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Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing *in vivo*

Raoul J.de Groot, W.Reef Hardy, Yukio Shirako[1] and James H.Strauss[2]

Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA

[1]Present address: Center for Agricultural Molecular Biology, Cook College, Rutgers University, New Brunswick, New Jersey 08903

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[2]Corresponding author

The non-structural proteins of Sindbis virus, nsP1, 2, 3 and 4, are produced upon cleavage of polyproteins P123 and P1234 by a proteinase residing in nsP2. We used cell free translation of SP6 transcripts to study the proteolytic activity of nsP2 and of nsP2-containing polyproteins. To generate polyprotein enzymes, a set of plasmids was made in which cleavage sites were eliminated and new initiation and termination codons introduced by *in vitro* mutagenesis. As a substrate, we used a polyprotein in which the nsP2 proteinase had been inactivated by a single amino acid substitution. All nsP2-containing polyproteins cleaved the nsP1/2 site *in trans*. However, proteinases containing nsP1 were unable to cleave the nsP2/3 site. Furthermore, only proteinases containing nsP3 could cleave the nsP3/4 site. These differences in cleavage site specificity result in a temporal regulation of processing *in vivo*. At 1.7 h post infection P123 and nsP4 accumulated and only small amounts of P34 were found. However, at 4 h post infection P123 was processed rapidly and P34 was produced rather than nsP4. Since nsP4 is thought to be the viral RNA polymerase, the temporal regulation of the nsP4/P34 ratio may be responsible for the temporal regulation of RNA synthesis.

Key words: alphavirus/non-structural proteins/polyprotein/proteinase

Introduction

Sindbis virus (SIN) is an enveloped, plus-stranded RNA virus belonging to the genus alphavirus of the family Togaviridae. The 11.7 kb genome, which is capped and polyadenylated, acts as a messenger for the production of four non-structural proteins (nsP1, nsP2, nsP3 and nsP4, ordered from the NH_2-terminus), which are thought to form the viral transcriptase–replicase complex. During replication, the genomic RNA is transcribed into full-length minus-strand RNA, which in turn serves as a template for the synthesis of new genomic RNA and 26S subgenomic RNA. The latter RNA species is the messenger for the structural proteins (Strauss and Strauss, 1986). The synthesis of minus-strand RNA ceases 3–4 h post infection, while the production of genomic and subgenomic RNA continues throughout the infectious cycle (Sawicki and Sawicki, 1980; Sawicki *et al.*, 1981a,b). The mechanism by which minus-strand synthesis is switched off is unknown.

The non-structural proteins are made as two polyprotein precursors (Strauss *et al.*, 1983, 1984). For both precursors translation starts at the same initiation codon. In most instances translation stops at an opal termination codon downstream of the nsP3 gene, resulting in polyprotein P123 (200 kd). However, at a rather high frequency, read-through of the termination codon occurs leading to the synthesis of a larger polyprotein (250 kd) which includes nsP4 (P1234).

Although the functions of the non-structural proteins have not been fully elucidated, the characterization of temperature sensitive mutants and protein sequence comparisons have provided important clues. nsP4 is hypothesized to be the actual RNA polymerase (Kamer and Argos, 1984; Hahn *et al.*, 1989a). nsP1 is involved in minus-strand synthesis (Hahn *et al.*, 1989b) and also contains a methyl-transferase activity needed for the capping of viral RNAs (Mi *et al.*, 1989). nsP2 is required for the synthesis of the 26S subgenomic RNA and for the shut-off of minus-strand synthesis (Hahn *et al.*, 1989b). Interestingly, nsP2 also contains the proteinase responsible for the processing of the non-structural polyproteins (Ding and Schlesinger, 1989; Hahn *et al.*, 1989b; Hardy and Strauss, 1989), and thus in principle could regulate the synthesis of minus-strand and subgenomic RNA through proteolytic cleavages. The proteolytic activity was localized to the carboxy-terminal half of nsP2. This domain shows a limited sequence similarity to the papain-like thiol proteinases, implicating Cys 481 as the active site residue (Hardy and Strauss, 1989).

