Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein

Kristiina Takkinen,† Johan Peränen and Leevi Kääriäinen*

Institute of Biotechnology, University of Helsinki, Valimotie 7, SF-00380 Helsinki, Finland

The processing and stability of the non-structural (ns) proteins of Semliki Forest virus were studied in vivo. Virus-specific proteins from infected cells were identified by immunoprecipitation with monospecific antisera. The complete ns precursor, P1234, translated within 7 to 9 min of the start of translation, was processed before the completion of translation into P123 and nsP4. Pulse-chase experiments showed that the mature ns proteins were relatively stable for at least 2 h. Interestingly, the decrease in the amount of the P34 precursor during chase is accompanied by an increase only in the amount of nsP3, which could explain the observed lower amount of nsP4 in infected cells. In cells infected with SFV RNA− mutants ts4 and ts6 maintained at the restrictive temperature, nsP4 but no nsP1, nsP2 or nsP3 accumulated in addition to the ns precursor proteins P1234, P123, P12 and P34. Translation in vitro of mRNAs from a cDNA clone, encoding P1234 with a deletion in the carboxy-terminal protease domain of nsP2, did not yield nsP1, nsP2 or nsP3 but only nsP4, indicating that cleavage at the nsP3/4 sites is independent. Evidently, nsP4 is produced by a nascent cleavage of the growing P1234, catalysed by its own protease activity; the proteolytic cleavages at the nsP1/2 and nsP2/3 sites are catalysed by the protease moiety of nsP2.

Introduction

The 5' two-thirds of the 42S RNA genome of Semliki Forest virus (SFV) is translated as a long non-structural (ns) polyprotein, P1234, from which the mature ns proteins, designated nsP1 to nsP4, are released by proteolytic cleavage. All four alphavirus-specific ns proteins are involved in the replication of virus RNA (Schlesinger & Schlesinger, 1990; Kääriäinen et al., 1987; Strauss & Strauss, 1986). The full-length ns polyprotein P1234 of SFV is processed through two alternative pathways yielding either P123 (nsP1 + nsP2 + nsP3) and nsP4, or P12 (nsP1 + nsP2) and P34 (nsP3 + nsP4). These short-lived processing intermediates have been identified in cells infected with different temperature-sensitive (ts) mutants; their precursor–product relationships have been analysed by peptide mapping (Lachmi & Kääriäinen, 1976, 1977; Kääriäinen et al., 1978; Keränen & Kääriäinen, 1979; Glanville et al., 1978). All the intermediates have been identified in prototype SFV-infected cells, as well as in the in vitro translation products of the 42S RNA (Lehtovaara et al., 1980; Takkinen et al., 1990).

The expression strategy of the ns polyprotein of Sindbis virus (SIN), a closely related alphavirus, is different. Full-length P1234 is produced in low amounts by readthrough of the opal codon located between nsP3 and nsP4, and therefore nsP4 is produced in very low amounts (Strauss et al., 1983, 1984). The proteolytic cleavages at the nsP1/P2 and nsP2/P3 sites in the major ns precursor, P123, are carried out by the protease activity of nsP2 (Ding & Schlesinger, 1989; Hardy & Strauss, 1989; Shirako & Strauss, 1990; de Groot et al., 1990). The protease domain is located in the carboxy-terminal region of SIN nsP2, which shows limited sequence identity with the active site sequence of the cysteine proteinases of the papain family (Hardy & Strauss, 1989).

We have recently studied the processing of the SFV nsP3/P4 site by in vitro translation of mRNAs encoding the P34 protein and its truncated derivatives. It was shown that nsP4 has autoprotease activity, which resides within the first 102 amino-terminal residues, and that this activity can cleave at the nsP3/4 site, but seemingly only in cis (Takkinen et al., 1990).

In this paper we have studied the processing of P1234 in vivo and in vitro using prototype SFV and ts RNA− mutants as well as mRNA constructs transcribed in vitro.

† Present address: VTT, Biotechnical Laboratory, P.O. Box 202, SF-02151 Espoo, Finland.
Methods

Cells and viruses. The origin and cultivation of prototype SFV and the ts mutants, as well as BHK cells, have been described previously (Keränen & Kääriäinen, 1974, 1979).

