Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease

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The RNA replicase proteins of Semliki Forest virus (SFV) are translated as a P1234 polyprotein precursor that contains two putative autoproteases. Point mutations introduced into the predicted active sites of both proteases nsP2 (P2) and nsP4 (P4), separately or in combination, completely abolished virus replication in mammalian cells. The effects of these mutations on polyprotein processing were studied by in vitro translation and by expression of wild-type polyproteins P1234, P123, P23, P34 and their mutated counterparts in insect cells using recombinant baculoviruses. A mutation in the catalytic site of the P2 protease, C478A, (P2 CA) completely abolished the processing of P12 CA34, P12CA3 and P2CA3. Co-expression of P23 and P12 CA34 in insect cells resulted in in trans cleavages at the P2/3 and P3/4 sites. Co-expression of P23 and P34 resulted in cleavage at the P3/4 site. In contrast, a construct with a mutation in the active site of the putative P4 protease, D6A, (P1234DA) was processed like the wild-type protein. P34 or its truncated forms were not processed when expressed alone. In insect cells, P4 was rapidly destroyed unless an inhibitor of proteosomal degradation was used. It is concluded that P2 is the only protease needed for the processing of SFV polyprotein P1234. Analysis of the cleavage products revealed that P23 or P2 could not cleave the P1/2 site in trans.

Introduction

Semliki Forest virus (SFV) is a member of the Alphavirus genus of the Togaviridae family. The SFV 5′-proximal open reading frame is translated as a large polyprotein (P1234) of 2432 amino acid residues followed by rapid cleavage into non-structural proteins nsP1–nsP4, hereafter designated as P1–P4. As intermediates, polyproteins containing P1 plus P2 (P12) and P3 plus P4 (P34) as well as P123 have been detected (Lachmi & Kääriäinen, 1976; Keränen & Ruohonen, 1983; Takkinen, 1986; Kääriäinen et al., 1987; Takkinen et al., 1991). The expression strategy of the non-structural proteins of the related alphavirus Sindbis (SIN) is somewhat different. The open reading frame of P1234 is interrupted by an opal termination codon, which must be suppressed in order to achieve translation of P4. Thus, the major translation product is a shorter P123 polyprotein, which is produced only at a low level by proteolytic cleavage from P1234 (Strauss et al., 1983; Li & Rice, 1989, 1993; Strauss & Strauss, 1994). Evidently, a fraction of the P4 protein expressed is rapidly degraded in the infected cells by the proteosomal system (de Groot et al., 1991), which makes the detection of P4 problematic in SIN virus-infected cells (Hardy & Strauss, 1988).

The processing of SIN virus P123 and P1234 has been studied extensively (Hardy & Strauss, 1988, 1989; Ding & Schlesinger, 1989; Hardy et al., 1990; Shirako & Strauss, 1990; Strauss et al., 1992; Strauss & Strauss, 1994). According to these studies, P2 is responsible for all proteolytic cleavages of P1234 and P123. Experiments performed in vitro indicate that P2, P123, P23 and P2 are proteases with slightly different substrate specificities (Shirako & Strauss, 1990). Protease activity has been mapped to the carboxy-terminal half of P2, which has a domain similar to papain-like proteinases with active site cysteine (C^CS^) and histidine (H^HH^) residues (Ding & Schlesinger, 1989; Hardy & Strauss, 1989; Strauss et al., 1992). Mutation of these residues results in the loss of P2 protease activity and failure to produce infectious RNA when transcribed from the infectious SIN virus cDNA clone (Strauss et al., 1992). Construction of cleavage site mutants of the SIN virus...
polyprotein, which express P123 and P4 or P1, P23 and P4, results in low yields of virus but the early minus-strand RNA synthesis is as efficient as that of the wild-type virus (Shirako & Strauss, 1994). The specific role of P123 and possibly P23 in SIN virus minus-strand RNA synthesis has also been demonstrated by expressing SIN virus P123 and P4 using recombinant vaccinia viruses (Lemm & Rice, 1993a, b; Lemm et al., 1994). Only a low level of plus-strand RNA synthesis can be carried out by P123 together with properly cleaved P4, with tyrosine as the amino-terminal residue (Lemm & Rice, 1993 b; Lemm et al., 1994). Thus, SIN virus-directed RNA synthesis is controlled by expression and cleavage of the non-structural polyprotein.

