

# Co-translational folding of an alphavirus capsid protein in the cytosol of living cells

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focus on  
MEMBRANE TRAFFIC

**The Semliki Forest virus capsid protein contains a chymotrypsin-like protease domain that must fold before it can autocatalytically cleave the protein from a larger polyprotein precursor. Here we analyse this cleavage in living mammalian and prokaryotic cells, and find that it occurs immediately after the emergence of the protease domain from the ribosome during protein synthesis. The acquisition of the native conformation of this domain thus occurs rapidly and at the same time as translation. It does not require termination of translation or release from the ribosome, and nor does it involve Hsp70 binding. These results provide direct evidence that protein folding can occur co-translationally in the cytosol of both prokaryotes and eukaryotes.**

One of the open questions in the field of protein folding is to what extent results obtained using *in vitro* approaches reflect the *in vivo* situation. Most of the available information regarding the biophysical principles of folding and the mechanisms of action of individual chaperones has been obtained using full-length proteins and the classical *in vitro* refolding approach<sup>1</sup>. There is increasing evidence, however, that the initial folding of proteins in living cells involves not full-length polypeptide chains but rather ribosome-bound, growing nascent chains (for reviews see refs 2–4). This evidence adds to the complexity of folding studies, because the length of the polypeptide chain is not fixed during co-translational folding, a variety of co-translational modifications occur that may influence the folding process, and folding takes place in an environment at the ribosome about which little is known.

The evidence for co-translational (and co-translocational) folding of proteins in the endoplasmic reticulum (ER) is convincing. For several proteins in living cells, it has been shown that, as the growing polypeptides enter the ER lumen, disulphide bonds and conformational epitopes form<sup>5–8</sup>. For cytosolic proteins, the evidence is much less direct. It rests mainly on results obtained by analysing arrested nascent chains associated with ribosomes after cell lysis<sup>9,10</sup>, and on observations made using eukaryotic and bacterial *in vitro* translation systems<sup>11–15</sup>. On the basis of such experiments, it has been reported that the folding of a model protein was co-translational in mammalian translation systems but post-translational and inefficient in *Escherichia coli* cytosol<sup>10</sup>. This interesting result indicated that eukaryotic and prokaryotic translation/folding machineries may differ in their ability to support co-translational folding.

To circumvent the uncertainties introduced by the slower elongation rate and non-physiological conditions of *in vitro* translation, and the uncontrollable effects of elongation arrest, we have designed an *in situ* approach to study co-translational folding in the cytosol. We analyse the *in vivo* folding of Semliki Forest virus (SFV) capsid (C) protein in infected and transformed mammalian and bacterial cells.

SFV is a well-studied, enveloped virus of the Alphavirus genus<sup>16</sup>. The five structural proteins are translated from a single subgenomic viral RNA as a polyprotein, which is processed by viral and cellular proteases into its component proteins. The C protein (267 amino acids) is the most amino-terminal of the five proteins (Fig. 1a). It contains a positively charged N-terminal domain that interacts with the viral RNA in the nucleocapsid, and a globular carboxy-terminal

domain (residues 112–267, relative molecular mass ( $M_r$ ) 19,000 (19K)) that forms the capsid shell<sup>17</sup>. The X-ray structure of the C-terminal domain (which has dimensions of about  $3.8 \times 2.0 \times 3.4$  nm) shows two  $\beta$ -barrel subdomains reminiscent of those of chymotrypsin<sup>17,18</sup>.