Radiolabelling of cells. BHK cells in 60 mm dishes were infected with prototype SFV (50 p.f.u./cell) and incubated at 37 °C. The medium was replaced with methionine-free MEM 30 min before labelling at 2.5 h post-infection (p.i.); at this time cells were exposed to 200 µCi/dish [35S]methionine (1000 Ci/mmol; Amersham) for various times. In the early and late phases of infection the labelling was done 1 h 45 min and 4 h p.i., respectively. When samples were chased after labelling, the cells were incubated in MEM containing a 20-fold excess of unlabelled methionine.

Hypertonic treatment of the cells, for synchronization of the initiation of translation, was done essentially as described (Kääriäinen et al., 1978; Keränen & Ruohonen, 1983). Infected cells were treated with MEM containing 335 mm-NaCl for 30 min before labelling, the hypertonic MEM was replaced by MEM containing 0.1 M-sucrose and [35S]methionine 3 h p.i., and the cells were labelled for various times. Cells infected with ts4 or ts6 were first incubated at the permissive temperature (28 °C) to allow the synthesis of virus-specific RNA. At 6 h p.i. the cells were transferred to the restrictive temperature (39 °C) and labelled for 15 min, followed by a 2 min chase. In all experiments the cells were lysed in 2% hot (60 °C) SDS.

Immunoprecipitation. Cell lysates were passed through a 20-gauge needle 10 times to shear the DNA, boiled for 2 min and diluted (1/10) with NET buffer [1% NP40, 50 mm-Tris·HCl pH 8.0, 400 mm-NaCl, 5 mm-EDTA, 0.02% NaN3 and 100 units/ml Trasylol]. An aliquot (700 µl) of the diluted lysate was incubated with 10 µl of the respective antiserum at 4 °C overnight and Protein A-Sepharose (Pharmacia), 250 µl of 2.5% solution, was used to collect the immune complexes by incubation in an end-over-end shaker for 60 min. The beads were washed five times with NET buffer and once with 10 mm-Tris·HCl pH 6.8, and the immunoprecipitates were dissociated from the beads with Laemmli sample buffer and analysed by SDS-PAGE in 7.5% or 7.5 to 15% gels (Laemmli, 1970). The gels were fluorographed according to Bonner & Laskey (1974) and densitometric tracing of the autoradiograms was carried out using Ultrascan XL Enhanced Laser Densitometer (LKB).

In vitro transcription-translation constructions. A cDNA clone encoding the P1234 precursor (pTSF1234) has been cloned under the control of the T7 promoter in a pGEM vector (Promega) (unpublished data). The pTSF15234 construct derived from pTSF1234 contains a Scal [nucleotide (nt) 3269 in SFV cDNA]-SalI (nt 3791) fragment deletion within the carboxy-terminal coding region of nsP2. The deletion was produced using a subclone containing the HindIII (nt 1944)-XhoI (nt 5303) cDNA fragment of SFV 42S RNA (Takkinen, 1986). This clone was first cleaved with SalI (nt 3791), the 5' protruding SalI ends were filled in with the Klenow fragment of DNA polymerase I to allow in-frame fusion with the blunt-ended Scal fragment, and then cleaved with HindIII. The HindIII (nt 1944)-SalI (nt 3791) fragment was replaced by the HindIII (nt 1944)-SalI (nt 3269) fragment from the same cDNA clone, resulting in a deletion of 522 bp, encoding 174 amino acid residues. The deletion was transferred to pTSF1234 by replacing its HindIII-XhoI fragment with that of the deletion clone. For transcription, both clones were linearized at the multi-linker region of the vector using BamHI; in vitro transcription and translation, and immunoprecipitation analysis, were done as described earlier (Takkinen et al., 1990).

Results

Stability of SFV-specific ns proteins

BHK-21 cells were infected with prototype SFV at 37 °C and 2.5 h p.i. exposed to [35S]methionine for 30 min and chased for 0, 30, 60 or 120 min (lanes 1 to 4, respectively). Virus-specific proteins were detected by immunoprecipitation with anti-nsP1 (a-1), anti-nsP2 (a-2), anti-nsP3 (a-3) and anti-nsP4 (a-4) antisera and analysed by SDS–PAGE in 7.5% gels. Lane M, M, markers. The positions of the precursors and mature ns proteins are indicated.