In SFV-infected cells, translation of the non-structural polyprotein should yield equimolar amounts of P1–P4. However, only about 20% of the expected amount of P4 is found in pulse-labelling experiments (Takkinen et al., 1991). The appearance of P4 as the first mature protein, during in vitro translation and in SFV-infected cells, suggested that it may be cleaved immediately after completion of the translation of P1234. Hypertonic synchronization of translation initiation in cells infected with polyprotein cleavage mutants ts4 and ts6 of SFV supported immediate P4 cleavage (Takkinen et al., 1991). There was also rapid processing of the P34 precursor, which yielded prevalent amounts of mature P3, while P4 seemed to be degraded. SFV P4 has at its amino terminus a series of amino acids that have been utilized for RNA polymerase production but would be lethal for the virus, processing of P1234 was carried out exclusively by the P2 protease.

Similarly, the P2 5′ end and the P3 5′ end were joined. Appropriate sequences were subsequently cloned between these ends from the infectious SFV cDNA (icsSFV4, Liljestrom et al., 1991) to ensure the creation of functional polyproteins, P1234, P123 and P23. The P2 cysteine 478 to alanine (C78A) and P4 aspartic acid 6 to alanine (D6A) mutants were generated using the unique site-elimination method (USE mutagenesis kit, Pharmacia). The EcoRI restriction fragment from the plasmid encoding P2 C78A (P278A) was transferred into EcoRI-digested plasmids encoding P1234, P123 and P23 to create plasmids encoding P1234, P123 and P23, respectively. The Nelf/Xhol restriction fragment from the plasmid encoding P47A (P47A) was transferred into Nelf/Xhol-digested plasmids encoding P1234 or P1234 to create constructs designated P1234DA and P1234DA. The Xhol/HindIII-digested fragments containing coding sequences for the 77 C-terminal amino acids of P3 and full-length P4 from clones P1234 and P1234DA were transferred into plasmid pTSF3 (Peranen et al., 1988) to create plasmids encoding P34 and P34DA. The same fragments, cloned into the EcoRV-linearized vector plasmid pGEM-5 (Promega), were used to create plasmids designated P34 and P34DA. To create DNA constructs encoding full-length P3 and the first 102 amino acids of P4 (plasmids P34A and P34A4), the KpnI/HindIII-digested fragment was deleted from P34 and P34DA. Finally, a SnaI/BglII-digested fragment (nt 1799–6713) from icsSFV4 (Liljestrom et al., 1991) was substituted with corresponding fragments from plasmids P1234DA and P1234DA or P1234DA to create full-length mutant cDNA-containing plasmids designated icSFVMut2DA, icSFVMut4DA and icSFVMut2DA, respectively.

### Methods

**DNA constructs.** In order to make constructs encoding P1234, P123 and P23, the 5′ ends of P1 and P2 were mutated to create a site for BamHI using PCR. The 3′ ends of the coding sequences for P4 and P3 were altered by PCR mutagenesis to form a site for SphI. The sequence was verified and the DNA fragment encoding P1 was joined to those encoding P3 or P4 using suitable restriction sites and blunt-end ligation.

**In vitro transcription/translation of full-length cDNA clones and transfection of cells.** DNA from icSFV4, icSFVMut2DA, icSFVMut4DA and icSFVMut2DA was linearized by digestion with SphI and purified on QiAquick PCR purification column (Qiagen). Capped RNA was transcribed from 5 μg of linearized plasmid in 100 μl reaction mix comprising 20 μl of 5 × transcription buffer (Promega), 1 mM mGpppG (Pharmacia), 5 mM DTT, 3 mM each of UTP, ATP and CTP, 0.5 mM GTP (Pharmacia), 100 U RNasin (Promega) and 30 U SP6 RNA polymerase (Promega). The mixture was incubated at 37 °C for 2.5 h, GTP was added to a final 3 mM concentration and incubation was continued for a further 1 h at 37 °C. RNA transcripts were used for in vitro translation with a coupled T7-transcription/translation kit (Promega), without the T7 polymerase. The standard 25 μl reaction mixture contained 1 μg of RNA and 10 μCi of [35S]methionine (Amersham). Reaction products were analysed by SDS–PAGE in 10% gels and by autoradiography. The same transcripts were used to transfect HeLa and BHK cells, essentially as described (Peranen & Kääriäinen, 1991). Infection was monitored at 7 and 16 h post-transfection by immunofluorescence with polyclonal antiserum against P1 and the envelope protein E2 (Laakonen et al., 1998).

