2A^{pro}$ in 500 μl reaction volume containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM dithiothreitol, 1 mM ethylenediaminetetra-acetate, and 5%

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Abbreviations: eIF, eukaryotic initiation factor; HRV2, human rhinovirus serotype 2; $2A^{pro}$, 2A proteinase; pNA, *para*-nitroanilide; eIF4Gase, cellular eIF4GI cleavage activity; IRES, internal ribosomal entry site

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glycerol (buffer A). The cleavage reaction was performed at 20°C for the time indicated and was monitored at 405 nm in a Hitachi U2000 spectrophotometer. To measure the thermosensitivity of ts-2A^{pro}, the proteinase was pre-incubated for 15 min either at 42°C (restrictive temperature) or at 20°C (permissive temperature) and then added to the pNA peptide.

2.3. Preparation of HeLa cell cytoplasmic extract

All manipulations were performed at 4°C. 8×10^7 HeLa cells grown in four 15 cm Petri dishes were washed twice with ice cold phosphate-buffered saline (PBS) containing 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ and once with PBS without Ca^{2+} and Mg^{2+}. The monolayer was scraped off, taken up in PBS and centrifuged at $300\times g$ for 5 min. The pellet was washed with 2 ml of homogenisation buffer HB (250 mM sucrose, 3 mM imidazole, pH 7.4) and centrifuged again. Cells were resuspended in 1 ml HB using a 1 ml pipette tip and were lysed by passing through a 22G needle attached to a syringe for several times. Disruption of cells was monitored by phase-contrast microscopy. Nuclei were pelleted by centrifugation at $1000\times g$ for 10 min. The supernatant was then centrifuged for 30 min at $100\,000\times g$ in a Beckman TLA100.3 rotor. The clear cytoplasmic supernatant was frozen at −70°C or used for in vitro cleavage assays directly. Protein concentration was determined using the Pierce BCA system [32].

2.4. eIF4GI and eIF4GII cleavage

Cleavage reaction of eIF4GI and eIF4GII was performed at 20°C in buffer A. To inactivate thermolabile proteins, HeLa cell cytoplasmic extract was pre-incubated for 15 min at 42°C prior to the cleavage reaction. Where indicated, wild-type 2A^{pro} (wt-2A^{pro}) and ts-2A^{pro} were incubated for 15 min at 42°C. For each cleavage reaction, 40 μg HeLa cell extract was mixed either with 0.5 μg wt-2A^{pro} or with 1.0 μg ts-2A^{pro} in a final volume of 20 μl and incubated at 20°C for 1 h. After treatment for 15 min either at 42°C or at 20°C, 40 μg of fresh HeLa cell extract was added and the samples were incubated for 1 h at 20°C. The reaction was terminated by adding 7 μl 5×Laemmli sample buffer [33].

2.5. Western blot

Cleavage of eIF4GI and eIF4GII was analysed by Western blots. Briefly, aliquots of the cleavage reactions were separated on 6% polyacrylamide gels (SDS–PAGE) and blotted onto Immobilon P membranes (Millipore, Bedford, MA, USA) [34]. The membranes were blocked with 5% fat-free milk powder, 0.5% Tween 20 in PBS and subsequently probed either with rabbit anti-eIF4GI antiserum (1:1000 dilution; raised against the C-terminal region of eIF4GI) or with rabbit anti-eIF4GII antiserum (1:500 dilution; raised against the N-terminal region of eIF4GII as previously described [35]).

3. Results

3.1. Thermosensitivity of the F130Y mutant of 2A^{pro}

To assay the mode of eIF4GI and eIF4GII cleavage, use was made of a thermolabile variant of 2A^{pro} (ts-2A^{pro}) of HRV2 previously identified using a colorimetric screening assay [36]. This 2A^{pro} contains a single amino acid replacement at position 130, the phenylalanine residue of wt-2A^{pro} being changed to a tyrosine [31]. As a consequence of this F130Y change the enzyme is rapidly inactivated at the restrictive temperature of 42°C, but retains its full activity upon incubation at 20°C (permissive temperature).