In Fig. 1, the broadness of the nsP3 band is evidently due to differences in the phosphorylation of the protein (Peränen et al., 1988). The amount of nsP4 clearly was lower than that of nsP3 throughout the chase period. Less nsP4 relative to nsP1, nsP2 and nsP3 in SFV-infected cells has also been observed by Keränen & Ruohonen (1983) and by Sawicki & Sawicki (1986).

The processing of P34 and the accumulation of mature nsP4 was studied also in the early (1 h 45 min p.i.) and late (4 h p.i.) phases of infection (Fig. 2). Infected cells were labelled with a 5 min pulse to allow the detection of the precursor proteins, chased for 0, 5, 15, 30 or 60 min, and cell lysates were immunoprecipitated with anti-nsP3 and anti-nsP4 antisera. P1234 and P123 were clearly present in the early phase of infection whereas late in infection they were hardly detectable.

In the early phase, P1234 was processed within 15 min, and P123 and P34 within 60 min. There was an obvious
increase in the amount of nsP3 during the chase, whereas negligible change was seen in the amount of nsP4 (Fig. 2). Quantification of the precursor protein as well as nsP3 and nsP4 bands by densitometry showed that there was an almost quantitative transfer of radioactivity from nsP3 sequences of P1234 (nine of 53 methionine residues) and P34 (nine of 27 methionine residues) to nsP3, but none to nsP4 (Fig. 3). The same was true for samples derived from cells labelled late in infection (Fig. 2). These results strongly suggest that nsP4 is not derived from the P34 precursor. The amount of nsP4 was always highest immediately after the pulse and instead of increasing during the chase it decreased slightly. Exactly the opposite was seen with nsP3, indicating that P34 is a precursor only for nsP3.

**Processing kinetics of SFV ns proteins**

Initiation of translation in cells infected with prototype SFV was synchronized 2.5 h p.i. by hypertonic treatment. The cells were labelled thereafter for increasing time periods in a slightly hypertonic medium (Kääriäinen et al., 1978), followed by immediate lysis in hot SDS to prevent further processing during treatment of the samples. This labelling allowed visualization of the synthesis of nascent polypeptides and their early intermediates (Fig. 4). After a 3 min pulse, nascent polypeptides, derived from the amino terminus of the P1234 polyprotein, could be precipitated only with anti-nsP1 antiserum. Anti-nsP3 antiserum precipitated a slightly smaller protein than P123 2 min later, whereas anti-nsP2 antiserum precipitated a substantial amount of protein migrating at the position of P12, suggesting that...
K. Takkinen, J. Peränen and L. Kääriäinen

Fig. 4. Translation and processing of SFV ns proteins in vivo. BHK cells infected with SFV were treated with hypertonic salt solution 2.5 h p.i. for 30 min to synchronize the initiation of translation and were then labelled for increasing times with [35S]methionine. SFV precursors and mature proteins were identified by immunoprecipitation (lanes 1 to 4, anti-nsP1, anti-nsP2, anti-nsP3 and anti-nsP4 antisera) and analysed by SDS–PAGE in a 7.5 to 15% gel. Lane M, M', markers. The positions of the different ns proteins are indicated.

this was cleaved before the completion of the translation of P123. Anti-nsP4 antiserum precipitated low amounts of labelled proteins migrating at positions of P1234 and P34 7 min after synchronous initiation; at the same time, anti-nsP2 and anti-nsP3 antisera precipitated heavily labelled protein migrating at the position of P123, suggesting that it was cleaved prior to or at the completion of translation of P1234. Within 9 min all precursors and mature nsP4 could be clearly visualized, as could a low amount of nsP2. Mature nsP1 and nsP3 were clearly distinguishable only after a 16 min labelling period. The high M, products, which were precipitated after 11 min with anti-nsP3 antiserum, were probably aggregates formed during cell lysis. The complete ns protein-encoding region was translated within 7 to 9 min, giving a maximum translation rate of about 270 to 350 amino acids/min for P1234, which is 2431 amino acids long. This value is in the same range as the earlier estimation by Keränen & Ruohonen (1983).