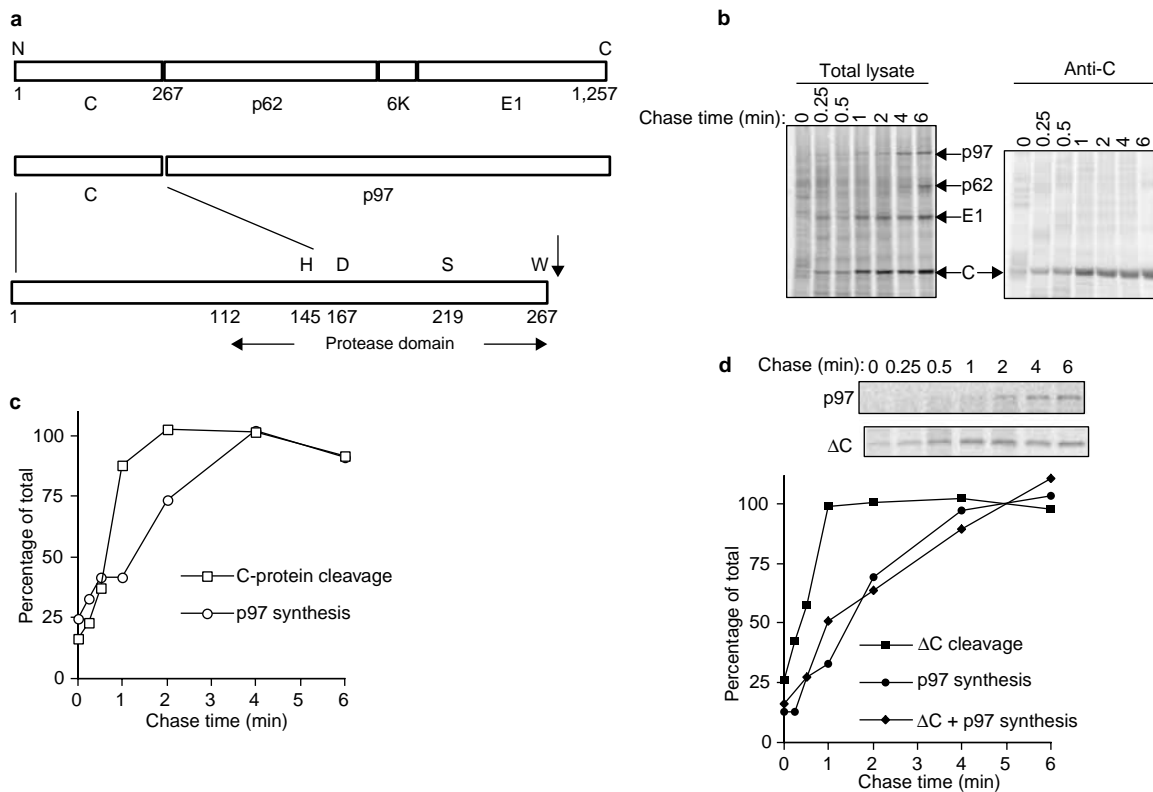
The C protein does, in fact, possess autocatalytic protease activity which it uses to cleave itself off from the polyprotein precursor<sup>19,20</sup>. The three catalytic residues are dispersed in the linear sequence (Fig. 1a). During protein folding these residues must come together with each other and with the C-terminal substrate sequence. Hence, only a folded C-protein protease domain can exhibit autocatalytic activity. To monitor folding of this protein domain in live cells, we took advantage of the fact that the protein only acts *in cis*<sup>21</sup>. After cleavage, the C terminus is left in the active site, preventing further proteolytic activity<sup>17,18</sup>.

## Results

***In vivo* co-translational folding of C protein.** To determine whether folding of the C-protein protease domain occurs before completion of the polyprotein (that is, on a growing nascent chain), we gave SFV-infected Chinese hamster ovary (CHO) cells a 45-s pulse of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, and followed the generation of cleaved C protein during chase. We used *N*-ethylmaleimide (NEM), a membrane-permeable alkylating agent, to inhibit post-lysis cleavage (see below). As the average elongation rate is about 4.5 amino acids s<sup>-1</sup> in these cells<sup>22</sup>, the synthesis of a complete polyprotein chain takes about 4 min. If C-protein cleavage were to occur post-translationally, a labelled C-protein band would not be detected before 3 min of chase.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) showed that a labelled, 33K C-protein band was present immediately following the pulse (Fig. 1b). During subsequent chase, the amount of labelled C protein increased rapidly (Fig. 1b, c). More than half of total C protein was visible by 1 min of chase and complete cleavage was reached before 2 min of chase (Fig. 1c). This indicated that the labelled C protein was separated from the growing chain well before chain termination of the polyprotein had occurred.