To examine the activity of 2A^{pro} a fast and convenient in vitro assay has been established for rhinoviral 2A^{pro} using a chromogenic peptide resembling the cleavage sequence of HRV2 2A^{pro} in the HRV2 polyprotein (P8–P1) [28–30]. The proteolytic enzyme activity can be followed by monitoring the absorbance at 405 nm. The chromogenic peptide carries a pNA group at its C-terminus which is cleaved off by 2A^{pro}. Free pNA is determined by the absorbance at 405 nm. Wt-2A^{pro} and the F130Y mutant of 2A^{pro} were incubated with the peptide TRPIITTA-pNA and the kinetics of the cleavage reaction were followed at 20°C. In order to test for thermosensitivity, wt-2A^{pro} and the F130Y mutant were first pre-incubated for 15 min at 42°C or at 20°C before assaying for proteolytic activity with the TRPIITTA-pNA peptide at 20°C. As seen in Fig. 1, the proteolytic activity of wt-2A^{pro} was only slightly affected by pre-incubation at 42°C. After 15 min more than 80% of the 2A^{pro} cleavage activity could still be observed as compared to the control. However, in contrast to pre-incubation at 20°C the F130Y mutant of 2A^{pro} did not exhibit any peptide cleavage activity following a 15 min pre-incubation at 42°C. Even after a prolonged in-

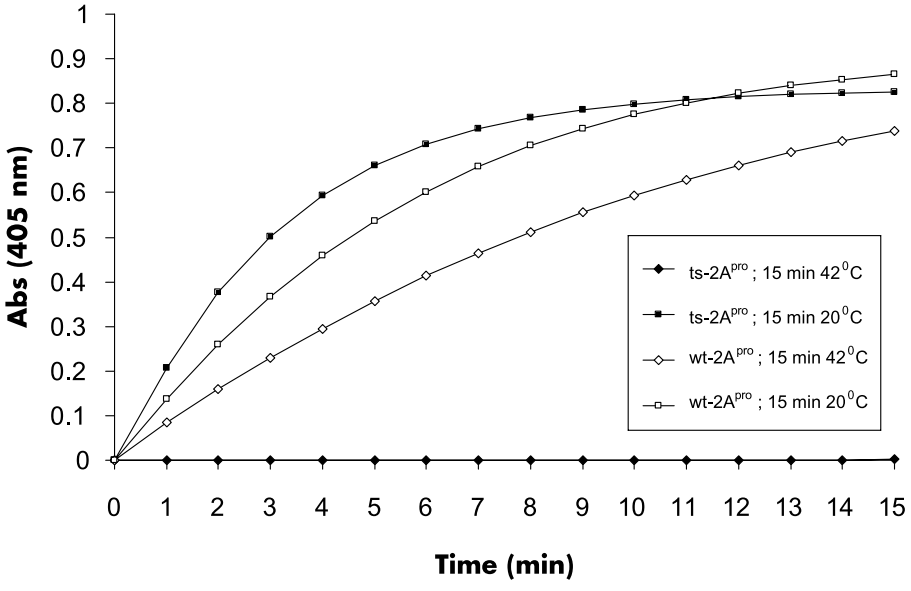

Fig. 1. Rate of cleavage of pNA peptide by 2A^{pro}. 30 nmol wt-2A^{pro} or ts-2A^{pro} were incubated with 50 nmol pNA peptide at 20°C and cleavage was followed as change of absorbance at 405 nm. Both proteinases were pre-incubated for 15 min either at 20°C or at 42°C prior to pNA peptide cleavage.

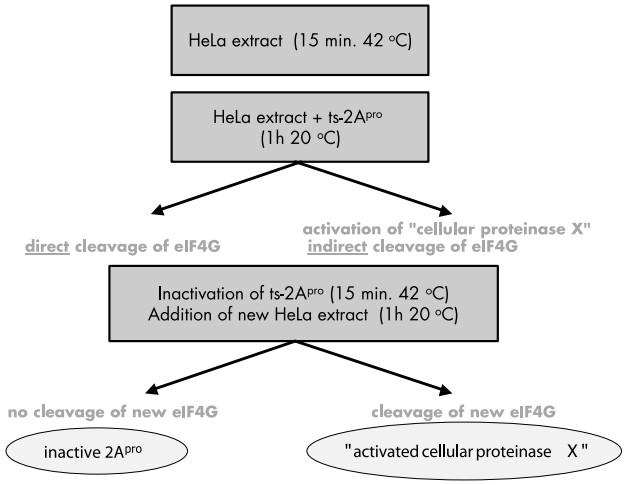

Fig. 2. Scheme of the temperature shift experiment.