Processing of P123 *in vitro* was shown to be sensitive to dilution, indicating that cleavage of this protein occurs predominantly in a bimolecular reaction (*in trans*); in contrast, processing of P12 was not influenced by dilution, strongly suggesting autoproteolysis (cleavage *in cis*) (Hardy and Strauss, 1989). *In vivo*, the kinetics of processing indicated that at 3–4 h after infection, P123 is first cleaved to P12 and nsP3, followed by cleavage of P12 (Hardy and Strauss, 1988). Paradoxically, however, the elimination of the nsP2/3 cleavage site by site specific mutagenesis did not influence the cleavage at the nsP1/2 and nsP3/4 sites, as studied *in vitro*, but the elimination of the nsP1/2 site prevented the cleavage at the nsP2/3 site and resulted in the accumulation of P123, i.e. cleavage of the nsP1/2 site appears to be essential for initiation of the processing pathway (Shirako and Strauss, 1990). These conflicting observations can be rationalized by postulating that (i) there are proteolytically active polyproteins containing the nsP2 region; (ii) the different polyprotein proteinases differ in their preferences for the three cleavage sites; and (iii) at 3–4 h after infection proteinases with a preference for the nsP2/3 site predominate. In this paper we show by cell free translation of synthetic transcripts that the different polyproteins are indeed active proteinases that differ in their cleavage site

preferences. Furthermore, we provide evidence that these differences result *in vivo* in a temporal regulation of polyprotein processing. The possible consequences for regulation of viral replication are discussed.

Results

Construction of cDNA clones for expression of nsP2-containing polyproteins

In vitro transcription of cDNA clones, followed by *in vitro* translation, provides a powerful approach to the study of the processing of viral polyproteins (Ympa-Wong and Semler, 1987). We have employed this strategy to determine the *trans*-cleavage activities of SIN nsP2 and of polyproteins containing nsP2. For this purpose we constructed a set of cDNA clones that when transcribed and translated *in vitro* would give rise to nsP2-containing polyproteins that could be used either as enzymes or substrate in *trans*-cleavage assays. Construction of these clones required creation of new initiation or termination codons, mutagenesis of cleavage sites to render them non-cleavable, mutagenesis of the enzymatic domain to inactivate the proteinase, and replacement of the opal codon following nsP3 with a serine codon. This set of clones and the terminology used is illustrated schematically in Figure 1A and described in more detail below.

Since nsP2 is formed by proteolytic cleavage of a polyprotein, the nsP2 gene does not have an initiation or termination codon. In plasmid pToto.2, we have provided the nsP2 gene with an initiation codon preceding the Ala1 codon, and an amber stop codon immediately downstream of the 3′ terminal Ala807 codon. The gene is located downstream of an SP6 RNA polymerase promoter and the 5′-terminal noncoding 60 nucleotides of the SIN genome, such that transcription with SP6 polymerase leads to an RNA with the authentic SIN leader immediately coupled to the nsP2 gene. *In vitro* translation of such transcripts resulted in the synthesis of an 80 kd polypeptide, which in SDS−PAGE gels comigrated with the authentic SIN nsP2 protein (Figure 1B, lane 3). To distinguish this protein product from the nsP2 derived by normal proteolytic processing of polyproteins, we will refer to it as N2.

To produce non-cleavable polyproteins containing nsP2, we took advantage of observations that the 1/2, 2/3 and 3/4 cleavage sites can be eliminated by changing them from Gly-Ala-Ala, Gly-Ala-Ala and Gly-Gly-Tyr, respectively, to Glu-Ala-Ala, Glu-Ala-Ala and Gly-Val-Tyr, respectively (Shirako and Strauss, 1990; R.J.de Groot, unpublished results). The series of cDNA plasmids illustrated in Figure 1A when transcribed and translated *in vitro* yield all possible nsP2-containing polyproteins. *In vitro* translations of SP6 transcripts derived from these plasmids are shown in Figure 1B. To distinguish these non-cleavable polyproteins from those produced by translation of wild-type RNA, we will refer to them with an N rather than a P. Thus, for example, N123 refers to the polyprotein containing the sequences of non-structural proteins 1, 2 and 3 in which the cleavage sites have been eliminated by mutagenesis, whereas P123 is the polyprotein translated from wild-type RNA.