This was confirmed by determining the translation time of the cytosolic polyprotein p97 (Fig. 1c). Steady-state accumulation of labelled p97 was reached at 4 min of chase, indicating that C-protein folding and cleavage clearly out-competed polyprotein synthesis. The presence of the other labelled full-length viral proteins



**Figure 1 Co-translational folding and cleavage of C protein in living cells.**  
**a**, Diagram of the SFV structural polyprotein and C protein. Top, following synthesis, C cleaves itself and releases a signal peptide that targets the remaining polyprotein to the ER. E1, 6K and p62 are viral envelope proteins that are cleaved and processed by cellular enzymes. Vertical lines indicate proteolytic cleavage sites. Middle, p97 represents unprocessed, cytosolic, envelope protein sequences that have not been properly targeted to the ER after C-protein cleavage. Bottom, C-protein amino acids 1–112 constitute the SFV genomic RNA-binding domain, which is dispensable for autoprotease activity. The protease domain (residues 112–267) and the catalytic triad, residues H145, D167 and S219, are also indicated. The vertical arrow indicates the site of autocatalytic cleavage (after W267). **b**, Pulse–chase analysis of SFV-infected CHO cells. Following infection, cells were pulsed for 45 s with

$[^{35}\text{S}]$ methionine and  $[^{35}\text{S}]$ cysteine then chased for the indicated times at 37 °C; the reaction was stopped with ice-cold PBS containing 20 mM NEM. Detergent lysates were either analysed directly by SDS–PAGE (left) or first immunoprecipitated with antibody to SFV C protein (right). **c**, The cleaved capsid and p97 bands were quantified by phosphorimaging and plotted as a percentage of maximum. **d**, Folding and cleavage of the C-protein protease domain. CHO cells infected with recombinant vaccinia virus containing the T7 polymerase gene were transfected with a wild-type ( $\Delta\text{wt}$ ) polyprotein construct lacking the N-terminal domain of the C protein. Cells were pulse-chased as in **b** then immunoprecipitated with either anti-SFV (p97) or anti-C ( $\Delta\text{C}$ ) antibody. The time taken to synthesize a cleavage-negative mutant polyprotein ( $\Delta\text{C}$  + p97) was also determined. Further analysis was as in **b**, **c**.

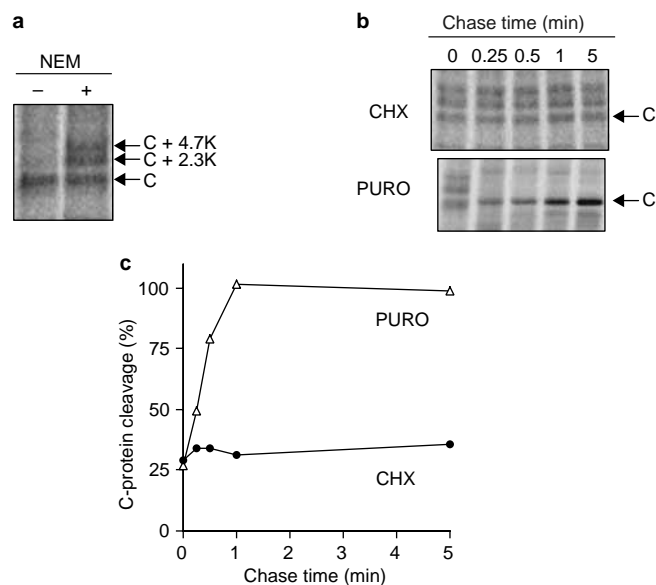
(p97, p62 and E1) at early chase times (Fig. 1b) was due to continued incorporation of label into nascent chains after the C protein had already been cleaved. Note that, at early chase times, no 130K band corresponding to a full-length polyprotein was visible (Fig. 1b, left panel). This provided independent evidence that the C protein is cleaved off before chain termination and release. Taken together, the results show that folding of the C-terminal domain of C protein is co-translational.