*In vitro* translation was performed using the T7-transcription/translation kit (Promega), according to the manufacturer’s instructions. The translation mixture was incubated at 30 °C for 40 min. In some experiments, the *in vitro* translation reaction was stopped by the addition of cycloheximide to a final concentration of 0.6 μg/ml. For *in trans* cleavage experiments, substrate and protease proteins produced in *in vitro* were incubated, mixed and incubated for a further 40 min at 30 °C to allow cleavages to occur.

**Construction of recombinant baculoviruses expressing SFV polyproteins.** The Bac-to-Bac Recombinant Baculovirus system (Gibco BRL) was used according to the manufacturer’s protocols. The SFV polyprotein-coding fragments were released by digestion with BamHI/ SphI from plasmids P1234, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA, P1234DA and cloned into the plasmid vector pFastBac1 (Gibco BRL), which was
digested with the same enzymes. Recombinant baculoviruses expressing these constructs were created and designated Bac1234, Bac1234DA, Bac1234DA-3, Bac1234DA-3, Bac1234DA-3, Bac1234DA, Bac34, Bac34DA, BacA34DA, Bac34A and Bac34DA, respectively. The correct size of the expressed polyproteins was verified by SDS–PAGE and Western blotting with SFV non-structural protein-specific antisera (Peränen et al., 1988; Ahola et al., 1997). Baculovirus stocks were amplified and titrated using an insect cell culture (Sf9, Gibco BRL) and SF-900 II culture media (Gibco BRL) containing 10% foetal calf serum and 50 mg/l gentamycin. However, for polyprotein expression and pulse–chase experiments, HighFive cells (BTI-TN-5B1-4) (Invitrogen) and HighFive medium (Invitrogen) were used. Optimal expression time was determined to be 40–48 h post-infection (p.i.) at 27 °C with 10 p.f.u. per cell. These conditions were used in all experiments.

Pulse–chase experiments. Approximately 2 × 10⁶ HighFive cells per 35 mm dish were infected with each recombinant baculovirus. The original medium was changed to a methionine-free Grace medium (Invitrogen) at 40 h p.i. for 60 min followed by a 15 min pulse (500 μCi/ml [³⁵S]methionine, Amersham). Samples were collected after a short ‘pulse’ (< 1 min) or 90 min ‘chase’ with HighFive medium containing a tenfold excess of unlabelled methionine. In some experiments, proteosome inhibitor MG132 (Calbiochem) was added to the medium at 50 μM. Samples were collected and immunoprecipitated with rabbit polyclonal antisera raised against SFV P1, P2, P3 or P4, essentially as described elsewhere (Kuula et al., 1997; Suopanki et al., 1998). Samples were then run on SDS–PAGE gels, which were dried and then exposed to phosphorimaging plates. Data were analysed using a BAS-1500 phosphorimager (FujiFilm) and TINA program, version 2.09c.

Results

Effects of mutations in the active sites of P2 and P4 putative proteases on translation and infectivity

Capped in vitro transcripts derived from plasmids encoding genomes of wild-type SFV (icSFV4) and mutants icSFVMut2CA, icSFVMut4DA and icSFVMut2CA4DA were used for in vitro translation in the rabbit reticulocyte system. Only uncleaved non-structural polyprotein P1234 was seen in the translation products of genomes with the C⁴⁸⁷A mutation in the putative active site of the P2 protease (Fig. 1, lanes 1, 2), whereas cleavage of P1234 took place in the products of the wild-type SFV (lane 4) and P4 mutant D⁹A genomes (lane 3). These results showed that the full-size genomes of the three mutants directed synthesis of SFV-specific proteins and that mutation in the putative active site of P4 did not prevent the processing of P1234 in vitro. The smaller derivative of P1 (ΔP1) observed in in vitro translation experiments is probably produced through the use of an alternative downstream in-frame initiation codon.

To study the effects of the mutations in vivo, capped in vitro transcripts derived from plasmids icSFV4, icSFVMut2CA, icSFVMut4DA and icSFVMut2CA4DA were used to transfect HeLa and BHK cells. Immunostaining of the transfected cells at 7 and 16 h post-transfection revealed that only cells transfected with wild-type RNA (icSFV4) were infected. The results were confirmed by Western blotting using specific antisera against

SFV non-structural and structural proteins. Thus, both point mutations were individually lethal for SFV replication.