To study the *trans*-cleavage activities of these nsP2-containing polyproteins, a substrate was required. In previous experiments, we have used cDNA clones containing deletions in the protease domain of nsP2 gene to produce

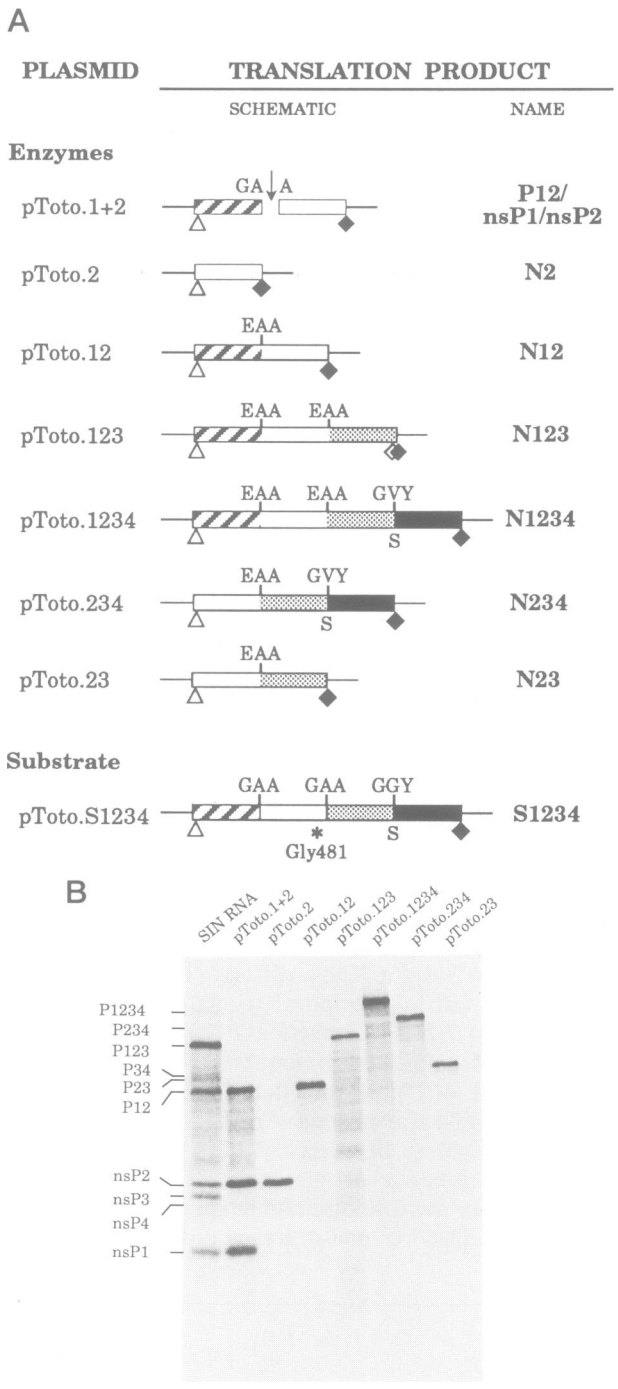

Fig. 1. (**A**) Schematic representation of the constructs used for expression studies. The nomenclature used for the plasmids and for the expression products obtained are indicated to the left and right, respectively. In the diagrams the non-structural protein coding regions are depicted as boxes with different shading used for each protein; the cleavage sites, either unaltered or mutagenized, are shown in the one letter amino acid code. Initiation codons are indicated by open triangles. The white diamond indicates the opal codon read through with high efficiency, which is replaced by a serine codon in the constructs pToto.1234, pToto.234 and pToto.S1234 as indicated; the solid diamonds depict termination codons resulting in efficient termination. The asterisk indicates the Cys481 to Gly substitution. (**B**) *In vitro* translation of SP6 transcripts derived from constructs shown in A. Rabbit reticulocyte lysate was supplemented with [^{35}S]methionine and RNA and incubated for 1 h at 30°. Translates were analyzed by 7.5% SDS−PAGE. A translate of SIN strain HR RNA served as a marker (lane 1). The positions of the SIN non-structural proteins and their precursors are indicated.