The positively charged N-terminal domain of C protein (amino acids 1–112) is dispensable for activation of the C-terminal protease domain<sup>23</sup>. To determine whether the N-terminal domain had any effect on the co-translational folding of C protein, we analysed an SFV structural polyprotein with the N-terminal 112 residues deleted ( $\Delta\text{wt}$ ) in transiently transfected CHO cells. Pulse–chase analysis followed by anti-C-protein immunoprecipitation and SDS–PAGE showed that the protease fragment ( $\Delta\text{C}$ ) was generated immediately following the pulse (Fig. 1d).  $\Delta\text{C}$  cleavage was completed by 1 min, while p97 elongation continued until 4 min. The cleavage time was similar to that of the full-length C protein in SFV-infected cells (Fig. 1b, c). Thus, the N-terminal sequence did not influence the co-translational nature of protease-domain folding. As an extra control, a SFV polyprotein construct,  $\Delta\text{ile}$ , which is

identical to  $\Delta\text{wt}$  except that it has a point mutation (S219I) that inhibits protease activity of the C protein<sup>20</sup>, was expressed. Elongation of the uncleaved polyprotein was not completed until 4–6 min of chase (Fig. 1d). As expected, no  $\Delta\text{C}$  protease fragment was generated in this case (data not shown).

**Folding before termination and release.** To analyse more precisely the time course of C-protein folding, we determined the length of the nascent chains at the time of cleavage using the wild-type virus. We expected the longest nascent chains that still contained the C-protein sequences to be longer than the mature C protein. The difference in length could be used to estimate the time of cleavage. As we expected nascent chains to be transient and of low abundance, it was important to inhibit C protein’s protease activity to minimize the risk of post-lysis cleavage. Although serine protease inhibitors fail to block the autocatalysis of alphavirus C proteins<sup>24</sup>, we found NEM to be a fast-acting and effective inhibitor. Alkylation with iodoacetamide also prevented cleavage, as did diamide, a powerful oxidizing agent (data not shown). Apparently, interference with sulphhydryl groups, of which there are four in the C protein, results in efficient inhibition.

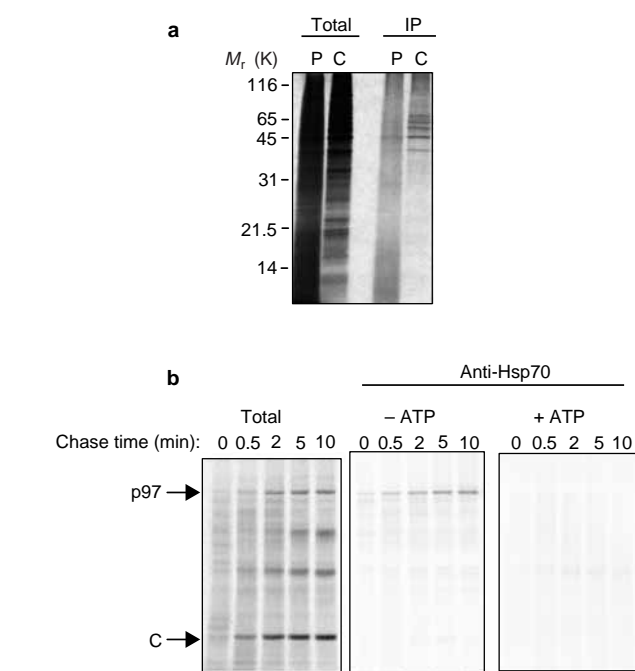
When cells, after a short radioactive pulse, were treated with NEM, lysed, immunoprecipitated with anti-C-protein antibodies,



**Figure 2 Effect of translation inhibitors on C-protein cleavage.** **a**, Detection of uncleaved, C-protein-containing nascent chains. Immediately following a 45-s pulse with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, infected cells were flooded with ice-cold PBS (-NEM) or PBS containing 20mM NEM (+NEM) (no chase). Lysates were immunoprecipitated with anti-C-protein antibody and analysed by SDS-PAGE. The amount of total label in the +NEM lane is greater than that in the -NEM lane because of the density of methionine and cysteine residues in the sequences downstream of the C-protein cleavage site. Relative molecular masses (in thousands) are indicated at the right. *M<sub>r</sub>* of C protein, 33K. **b**, Following radiolabelling, infected cells were chased for the indicated times in the presence of 0.5mM cycloheximide (CHX) or 0.5 mM puromycin (PURO). Samples were processed as in Fig. 1. C, cleaved capsid. **c**, The cleaved capsid bands were quantified by phosphorimaging and plotted as a percentage of the maximum.