cubation at 20°C no increase of the absorbance at 405 nm could be detected (data not shown). Thus the activity of the F130Y mutant of 2A^{pro} could be abolished completely by a shift to the restrictive temperature of 42°C. Therefore, the thermosensitive F130Y mutant of 2A^{pro} (ts-2A^{pro}) was considered an ideal tool to examine the mode of eIF4GI and/or eIF4GII cleavage.

3.2. Cleavage of eIF4GI and eIF4GII

The experimental approach for the elucidation of the mode of eIF4GI and/or eIF4GII cleavage is based on the following experimental protocol (Fig. 2): HeLa cell cytoplasmic extracts were first pre-incubated for 15 min at 42°C in order to inactivate any endogenous thermosensitive proteins. The extracts were then incubated with ts-2A^{pro} for 1 h at the permissive temperature of 20°C in order to achieve complete cleavage of eIF4GI and eIF4GII. Subsequently the mixture was shifted to the restrictive temperature (42°C) for 15 min in order to inactivate ts-2A^{pro}. If the ts-2A^{pro} would have activated a hypothetical 'cellular proteinase X' responsible for cleavage of eIF4GI and/or eIF4GII, addition of new HeLa cell extract and incubation at 20°C would still lead to cleavage of the newly added eIF4GI and eIF4GII, as the temperature shift would have destroyed specifically the activity of ts-2A^{pro}. If, on the other hand, ts-2A^{pro} cleaves eIF4GI and eIF4GII directly, no cleavage of newly added eIF4GI and eIF4GII should occur as the ts-2A^{pro} has been inactivated at the restrictive temperature. Since wt-2A^{pro} remains active at 42°C, eIF4GI and eIF4GII cleavage should occur under all conditions in the control experiments with wt-2A^{pro} carried out in parallel.

The results of these experiments are presented in Fig. 3A,B. Wt-2A^{pro} used in lanes 1, 4 and 6 as well as ts-2A^{pro} used in lane 2 were pre-incubated for 15 min at 42°C. Wt-2A^{pro} or ts-2A^{pro} were added to the HeLa cell extracts as indicated and the samples were incubated for 1 h at 20°C. Under these conditions more than 90% of eIF4GI and eIF4GII was cleaved by wt-2A^{pro} (lanes 1A and 1B) as well as by ts-2A^{pro} (lanes 3A and 3B). As expected, ts-2A^{pro} inactivated by pre-incubation at 42°C for 15 min did not give rise to any eIF4GI and eIF4GII cleavage product (lanes 2A and 2B). The small band close to the position of the eIF4GII cleavage product in lane 2B is presumably due to cross-reactive material as it is also present in the control sample not treated with 2A^{pro} (lane 8B). Addition of fresh HeLa cell extract and incubation for 1 h at 20°C resulted in cleavage of the newly added eIF4GI and eIF4GII both with wt-2A^{pro} (lanes 4A and 4B) and with ts-2A^{pro} (lanes 5A and 5B). A temperature shift to 42°C for 15 min of the sample with wt-2A^{pro} prior to the addition of new HeLa cell extract did not affect the enzymatic activity as newly added eIF4GI and eIF4GII were cleaved at 20°C for 1 h (lanes 6A and 6B). In con-