truncated polyproteins that are unable to process by themselves (Hardy and Strauss, 1989). However, these deletions may affect the overall conformation of the polyprotein and thereby influence the results of a *trans*-cleavage assay. Recently, we have found that by changing Cys 481 into Gly the proteolytic activity of nsP2 is completely abolished (R. Levinson, R.J. de Groot and J.H. Strauss, unpublished results). A plasmid was constructed in which the Cys to Gly substitution in nsP2 was combined with the opal to Ser substitution at the C-terminus of nsP3 (position 1897 of the SIN non-structural open reading frame; Li and Rice, 1989) (Figure 1). Transcription of this plasmid, pToto.S1234, followed by translation *in vitro*, resulted in the synthesis of the uncleaved 250 kd precursor (Figures 2 and 3). This product, designated S1234, was considered an ideal substrate, since the single amino acid Cys to Gly substitution is less likely to change the folding of the polyprotein than deletions in the nsP2 gene.

Trans-cleavage assays

For *trans*-cleavage assays, the nsP2-containing polyproteins used as enzymes were synthesized by *in vitro* translation using unlabeled methionine, while the substrate S1234 was radioactively labeled by performing the translation in the presence of [^{35}S]methionine. The translation reactions were stopped by adding cycloheximide and excess unlabeled methionine, after which the enzymes and substrate were incubated together. Translates of wild-type SIN genomic RNA were used as positive controls. Incubation of S1234 with the wild-type translate resulted in the production of all four non-structural proteins as well as of the polyproteins P12, P123 and P34 (Figure 2, lane 2). P12 and nsP3 were the most abundant products, indicating that cleavage occurred predominantly at the 2/3 and 3/4 sites. After incubation of S1234 with N123 or N1234 as enzymes, only the products P123, P23, nsP4 and nsP1 were found. Apparently, N123 and N1234 can cleave only the 1/2 and 3/4 sites and are unable to cleave the 2/3 site. Visual inspection of the autoradiogram suggested that most S1234 was converted into P123, indicating that the 3/4 site was predominantly cleaved (Figure 2). To assess the difference in the efficiency of cleavage of the 1/2 and 3/4 sites more precisely, we determined the amounts of input S1234 (lane labeled 'Blank') and the cleavage products P123 and P23 (lane '+N123') by densitometry (P234 was not detected). An endogenous reticulocyte protein labeled during translation served as an internal control for the amount applied to each lane. N123 cleaved 90–95% of the substrate at the 3/4 site, whereas only 35–38% of the 1/2 sites were cleaved. nsP4 was underrepresented in Figure 2. The amounts of nsP4 found in translates varied between experiments for unknown reasons. Loss of nsP4 was not always observed and appeared to depend on storage conditions and the batch of reticulocyte lysate used (not shown).

Upon incubation of S1234 with the enzymes N23 and N234, all four non-structural proteins were found, in addition to the polyproteins P123, P12, P23, P34 and P234 (Figure 2). Thus these enzymes are able to cleave all three sites. In contrast, processing of S1234 by N12 yielded only low amounts of nsP1 and P234 (Figure 3). Apparently, cleavage at the 2/3 and 3/4 sites did not occur, and enzyme N12 is able to cleave only the 1/2 site.