and analysed by SDS-PAGE (Fig. 2a), three major bands were seen. The most quickly migrating band corresponded to the authentic C protein. The more slowly migrating bands were converted to the C-protein band during a short chase (Fig. 1b, right panel, compare 0 and 0.25 min of chase), and so represented transient nascent chains of C protein that were longer than the full-length protein. The appearance of the uncleaved nascent chains as bands instead of uniform smears was probably caused by ribosomal pausing<sup>25</sup>. The *M<sub>r</sub>* value of 37.7K for the largest of the nascent-chain bands indicated that C-protein cleavage occurred when the growing nascent chains had reached a length of ~310 amino acids. Autocatalytic cleavage thus occurred when the ribosome had proceeded about 43 amino acids beyond the C terminus of the C protein. As the 60S ribosomal subunit covers the 30–40 most C-terminal residues of a nascent polypeptide<sup>26,27</sup>, we concluded that C protein cleaves itself off almost immediately when its C-terminal end (the cleavage site) exits the ribosome. Cleavage does not seem to occur inside the polypeptide channel of the ribosomal subunit, but rather in the cytosol immediately after extrusion.

The effects of the protein-synthesis inhibitors cycloheximide and puromycin support these conclusions. Cycloheximide arrests elongation without releasing nascent chains from ribosomes<sup>9</sup>, whereas puromycin induces premature chain release from the ribosome<sup>28</sup>. After chase with cycloheximide, no further elongation of nascent chains or further increase in C-protein cleavage was observed (Fig. 2b top panel, Fig. 2c). This indicated that, even with extra time, chains shorter than 37.7K would not be cleaved as long as they were arrested in a ribosome-bound form. In contrast, chase with puromycin resulted in rapid cleavage of all C-protein-contain-



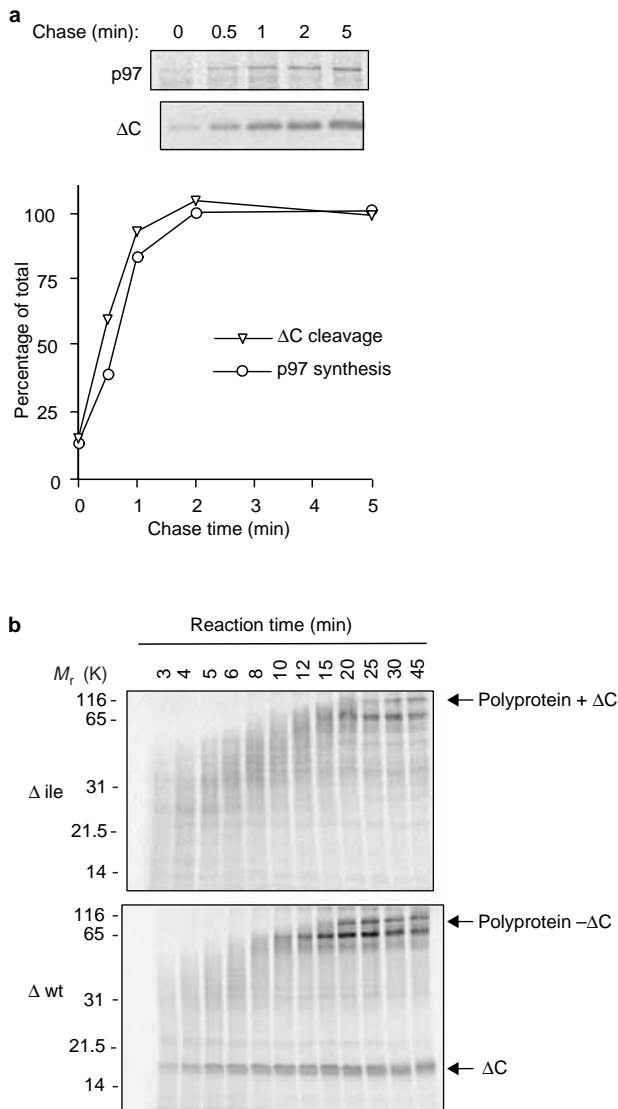
**Figure 3 Association of Hsp70 with SFV structural proteins.** **a**, Precipitation of cellular nascent chains with anti-Hsp70 antibody. Following a 15-s pulse with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, uninfected CHO cells were lysed immediately (pulsed, P) or first chased (C) for 2h. Detergent lysates contained 20U ml<sup>-1</sup> apyrase and were either analysed directly by SDS-PAGE (Total) or first immunoprecipitated with antibody to Hsp70 (IP). **b**, Following a 45-s pulse, SFV-infected cells were chased for the indicated times, and stopped with PBS containing 20 mM NEM. Detergent lysates containing 20U ml<sup>-1</sup> apyrase (-ATP) or 2.5mM Mg-ATP (+ATP) were analysed by SDS-PAGE before (Total; ATP-depleted) and after (anti-Hsp70) immunoprecipitation with anti-Hsp70 antibody.