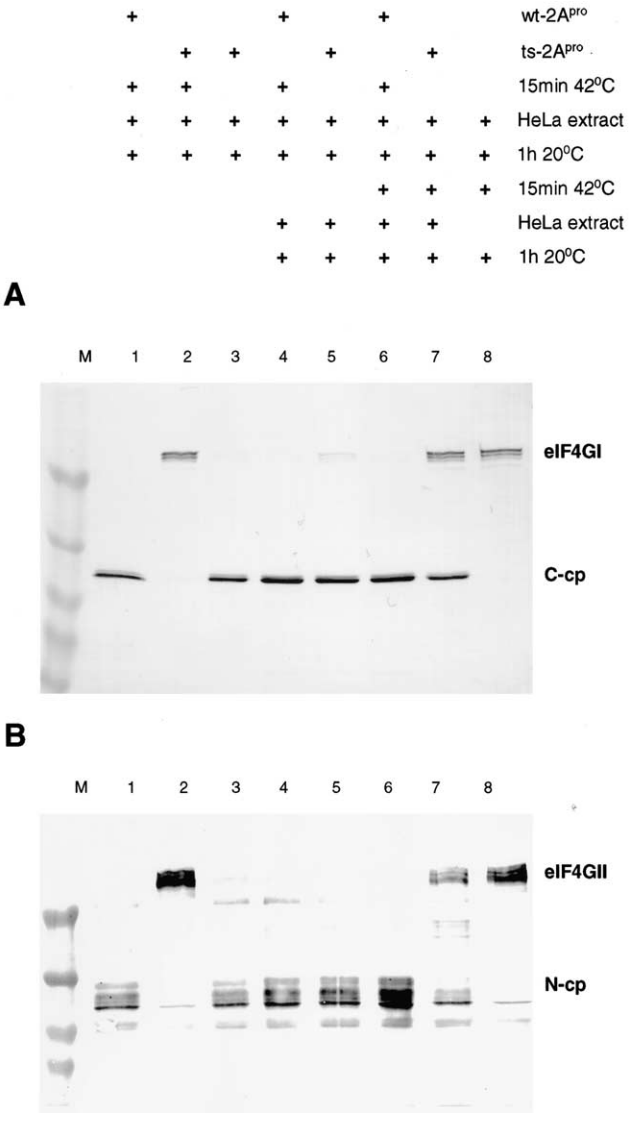

Fig. 3. Direct cleavage of eIF4GI (A) and eIF4GII (B) by HRV2 2A^{pro}. Wt-2A^{pro} used in lanes 1, 4 and 6 and ts-2A^{pro} used in lane 2 as well as HeLa cell cytoplasmic extract were pre-incubated at 42°C for 15 min. 40 μg of HeLa cell extract was then incubated with wt-2A^{pro} or ts-2A^{pro} at 20°C for 1 h. Samples corresponding to lanes 6, 7 and 8 were then heated at 42°C for 15 min, while samples in lanes 3, 4 and 5 were kept at 20°C. Subsequently, 40 μg of new HeLa cell extract was added as indicated (lanes 4–7) and incubation continued for 1 h at 20°C. HeLa cell extract without addition of 2A^{pro} served as control (lane 8). The samples were analysed on 6% SDS–PAGE, blotted onto Immobilon P membranes and probed either with rabbit anti-eIF4GI antiserum (A) or with rabbit anti-eIF4GII antiserum (B). Key: M, molecular weight marker; C-cp, C-terminal cleavage product of eIF4GI; N-cp, N-terminal cleavage product of eIF4GII.

trast, in the parallel experiment with ts-2A^{pro} using temperature shift to 42°C, no new eIF4GI and/or eIF4GII cleavage products were obtained upon addition of new HeLa cell extract and incubation at 20°C for 1 h (lanes 7A and 7B). As shown by comparison of the bands in lanes 7A and 7B with those in lanes 3A and 3B, the amounts of the cleavage products were the same, indicating that they were already produced during the first incubation at 20°C. Thus, the shift to the restrictive temperature of 42°C of the incubation mixture containing ts-2A^{pro} prevented the production of new eIF4GI and/or eIF4GII cleavage products in newly added HeLa cell extract during the second incubation at the permissive temperature. This lack of cleavage was obviously caused by the inactivation of ts-2A^{pro} at 42°C. No evidence for the activation of a cellular proteinase during incubation with 2A^{pro} could be obtained. These data support the notion that 2A^{pro} cleaves eIF4GI and eIF4GII directly without an intermediate step of activation of a latent cellular proteinase.