Processing of SFV ns proteins in cells infected with ts4 and ts6

The phenotypes of several ts, RNA− SFV mutants with defects in RNA replication have been characterized previously (Keränen & Kääriäinen, 1975, 1979). Two of these, ts4 and ts6, synthesize less subgenomic 26S RNA and have a defect in the processing of P1234 at 39 °C. BHK cells infected with ts4 or ts6 were allowed to start virus replication at the permissive temperature (28 °C), shifted to the restrictive temperature (39 °C) 6 h p.i., and labelled with [35S]methionine for 15 min and chased for 2 min. Proteins in the cell lysates were immunoprecipi-

Fig. 5. Proteolytic processing in BHK cells infected with the ts4 and ts6 mutants. Infected cells were exposed to [35S]methionine for 15 min after hypertonic treatment and were chased for 2 min. Virus-specific proteins were identified by immunoprecipitation (lanes 1 to 4, anti-nsP1, anti-nsP2, anti-nsP3 and anti-nsP4 antisera) and analysed by SDS–PAGE in a 7.5 to 15% gel. The total cell lysates (lane T) before immunoprecipitation are shown; the heavily labelled proteins migrating faster than the ns proteins represent the structural proteins of SFV. Lane M, M', markers.
Proteolytic processing in vitro of an SFV ns polyprotein with a deletion in the carboxy terminus of nsP2

The putative carboxy-terminal proteinase activity of nsP2 was studied by analysing the in vitro translation products of mRNAs transcribed from clones encoding complete P1234 (pTSF1234) and a protein with a deletion of 174 amino acid residues in the carboxy-terminal region of nsP2 (pTSF1Δ234) (Fig. 6a). The products were immunoprecipitated with all four antisera before analysis by SDS-PAGE (Fig. 6b). Identification of the precursors was hampered by non-specific proteolysis and premature termination. The anti-nsP1 antiserum and especially the anti-nsP4 antiserum immunoprecipitated contained smaller products, typical of the reticulocyte cell-free translation system used. However, the mature ns proteins could be identified with certainty.

No nsP1, truncated nsP2 (67K) or nsP3 was detected among the translation products of mRNA derived from pTSF1Δ234 (Fig. 6b, lanes 1 to 3). Lane 4 showed similar products irrespective of the deletion. The smaller bands were seen also in translation products lacking the amino-terminal proteinase region of nsP4, indicating that they are proteolytic products of the translation system itself.

Proteolytic processing of SFV polyprotein

Fig. 7. Translation and proteolytic processing of the SFV ns polyprotein. The open reading frame starting at the ATG (nt 86) and ending at the TAA stop codon (nt 7379) encodes the P1234 polyprotein of 2431 amino acids (aa), which is proteolytically cleaved to yield nsP1 (537 aa), nsP2 (798 aa), nsP3 (482 aa) and nsP4 (614 aa) (Takkinen, 1986). Proteolytic cleavage site sequences are indicated. The two alternative processing pathways of P1234 yielding either P12 and P34 (i), or P123 and nsP4 (ii), are schematically shown at half the scale of the upper map. The amino-terminal proteinase domain of nsP4 is marked with dots and the putative carboxy-terminal proteinase domain of nsP2 with diagonal lines. The broken line in (i) represents the hypothetical breakdown product of P34 processing.
cleavage does take place at the nsP3/4 junction. The events of the two alternative proteolytic processing pathways of the ns polyprotein, P1234, of SFV are shown in Fig. 7. The present results show that the first proteolytic cleavage takes place at some frequency between nsP2 and nsP3, producing P12 and P34 (Fig. 7, pathway i). If this cleavage at the nsP2/P3 site does not occur, the full length ns polyprotein P1234 is produced (Fig. 7, pathway ii).

The P12 precursor is obviously processed further by nsP2 proteinase, whereas the cleavage at the nsP3/P4 site of P34 is catalysed by nsP4 proteinase (Takkinen et al., 1990). An interesting discrepancy in the processing of P34 was seen both early and late after infection. During the chase there was a remarkable balance in the amount of radioactivity disappearing from P34 and that appearing in nsP3, suggesting that all nsP3 sequences from the P34 precursor were effectively transferred into nsP3 (Fig. 3). On the contrary there was no increase in the radioactivity of nsP4 during the chase period, suggesting that nsP4 sequences in the P34 precursor were degraded during the chase period. This would explain the observed lower amount of nsP4 relative to the other ns proteins in SFV-infected cells.