ing nascent chains larger than 33K (Fig. 2b bottom panel, Fig. 2c). This effect was probably due to the release of these proteins from the ribosome and the resulting premature exposure of the C terminus and the cleavage site.

In summary, activation of autocatalytic cleavage coincided roughly with emergence of the C-terminal sequences from the large ribosomal subunit. Assuming an average rate of elongation, one can estimate that folding of the protease domain was completed in less than 10 s after its synthesis. We conclude that folding of the C-terminal protease domain of C protein is rapid and co-translational. Folding is initiated and completed on ribosome-bound nascent chains in actively translating, unperturbed cells.

**Role of Hsp70.** Next, using co-immunoprecipitation, we investigated whether the ATP-dependent molecular chaperone Hsp70 is involved in the co-translational folding of C protein. Hsp70 is the most abundant chaperone in the mammalian cytosol, and has been shown to interact with elongation-arrested, nascent chains following ATP depletion<sup>9,14,29,30</sup>. Following a short (15-s) pulse of uninfected CHO cells, a smear of labelled polypeptides was immunoprecipitated by an Hsp70-specific monoclonal antibody (Fig. 3a, lanes P). These chains, particularly those between 10K and 40K in size, were no longer detected by precipitation after a 2-h chase (Fig. 3a, lanes C), indicating that they may have been nascent chains in complexes with Hsp70. This finding confirmed previous results obtained with this antibody<sup>9</sup>.

Following a 45-s pulse of SFV-infected CHO cells, C-protein nascent chains and the C protein itself failed to precipitate with the Hsp70-specific monoclonal antibody (Fig. 3b). Although labelled C protein continued to fold until 2 min of chase (Fig. 1b, c, 3b), none



**Figure 4 Folding of C-protein protease domain in the bacterial cytosol.**  
**a**, Pulse-chase analysis of *E. coli* BL21 cells transformed with the SFV polyprotein ( $\Delta$ wt) construct. Spheroplasts were prepared, then pulsed with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 15 s at 30 °C and chased for the indicated times. Lysates were immunoprecipitated with anti-SFV (p97) or anti-C ( $\Delta$ C) antibody and analysed by SDS-PAGE. Bottom, phosphorimaging quantification plotted as a percentage of maximum. **b**, Synchronized *in vitro* translation of the C-protein protease domain in *E. coli* S30 lysate. At 0 min,  $\Delta$ wt or cleavage-negative mutant  $\Delta$ ile DNA was added to an S30 transcription and translation reaction containing [ $^{35}$ S]metabolic label at 25 °C. 100  $\mu$ M aurintricarboxylic acid was added at 2.5 min to allow only a single round of elongation. At the indicated times, reactions were stopped by dilution into ice-cold buffer and analysed by SDS-PAGE.

was detected in association with Hsp70 at early or later chase times in ATP-depleted samples (Fig. 3b). Thus, folding of the C-protein protease domain is apparently Hsp70-independent. In contrast, newly synthesized p97 co-precipitated with Hsp70. p97 is a cytosolic side product of viral protein synthesis and is composed of glycoprotein sequences that failed to be targeted to the ER after C-protein cleavage<sup>16</sup>.