Effects of P2CA and P4DA mutations on processing of polyprotein intermediates

To further analyse the processing of SFV non-structural polyprotein, we studied the in vitro cleavages of previously identified intermediates P123, P23 and P34 and the effects of active site mutations in P2 and P4 on them. No cleavage of P123CA3 or P23CA3 could be seen in the translation products (Fig. 2, A, lanes 2, 4), whereas the wild-type P123 and P23 were processed (Fig. 2, A, lanes 1, 3). By precipitation with antisera against P1, P2 and P3, it was possible to identify the cleavage products of P123 and P23. Thus, P123 yielded P12, P2, P3/P1 and a truncated form of P1 (ΔP1) (P1 and P3 migrate similarly on the gels used). Translation of construct P23 yielded both P2 and P3 (Fig. 2, A, lane 3). However, the translation products of both P34 and P34DA constructs remained uncleaved (Fig. 2 A, lanes 5, 6). Thus, we concluded that in vitro processing of P123 requires active P2 protease. In contrast, processing of P34 did not take place, irrespective of the mutation in the putative active site of the proposed P4 protease. In addition, we used truncated versions of P34 containing the P3/4 cleavage site (Takkinen et al., 1990): PA34 and PA34DA, with a complete P3 (482 residues) plus 102 residues from the N terminus of P4 (about 64 kDa), and PA4 and PA4DA, with only 78 residues from the C terminus of P3 joined to the complete P4 (about 76 kDa). Combined in vitro transcription/translation experiments were carried out with these constructs (Fig. 2, B, lanes 1–4). No cleavage of the putative precursors, which would yield either P3 (Fig. 2, B, lanes 1, 2) or P4 (Fig. 2, B, lanes 3, 4),
was observed as compared with the in vitro translation of P3 and P4 (Fig. 2B, lanes 5, 6).

### Processing of SFV non-structural polyproteins in recombinant baculovirus-infected cells

In order to obtain a reliable system for in vivo expression of SFV non-structural polyproteins, recombinant baculoviruses designated Bac1234, Bac12CA34, Bac12CA34DA, Bac123, Bac12CA3, Bac2CA3, Bac34, Bac34DA, Bac34DA, Bac3Δ4 and Bac3Δ4DA were constructed and used for infection of HighFive cells. All these baculoviruses expressed SFV-specific non-structural polyproteins similar to the in vitro translation products of the corresponding constructs. However, it should be noted that polyprotein expression levels were high in this system, which may, therefore, influence results. To study the stability of the polyproteins in insect cells, pulse–chase experiments were carried out.

The non-structural proteins produced by Bac1234 were identified in pulse–chase experiments. A 15 min pulse was followed by a 90 min chase, as described in Methods. Anti-P1 antibodies precipitated P12 (only faintly seen in this exposure) and P1 after the pulse and only P1 after the chase (Fig. 3, lanes 1, 2). Similar analysis with antibodies against P2, P3 and P4 revealed the precursors P12 and P34 as well as the final products (Fig. 3, lanes 3–8). Thus, all mature SFV non-structural proteins were detectable by immunoprecipitation (Fig. 3) as well as by Western blotting (data not shown).

In Bac12CA34- or Bac12CA34DA-infected insect cells, no cleavage of the P1234 polyprotein could be observed in pulse–chase experiments, as shown by immunoprecipitation with anti-P3 (Fig. 4A, lanes 1–4) or anti-P4 antibodies (Fig. 4B, C, lanes 1–4). Cells infected with P1234DA or P1234 showed the P34 precursor (Fig. 4A–C, lanes 5, 7), the amount of which decreased during the chase period (Fig. 4A–C, lanes 6, 8). The amount of P4 detected increased when proteasomal inhibitor was included in the medium during and after labelling with [35S]methionine (Fig. 4B, C, lanes 5–8). We conclude that mutation D'H in P4 had no effect on the processing of P1234, whereas mutation C178A in P2 prevented all proteolytic cleavages of the SFV polyprotein.