The early appearance of nsP4 together with P123 (Fig. 4, 9 min time point) suggests that the proteolytic cleavage of P1234 to P123 and nsP4 is the prevalent pathway in the production of nsP4 (Fig. 7, pathway ii). In pulse-chase experiments, carried out early and late in infection, the amount of nsP4 was maximal immediately after the pulse, whereas the amount of nsP3 increased steadily during the chase. Interestingly, densitometric quantification analysis of the early pulse-chase experiment (Fig. 3) revealed that there was a clear decrease in the amount of the complete ns polyprotein P1234 within the first 5 min of chase, which was accompanied by a respective increase in the radioactivity associated with nsP3 but not that with nsP4. Thus complete P1234 was not able to feed the pool of nsP4, indicating that nsP4 might be produced only from a nascent cleavage of the growing P1234. Therefore, we assumed that the proteinase of nsP2 was inactive at 39°C in ts4- and ts6-infected cells; the only cleavage occurring effectively was that catalysed by the nsP4 proteinase. Support for this was obtained from in vitro translation studies of an mRNA encoding an ns polyprotein with a deletion of 174 amino acid residues in the putative proteinase domain of nsP2. The translation products did not include mature nsP1, nsP2 or nsP3, whereas nsP4 was produced. This indicates that nsP4 is an independent proteinase, which catalyses its own cleavage from the ns polyprotein (Fig. 7, pathway ii), but perhaps only in cis, as suggested previously (Takkinen et al., 1990). The other cleavages are evidently carried out by the nsP2 proteinase, which can also act in trans (Ding & Schlesinger, 1989; Hardy & Strauss, 1989; Hardy et al., 1990; Shirako & Strauss, 1990; de Groot et al., 1990).

Based on sequence analysis of RNA- ts mutants of SIN, nsP4 is the catalytic subunit of the RNA-dependent RNA polymerase of alphaviruses (Hahn et al., 1989a). nsP4 also contains the Gly-Asp-Asp motif surrounded by hydrophobic sequences, which is conserved in RNA-dependent polymerases (Kamer & Argos, 1984; Argos, 1988). We have shown here that nsP4 of SFV is relatively stable during a 2 h chase, although the amount present is less than that of other ns proteins. This result is compatible with those showing that the SFV RNA polymerase is active for several hours in the presence of protein synthesis inhibitors (Kääriäinen & Söderlund, 1978); under these conditions 42S RNA positive strands and 26S RNAs are synthesized exclusively. On the contrary, the synthesis of 42S RNA minus strands, which takes place early in infection, requires continuous protein synthesis (Sawicki & Sawicki, 1980; D. L. Sawicki et al., 1981; S. G. Sawicki et al., 1981). It has been postulated that for the synthesis of minus strands, new polymerase complexes are assembled, perhaps one complex for each minus strand (Kääriäinen et al., 1987). If this hypothesis is true, the 'early RNA polymerase' would be nascent, whereas the 'late RNA polymerase' would have the same components as a stable complex.

In SIN-infected cells, nsP4 is synthesized only in low amounts by readthrough of a termination codon (Strass et al., 1984; Li & Rice, 1989; Hardy & Strauss, 1988; Lemm et al., 1990; Shirako & Strauss, 1990; de Groot et al., 1990); it is difficult, therefore, to detect nsP4 in SIN-infected cells. Even after replacing the opal codon with sense codons, the amount of nsP4 remains low and is often undetectable by immunoprecipitation (Li & Rice, 1989; Shirako & Strauss, 1990). de Groot et al. (1990) have recently suggested that in SIN-infected cells nsP4 is used specifically for the synthesis of minus strands during early infection; this protein would be replaced later by P34, which in SIN-infected cells is fairly stable. For SFV-infected cells this possibility seems improbable.
because nsP4 is relatively stable and disappears with a half-life of 10 to 20 min both early and late in infection, and thus could not serve as a stable catalytic subunit of the RNA polymerase. To explain the relatively low amount of nsP4 in SFV-infected cells, we would like to propose the following hypothesis. Only nsP4 sequences which are released from the nascent cleavage of the ns polyprotein produce nsP4. Only those nsP4 molecules which immediately become part of the RNA polymerase complex are stabilized and preserved from degradation.

We thank Dr Sirkka Käräinen for helpful discussions and Ms Annikki Kallio for excellent technical assistance. This work was supported by the Sigrid Jusélius Foundation.

References