**Co-translational folding in *E. coli*.** We next expressed the polyprotein lacking N-terminal amino acids 1–112 ( $\Delta$ wt) in *E. coli* to determine whether the cytosol of a living prokaryote supports co-

translational folding of the C-protein protease domain. Following a 15-s radioactive pulse of transformed spheroplasts, accumulation of labelled p97 was completed by 2 min at 30 °C (Fig. 4a). This corresponded to a translation rate of about 10 amino acids  $s^{-1}$ . We reasoned that, if C-protein protease-domain folding were post-translational in these cells, the cleaved, radiolabelled fragment would not appear until about 1.7 min after the pulse. The immunoprecipitation results in Fig. 4a show that a labelled protease domain ( $\Delta$ C) was, in fact, present immediately after the pulse. The intensity of the band continued to increase until about 2 min (Fig. 4a). Thus folding and cleavage of the autoprotease domain in live *E. coli* are co-translational.

We also performed *in vitro* experiments using the *E. coli* S30 lysate for transcription and translation. Translation was synchronized by addition of aurintricarboxylic acid, a re-initiation inhibitor that allows only a single round of protein synthesis<sup>31</sup>. To determine the rate of translation, we used the cleavage-negative polyprotein construct  $\Delta$ ile. At 25 °C, the uncleaved polyprotein (polyprotein +  $\Delta$ C), ~125K in size, appeared after about 20 min of transcription and translation (Fig. 4b, top panel), indicating an elongation rate of 1–2 amino acids  $s^{-1}$ . When we analysed the wild-type construct without the mutation, we observed that  $\Delta$ C appeared after 3 min and peaked by about 10 min of the reaction (Fig. 4b, bottom panel). In contrast, the larger polypeptide from which the capsid had been removed (polyprotein –  $\Delta$ C, ~95K in size) did not appear until 12 min. The results indicated that co-translational folding and cleavage of the C-protein protease domain was also supported by the bacterial lysate.

## Discussion

Our results show that the protease domain of the SFV C protein folds co-translationally in the cytosol of living mammalian and prokaryotic cells. Folding to the native, enzymatically active conformation is completed almost immediately after the sequences that constitute the protease domain have moved out of the ribosome. Moreover, folding of the domain appears to be independent of the molecular chaperone Hsp70.

The results are consistent with previous data, obtained using an *in vitro* translation system, that show that the C protein is released shortly after synthesis<sup>32</sup>. However, the rate of elongation during *in vitro* translation is generally ten times slower than the rate *in vivo*. This allows time for events that may not occur during translation in the cell under physiological conditions. The same objection applies to conclusions about co-translational folding drawn from studies in which rhodanese and luciferase were translated *in vitro* using eukaryotic and prokaryotic translation systems<sup>11,13,14,33</sup>. Taken together, however, these *in vitro* studies have shown that a polypeptide chain can fold and attain enzymatic activity while still bound to ribosomes. Our results extend this conclusion to conditions prevailing under normal protein synthesis in live cells, and prove that folding of an active domain occurs before translation terminates.

Although co-translational folding may be suppressed in the prokaryotic cytosol, as suggested by the results of ref. 10, the *in situ* folding of C protein showed that there are polypeptides that can fold co-translationally and attain their active, native structures in the cytosol of a living bacterial cell. Therefore, if there is a difference between the ability of eukaryotes and prokaryotes to support co-translational folding *in vivo*, it cannot be an absolute one. Further *in vivo* studies are needed with authentic bacterial and eukaryotic proteins to determine whether the C protein is exceptional in its capacity to fold so efficiently while still bound to ribosomes. □

## Methods

### Virus infection.

60-mm dishes containing 80–90% confluent CHO K1 cells were washed twice with phosphate-buffered saline (PBS). Cells were infected at a multiplicity of infection of 50 plaque-forming units of SFV (Helsinki strain) per cell in RPMI 1640 medium (Gibco) containing 0.2% BSA and 10 mM HEPES, pH 6.8, at

25°C. After 1 h, the infection medium was replaced with minimal essential medium- $\alpha$  (Gibco) containing 8% fetal calf serum, and the cells were shifted to 37°C.