The expression and processing of Bac34 and Bac34DA in insect cells was studied using pulse–chase experiment protocols similar to those used for complete polyproteins. No specific cleavage products were detected with antisera against P3 and P4 (Fig. 5A, lanes 1–4 and 5–6, respectively), indicating that wild-type P34 does not have protease activity. We also expressed the truncated P34 derivatives in insect cells (Fig. 2B). P34A and P34DA, with a complete P3 of 482 residues plus 102 residues from the N terminus of P4, were well recognized by anti-P3 antiserum but did not yield P3 in insect cells (Fig. 5B, lanes 1–4), whereas anti-P4 antiserum poorly recognized the
SFV polyprotein processing

Fig. 4. Analysis of SFV-specific proteins produced by recombinant baculoviruses Bac12CA34 (lanes 1, 2), Bac12CA34DA (lanes 3, 4), Bac1234DA (lanes 5, 6) and Bac1234 (lanes 7, 8). Infected cells were pulse-labelled (P) for 15 min and chased (C) for 90 min. The post-nuclear supernatants were immunoprecipitated with antisera against P3 (A) or P4 (B, C). Portions of the cultures were exposed to 50 µM MG132 proteosomal inhibitor prior to radioactive-labelling (A, C). Immunoprecipitates were separated by SDS–PAGE and visualized by autoradiography. The recombinant baculoviruses used are indicated at the top of the gels. 14C-labelled molecular mass markers (kDa) are shown on the right (lane 9).

Fig. 5. Analysis of SFV-specific proteins produced by recombinant baculoviruses: (A) Bac34 (lanes 1, 2, 5, 6) and Bac34DA (lanes 3, 4, 7, 8); (B) Bac3A4 (lanes 1, 2, 5, 6) and Bac3A4DA (lanes 3, 4, 7, 8); and (C) BacA34 (lanes 1, 2, 5, 6) and BacA34DA (lanes 3, 4, 7, 8). Infected cells were pulse-labelled (P) for 15 min and chased (C) for 90 min. The post-nuclear supernatants were immunoprecipitated with antisera against P3 (lanes 1–4) or P4 (lanes 5–8). Immunoprecipitates were separated by SDS–PAGE and visualized by autoradiography. The recombinant baculoviruses used are indicated at the top of the gels and the antibodies used for immunoprecipitation are indicated immediately beneath. 14C-labelled molecular mass markers (kDa) are shown on the right (lane 9).

fusion protein (Fig. 5 B, lanes 5–8). Expressed hybrid proteins P∆34 and P∆34DA, with only 78 residues from the C terminus of P3 joined to the complete P4, were well recognized by anti-P4 antiserum (Fig. 5 C, lanes 5–8) but poorly recognized by anti-P3 antiserum (Fig. 5 C, lanes 1–4). These results confirm those obtained with the complete P1234 and show specifically that junction P3/4 cannot be cleaved by any activity present in the P4 region.

Proteolytic cleavage in trans of SFV non-structural polyprotein

Processing of SFV P1234 in trans using recombinant baculoviruses Bac12CA34DA and Bac34DA as substrates and Bac23 as the putative protease was also studied. Paired infections were carried out using ‘substrate’- and ‘protease’-expressing viruses. When cells infected with Bac23 and Bac12CA34DA were subjected to pulse-labelling, followed by a 90 min chase, polyprotein processing intermediates P12 and P34 were detected by immunoprecipitation with antibodies against P1–P4 (Fig. 6 A, lanes 1–4). Interestingly, only P12 was precipitated with anti-P1 antiserum (lane 1), suggesting inefficient or absent cleavage at site P1/2. In contrast, anti-P4 precipitated not only P34 but also P4 (lane 4). Quantification by phosphorimaging revealed that the molar ratio of P12 to P34 was about 5 to 1, supporting the idea that only P34 was cleaved (data not shown). The amounts of P2 and P3 could not
be estimated as they were part of the P23 protease (Fig. 6A, lanes 2, 3), which was evidently cleaved quantitatively as also shown in cells infected with Bac23 alone (Fig. 6B, lanes 5, 6). Co-infection with BacP34DA and Bac23 resulted in the cleavage of the substrate as shown by efficient precipitation of P4 with the corresponding antiserum (Fig. 6A, lane 8). Co-infection with Bac23 and Bac12CA3 resulted in production of P12, but again no P1 was detectable, suggesting that only P3 was cleaved from P12CA3 during co-expression with viral protease P23 (Fig. 6B, lanes 1–3). We also studied the accumulation of P12 under steady-state conditions by Western blotting (Fig. 7A). We could clearly detect accumulated P12 in co-infections of Bac12CA34 or Bac12CA3 with Bac23 (Fig. 7A, lanes 3, 4). In long exposures, P12 precursor was also detectable in cells infected with Bac1234 or Bac123 alone (data not shown). We conclude that self-cleavage-deficient substrates P1234, P123 and P34 can be cleaved in trans in insect cells when co-infected with recombinant baculovirus expressing the P23 protease. However, only the P2/3 and P3/4 sites were processed under these conditions.