A translation mixture containing nsP1, nsP2 and P12

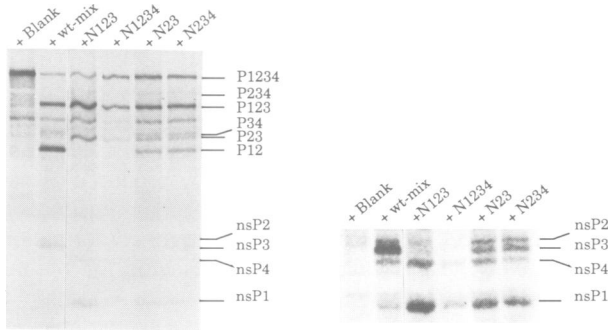

Fig. 2. *Trans*-cleavage assay: processing of the S1234 substrate by N123, N1234, N23 and N234 *in vitro*. ^{35}S-labeled S1234 was prepared as described in Figure 1. The polyproteins used as enzymes were synthesized similarly except that unlabeled methionine was used. Translation reactions were stopped by adding cycloheximide and excess unlabeled methionine after which the enzymes and substrate were mixed at a 1:1 (v/v) ratio and allowed to incubate for an additional 2 h at 30°. The cleavage products were analyzed by 7.5% SDS–PAGE. Translates without added RNA (Blank) and with SIN strain HR viral RNA (wt-mix) served as negative and positive controls, respectively. The right hand panel shows a longer exposure of the bottom half of the gel.

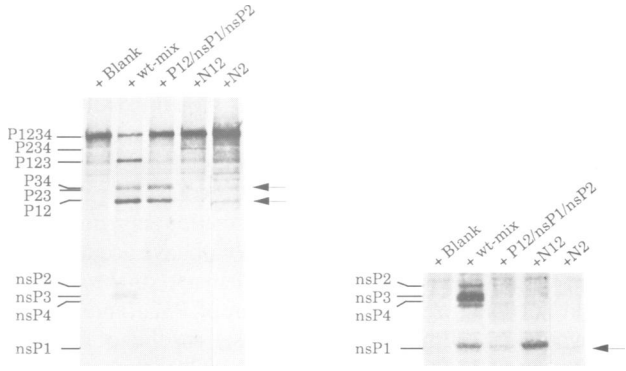

Fig. 3. *Trans*-cleavage assay: *in vitro* processing of the S1234 substrate by N2, N12 and a translate containing P12, nsP1 and nsP2 (derived from pToto.1+2). The experimental procedures were as in Figure 2. The right hand panel shows a longer exposure of the bottom half of the gel. The arrows point to P34, P12 and nsP1 in the N2 digest of S1234.

derived from pToto.1+2 (Figure 1B, lane 2) cleaved S1234 predominantly at the nsP2/3 cleavage site: the major products observed were P12 and P34. The 1/2 site was cut less efficiently, and cleavage of the 3/4 site was not detected (Figure 3). If we assume that P12 has the same cleavage specificity as N12, these findings indicate that nsP2 produced by cleavage of P12 is able to cleave the 2/3 site but not the 3/4 site, with possible activity at the 1/2 site unresolved. Essentially the same results were obtained upon incubation of S1234 with N2 derived from pToto.2, as this enzyme was able to cleave the 1/2 and 2/3 sites but not the 3/4 site. However, the proteolytic activity of N2 was surprisingly low (Figure 3). To rule out the possibility that these results were caused by mutations introduced into pToto.2 and pToto.12 during cloning, the nsP2 genes in constructs pToto.12 and pToto.2 were sequenced, and the sequence in both was identical to that in the parental cDNA clone pToto1101. In addition, the 2029 bp *Eco*RI–*Pst*I fragments (nucleotides 1920–3949 of the SIN genome; 83% of the nsP2 gene) of

pToto.2 and pToto.12 were used to replace the corresponding fragments in pToto1101. The non-structural polyproteins translated from transcripts of the resulting plasmids were processed normally (data not shown), and thus the protease domain appears to be normal. The reason for the poor proteolytic activity of N2 remains unclear. The most likely explanation is that nsP2, normally produced as part of a polyprotein, requires the flanking protein sequences to adopt its correct (i.e. proteolytically active) conformation. Alternatively, nsP1 could act as a cofactor for optimal cleavage by nsP2.

The results of the *trans*-cleavage assays are summarized in Table I. The specificities of the various polyproteins can be described with the following rules: (i) the presence of nsP4 sequences does not affect the specificity of the proteinase, i.e. polyproteins with or without nsP4 had the same cleavage specificity; (ii) if nsP1 is present in the polyprotein, the proteinase is unable to cleave the 2/3 site; proteinases lacking the nsP1 moiety cleave the 2/3 site efficiently; (iii) the presence of nsP3 in the proteinase is required for cleavage of the 3/4 site.