### Pulse-chase analysis of infected cells.

At 4.5 h post-infection, CHO cells were washed twice with PBS then incubated in cysteine- and methionine-free medium for 20 min at 37°C. Cells were then pulse-labelled for 45 s at 37°C with medium containing 0.5 mCi ml<sup>-1</sup> [<sup>35</sup>S]methionine plus [<sup>35</sup>S]cysteine (*in vivo* cell labelling mix, >1,000 Ci mM<sup>-1</sup>, Amersham). Chase was initiated by adding minimal essential medium- $\alpha$  containing 4.5 mM methionine plus cysteine. At various times, cells were rapidly cooled with ice-cold PBS and lysed in HEPES-buffered saline (HBS; 200 mM NaCl, 50 mM HEPES, pH 7.6) containing 2% CHAPS (Pierce) and chymostatin, leupeptin, antipain and pepstatin (10  $\mu$ g ml<sup>-1</sup> each). PBS and lysis buffer contained 20 mM NEM except where indicated. For subsequent detection of Hsp70 interactions, cells were lysed in the presence of 20 U ml<sup>-1</sup> apyrase (Sigma) or 2.5 mM exogenous Mg-ATP. Nuclei were pelleted by centrifugation for 5 min at 15,000g. Postnuclear supernatants were analysed by 10% SDS-PAGE.

### Immunoprecipitation.

Lysates were rotated at 4°C with anti-C-protein monoclonal antibody C12 (ref. 34) (a gift from I. Greiser-Wilke), anti-SFV polyclonal antibody or anti-Hsc70/Hsp70 monoclonal antibody SPA-822 (StressGen), together with protein A-Sepharose beads (Sigma), for 2 h. Protein-A-bound material was washed with 0.5% CHAPS in HBS and released by boiling in SDS-PAGE sample buffer.

### Plasmid construction.

The complementary DNA encoding the SFV structural polyprotein was derived from plasmid pSFV4 (provided by H. Garoff). Sequences coding for C-protein amino acids 1–112 were deleted using PCR mutagenesis (referred to as  $\Delta$ wt). An additional serine-to-isoleucine substitution at residue 219 was engineered into the  $\Delta$ le construct. Inserts were cloned into a modified pRSET A vector<sup>25</sup> (for *in vivo* experiments using *E. coli* BL21 cells) or pBluescript Ksp<sup>+</sup> vector (Stratagene) (for vaccinia virus co-transfection and S30 lysate experiments) under control of the T7 promoter.

### In vivo expression in E. coli.

*E. coli* BL21 (DE3) pLysS plasmid (Promega) transformed with the  $\Delta$ wt construct were grown in M9 minimal medium containing 0.5 mM isopropyl- $\beta$ -D-thiogalactoside for 3 h at 30°C. Spheroplasts were prepared<sup>26</sup> and resuspended in M9 medium containing 2  $\mu$ g ml<sup>-1</sup> rifampicin but lacking cysteine and methionine for 20 min. Spheroplasts were pulsed with 0.1 mCi ml<sup>-1</sup> [<sup>35</sup>S]metabolic label for 15 s at 30°C. Chase was initiated by adding 5 mM unlabelled cysteine and methionine. Cells were lysed at the indicated times in ice-cold 2% CHAPS buffer containing 20 mM NEM, and soluble supernatants were analysed by 15% SDS-PAGE.

### Expression in E. coli S30 lysate.

1  $\mu$ g  $\Delta$ wt or  $\Delta$ le plasmid DNA was added to the S30 T7 expression system for circular DNA (Promega) together with 0.5 mCi ml<sup>-1</sup> [<sup>35</sup>S]methionine and cysteine, 2  $\mu$ g ml<sup>-1</sup> rifampicin (Sigma) and 200 U ml<sup>-1</sup> recombinant Rnasin (Promega), at 25°C. 100  $\mu$ M aurintricarboxylic acid, an inhibitor of translation re-initiation, was added at 2.5 min to synchronize translation. At the indicated times post-synchronization, reactions were stopped by dilution into ice-cold buffer to a final concentration of 2% CHAPS and 20 mM NEM in HBS and analysed by 15% SDS-PAGE.

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