To address the possibility of in trans cleavage of the P1/2 site, the in vitro translation reaction P12CA3 was labelled with [35S]methionine, and in a parallel reaction, unlabelled P123 was produced. Both reactions were stopped after 40 min by the addition of cycloheximide. No further protein was produced after the reaction was stopped (data not shown). We observed the production of P123 (Fig. 7B, lane 1) when a sample of the originally labelled P12CA3 was analysed. When translation products of both labelled P12CA3 (as substrate) and unlabelled P123 (as protease) were mixed and incubated together for 40 min, P12 and P3 cleavage products were observed (Fig. 7B, lane 2). The identities of P12 and P3 were confirmed with immunoprecipitation with anti-P2 and anti-P3 antibodies (Fig. 7B, lanes 3, 4). Anti-P1 antibodies precipitated only P123 (Fig. 7B, lane 5) and P12 (Fig. 7B, lane 6) in untreated and treated samples, respectively. Disappearance of the P12 precursor was not observed in these assays, indicating that the P1/2 bond was not processed in trans under these conditions.
Discussion

Early experiments by Sawicki and co-workers showed that inhibition of protein synthesis during the first 3–4 h of infection specifically shut off minus-strand RNA synthesis in SFV- and SIN virus-infected cells (Sawicki & Sawicki, 1980; Sawicki et al., 1981a, b). Several years later, it was shown that the non-structural polyprotein P123 together with P4 could carry out minus-strand RNA synthesis. Thus, the speed of P123 processing plays a crucial role in the control of RNA synthesis in alphavirus-infected cells (Lemm & Rice, 1993; Lemm et al., 1994; Shirako & Strauss, 1994). Later in infection when the concentration of the P2 protease is higher, the half-life of P123 is too short to allow the synthesis of minus-strand RNAs, whereas synthesis of 26S and 42S plus-strand RNAs continues even in the absence of protein synthesis (Kääriäinen & Söderlund, 1978).

Here we have studied the processing of SFV polyprotein P1234 and its previously identified processing intermediates P123, P12 and P34 (Lachmi & Kääriäinen, 1976; Glanville et al., 1978; Keränen & Kääriäinen, 1979; Kääriäinen et al., 1987) as well as the putative intermediate P23 by in vitro translation and by expression through recombinant baculoviruses. To identify the responsible proteases for processing the polyprotein, we introduced a point mutation into the active site of the P2 protease (Strauss et al., 1992; Strauss & Strauss, 1994; ten Dam et al., 1999) and transferred it into constructs encoding the non-structural polyprotein and its cleavage intermediates. To specifically investigate the putative autoprotease activity of P4 (Takkinen et al., 1990, 1991), we prepared constructs encoding P34, and truncated forms thereof (P3A34, P3A4), representing major deletions of P3 or P4 but preserving the P3/4 junction sequence. Mutation in the putative active site aspartyl residue D9 in P4 was introduced into the above constructs as well as into P1234. In vitro translation and expression in insect cells through recombinant baculoviruses failed to support the previously proposed autoprotease activity of P4. In contrast, mutation in the active site of the P2 protease (cysteine residue C157) inhibited the processing of the non-structural polyprotein and its partial cleavage products. This confirms that P2 is the sole protease responsible for the complete processing of the polyprotein, as has been previously shown for SIN virus (Strauss & Strauss, 1994).