4. Discussion

After the discovery that mutations in the 2A^{pro} gene of poliovirus prevent the host cell shut-off of protein synthesis and cleavage of eIF4G, many studies were carried out to unravel the mechanism of eIF4G cleavage. Early experiments were hampered by the fact that viral enzymes and cellular initiation factors were not available in highly purified form. The situation changed dramatically when viral enzymes as well as initiation factors were expressed as recombinant proteins in *Escherichia coli* and purified to homogeneity. It was soon discovered that much of the controversy on the cleavage mechanism was due to the fact that eIF4G itself as used in several studies [37,38] is a poor substrate for 2A^{pro}. Addition of eIF4E increases the susceptibility to 2A^{pro} cleavage by about 100-fold [25]. This explains why eIF4G is a good substrate for 2A^{pro} when presented in the form of the eIF4F complex [23]. Nevertheless, the efficiency of eIF4G cleavage in the in vitro reconstituted system using highly purified recombinant proteins was still considerably lower than that found in the infected cell. Efficient eIF4G cleavage comparable to that found in vivo was only obtained upon de novo synthesis of 2A^{pro} in a coupled in vitro transcription/translation system [27]. Yet, from these experiments alone it was not possible to exclude that rapid 2A^{pro} cleavage of eIF4GI and eIF4GII requires the activation of an endogenous cellular proteinase. Furthermore, Zamora et al. [21] have described two cellular eIF4Gases in poliovirus-infected HeLa cells. These eIF4Gases were also found at low level in uninfected cells. Cellular eIF4Gase activity was increased upon induction of apoptosis. As infected HeLa cells are known to induce at least a partial apoptotic response, it is conceivable that eIF4Gases are also active upon poliovirus infection [39,40]. However, neither a relationship between 2A^{pro} and activation of the eIF4Gases nor the possible cleavage activity of the eIF4Gases on eIF4GII has been clarified.

In order to assign a specific function to 2A^{pro} in the mode of eIF4G cleavage a different approach has now been used employing a ts-2A^{pro}. A HeLa cell cytoplasmic extract was at first incubated with ts-2A^{pro} at the permissive temperature to allow complete cleavage of eIF4GI and eIF4GII. Following a shift to the restrictive temperature to inactivate ts-2A^{pro}, fresh HeLa cell extract was added and the incubation was continued at the permissive temperature. Indeed no cleavage of newly added eIF4GI and/or eIF4GII was observed. It can therefore be concluded that the cleavage reaction requires the continuous presence of active 2A^{pro} unless it is assumed that a hypothetical cellular proteinase becomes heat labile upon activation, which at present cannot be excluded. However, the most straightforward interpretation of the data is consistent with a model of direct cleavage of eIF4GI and eIF4GII by 2A^{pro}. The recently described cellular eIF4Gases are apparently not activated under our cleavage conditions as there is no cleavage in the absence of 2A^{pro}.

A mechanism of direct cleavage by 2A^{pro} also solves the problem of differential cleavage of eIF4GI and eIF4GII as observed during picornavirus infection. In contrast to HRV2, where both eIF4GI and eIF4GII are cleaved at a comparable rate [41], the rates of cleavage of eIF4GI and eIF4GII differ significantly during poliovirus and HRV14 infection [12,42]. Both viruses cleave eIF4GII much more slowly than eIF4GI. This difference in sensitivity would be difficult to reconcile in any model of indirect cleavage via activation of a latent cellular proteinase. However, this difference can easily be explained by the different cleavage specificities of the 2A^{pro}s of the various viruses.

eIF4GI and eIF4GII represent ideal targets for the shutdown of host cell protein synthesis by a virus. Both are scaffolding proteins involved in organisation of the protein synthesis machinery complex by binding different initiation factors involved in cellular mRNA recognition. Cleavage by 2A^{pro} sequesters the cap binding functional domain. By this direct eIF4G cleavage, host cell protein synthesis is shut down and IRES-dependent initiation of the picornaviral RNA can proceed without activation of an additional cellular proteinase. This strategy guarantees optimal expression of the picornaviral genome under conditions of maximal suppression of cellular protein synthesis.

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