Temporal regulation of the [nsP4]/[P34] ratio

The differences in site-preference of the proteinase precursors, observed *in vitro*, prompted us to consider the possibility of post-translational regulation of processing *in vivo*. In particular, our data predicted a temporal regulation of the nsP4 to P34 ratio. The rationale for this is that very early in infection, P123 and P1234 are expected to be abundant. Since these enzymes predominantly cleave the 3/4 site and do not cleave the 2/3 site, nsP4 would be generated rather than P34. However, later in infection, enzymes with a preference for the 2/3 site (Table I) will have accumulated (Hardy and Strauss, 1988). Rapid cleavage of the 2/3 site would not only prevent accumulation of P123 and P1234, but would also eliminate the proteinases capable of cleaving the 3/4 site (P123, P1234, P23 and P234), thus resulting in the accumulation of P34 rather than nsP4.

To test this hypothesis, primary chicken embryo cells infected with SIN strain HR were labeled during a 5 min pulse with [^{35}S]methionine early (1 h 45 min) and late (4 h) after infection, followed by a chase with excess unlabeled methionine (it should be noted that under these conditions ~15 min are required to synthesize a complete SIN polyprotein, and thus the first part of the chase period is involved with completion of initiated labeled chains; Hardy and Strauss, 1988). Cell lysates were immunoprecipitated with a rabbit antiserum specific for nsP4 (Hardy and Strauss, 1988). Early in infection, P1234 was predominantly processed to produce nsP4, as predicted (Figure 4, early). P34 accumulated to some extent during the 5 min chase, but then declined, most likely to yield nsP3 and nsP4. The amount of P1234 present could not be determined because of the presence of a 250 kd host protein which precipitated non-specifically. Conversely, at 4 h after infection P34 accumulated rather than nsP4 (Figure 4, late). The 250 kd host protein was not present, probably due to effective shut-off of host protein synthesis, allowing visualization of P1234. Since P1234 was not present in large amounts, cleavage at the 2/3 site occurred either cotranslationally or immediately after termination of translation (Figure 4, late). During the chase P34 was converted into a form with slightly lower mobility, most likely due to phosphorylation (Hardy and Strauss, 1988; Peränen *et al.*, 1988; G.Li, M.W.LaStarza, W.R.Hardy, J.H.Strauss and C.M.Rice, in preparation).

Table I. Enzymes that cleave each of the three different sites in S1234

Site 1/2	Site 2/3	Site 3/4
nsP2	nsP2	P123
P12	P234	P1234
P123	P23	P234
P1234		P23
P234		
P23		