The design of these experiments allowed us to make conclusions concerning polyprotein processing at the different cleavage sites. Co-infection with Bac23- and Bac12CA3A- or Bac12CA3 encoding substrate polyproteins, which cannot self-cleave, did not yield P1, indicating that the P1/2 junction could not be cleaved in trans by P23 or its processing product P2. On the other hand, the ability of wild-type P123 and P12 to generate P1 showed that the P1/2 site could be cleaved but neither cis nor trans cleavage could be distinguished. In SIN virus P123, the P1/2 bond could be cleaved in a bimolecular reaction by P123, but it could not be cleaved in cis (Shirako & Strauss, 1990; de Groot et al., 1990). Also in our in vitro translation experiments using P12CA3 as a substrate for proteolysis and P123 as a protease, we did not observe cleavage of the P1/2 bond in trans. However, the protease used is itself cleavable. Under similar conditions, the cleavage of SIN virus P1/2 is inefficient (Hardy & Strauss, 1989). However, we have not succeeded in demonstrating in trans cleavage of the P1/2 bond in our in vitro system.

P2 and P3 appeared rapidly in Bac23-infected insect cells. P123 yielded P1, P2 and P3 in both insect cells and in vitro translation. In the SIN virus non-structural polyprotein, cleavage at the P1/2 site has been shown to be necessary before the second cleavage at the P2/3 site can occur (Shirako & Strauss, 1990). Thus, we assume that SFV P123 is also cleaved first at the P1/2 site followed by rapid cleavage of the P2/3 site. Co-infection with recombinant viruses Bac12CA3 and Bac23 resulted in the production of P12, P2 and P3, but not P1, showing that cleavage had taken place at the P2/3 site in trans.

Co-infection with Bac23 and Bac12CA34 or Bac34 yielded P4 as an indication of in trans cleavage at the P3/4 junction. Cleavage at the P3/4 site produces the early RNA polymerase responsible for the synthesis of 42S RNA minus strands (Lemm et al., 1994; Shirako & Strauss, 1994). P123 is short-lived like minus-strand RNA synthesis in the presence of protein synthesis inhibitors (Sawicki & Sawicki, 1980). Later in infection, when the concentration of P2 protease increases, the probability of cleavage in trans at the P2/3 site increases, leading to shut-off of minus-strand RNA synthesis. At the same time, P12 and P34 are seen as short-lived intermediates of the processing pathway (Lachmi & Kääriäinen, 1976; Keränen & Kääriäinen, 1979; Lehtovaara et al., 1980; Keränen & Ruohonen, 1983). Thus, it is possible to explain how the previously identified intermediates (P123, P12 and P34) are processed from SFV P1234 non-structural polyprotein. The complete cleavage products P1–P4 serve as the late RNA polymerase, which synthesizes only 42 and 26S plus-strand RNAs (Suopanki et al., 1998).

Although all SFV non-structural proteins are synthesized principally in equimolar amounts, there is clearly less mature P4 (about 20%) as compared with P1–P3 (Takkinen et al., 1991). Addition of proteasomal inhibitor MG132 to the medium of insect cells infected with recombinant baculoviruses producing P4 resulted in an increased amount of P4, suggesting that this protein is normally degraded by proteosomes through the ubiquitination pathway, similar to SIN virus P4 (de Groot et al., 1991).

In addition to the central role in the timing of minus- and plus-strand RNA synthesis, P2 has other important functions in alphavirus RNA replication. It is an NTPase (Rikkonen et al., 1994; Rikkonen, 1996) and RNA helicase (Gomez de Cedron et al., 1999). We found recently that P2 has RNA 5’ triphosphatase activity needed in the capping of viral RNAs (Vasiljeva et al., 2000). Triphosphatase, NTPase and RNA
helicase activities are localized in the amino-terminal half of the molecule.

About 50% of P2 molecules are transported to the nucleus during virus infection (Peränen et al., 1990; Rikkonen et al., 1992). The role of this nuclear transport in virus replication is not known, but elimination of the nuclear localization signal of P2 resulted in viable virus; however, the virus had lost its pathogenicity for mice (Rikkonen, 1996). Finally, P2 regulates the synthesis of the subgenomic 26S mRNA of the structural proteins (Sawicki et al., 1978; Suopanki et al., 1998; Strauss & Strauss, 1994). Protease activity, nuclear localization signals and probably the ‘26S RNA regulatory domain’ are located in the carboxy-terminal half of P2. As shown previously (reviewed by Strauss & Strauss, 1994) and in this paper, P2 protease is also acting in the context of the non-structural polyprotein and its cleavage intermediates, which makes it a fascinating object for future studies. The availability of an in vitro system for alphavirus RNA replication (Lemm et al., 1998) enables further investigation into the regulation of the RNA replication process.

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