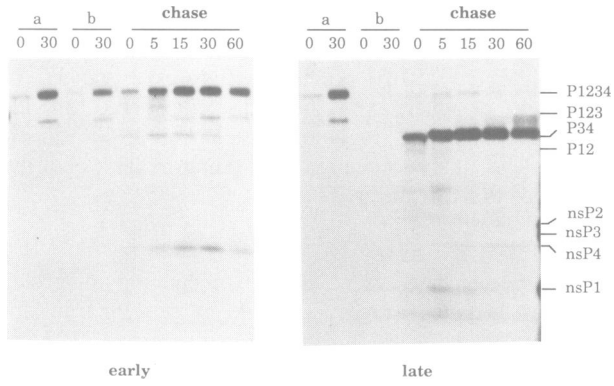

Fig. 4. Synthesis of P34 and nsP4 early and late in SIN infection. Confluent monolayers of secondary chicken embryo cells were infected with SIN strain HR at an m.o.i. of 50 p.f.u./cell. After 1 h incubation at 37°C, the inoculum was removed and replaced by Eagle's medium, containing 5 μM methionine. At 1 h 45 min p.i. ('early') or 4 h p.i. ('late') the viral proteins were labeled with [^{35}S]methionine during a 5 min pulse and then either lysed immediately or after a chase with excess unlabeled methionine for the periods of time indicated (in min). The lysates were immunoprecipitated with a non-specific rabbit antiserum raised against nsP4. Immunoprecipitates were analyzed by 7.5% SDS–PAGE. The following controls were included: (a) mock-infected cells were lysed after a 5 min pulse labeling and a 0 min or 30 min chase, and subjected to immunoprecipitation with αnsP4 serum; (b) SIN infected cells were lysed after a 5 min pulse labeling and a 0 or 30 min chase and subjected to immunoprecipitation with αnsP4 pre-immune serum. An *in vitro* translate of SIN strain HR viral RNA served as a marker. The positions of the SIN non-structural proteins and their precursors are given.

To study the processing of nsP2-containing polyproteins at the two different time-points, immunoprecipitations were also performed with a rabbit antiserum directed against nsP2 (Figure 5). Early in infection, P123 accumulated during the first 15 min of chase but declined thereafter, whereas the amount of nsP2 increased steadily during the chase (Figure 5, early). In contrast, P123 did not accumulate late in infection, but appeared to be processed rapidly to generate P12 and nsP2 (Figure 5, late).

Discussion

Many RNA viruses express their genetic information by synthesis of polyproteins that are post-translationally cleaved to generate the functional viral gene products. Host proteinases may be involved in the case of structural proteins that mature in subcellular organelles, but in the case of proteins processed in the cytosol the proteinases responsible are encoded by the virus itself (Rice and Strauss, 1981; Wellink and van Kammen, 1988). Alphaviruses produce both

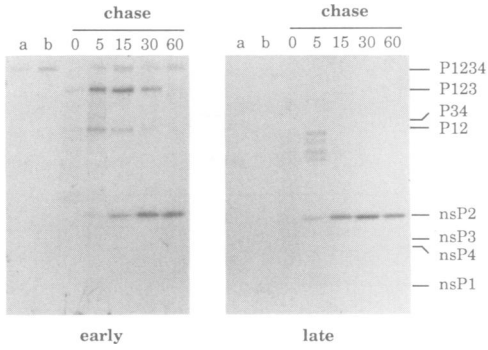

Fig. 5. Synthesis of nsP2 and nsP2-containing precursors early and late in SIN infection. The samples described in Figure 4 were immunoprecipitated with a monospecific rabbit antiserum raised against SIN nsP2. Material from an equivalent number of cells was loaded in each lane, and the samples were analyzed in the same gel. A 10 day exposure of the autoradiogram is shown for the panel labeled 'early' and a 1.5 day exposure for the panel labeled 'late'. The following controls are included: (a) mock-infected cells, lysed after a 5 min pulse labeling and a 30 min chase, subjected to immunoprecipitation with αnsP2 serum; (b) SIN infected cells, lysed after a 5 min pulse labeling and a 30 min chase, subjected to immunoprecipitation with αnsP2 preimmune serum.

their structural and non-structural proteins by processing of polyprotein precursors. Whereas the proteolytic cleavage of the structural polyprotein has been studied in considerable detail (Hahn *et al.*, 1985; Melancon and Garoff, 1986, 1987; Schlesinger and Schlesinger, 1986; Strauss and Strauss, 1986), only recently have details about the processing of the non-structural proteins become known. These studies on the non-structural proteins have mostly used SIN, and have been aided by the availability of a full-length cDNA clone (Rice *et al.*, 1987) and monospecific antisera raised against each of the non-structural proteins (Hardy and Strauss, 1988). It has now been firmly established that all three cleavage sites in P1234 are cut by a proteinase residing in the C-terminal half of nsP2 (Ding and Schlesinger, 1989; Hardy and Strauss, 1989; Shirako and Strauss, 1990).

Here, we have studied the proteolytic activities of nsP2-containing polyproteins by using *in vitro* mutagenesis techniques and cell free translation of synthetic transcripts. Our data show that all nsP2-containing polyproteins are proteolytically active and can cleave the 1/2 site *in trans*. However, the precursors differ strikingly in their cleavage site preference with respect to the 2/3 and 3/4 sites. Polyproteins containing nsP1 were unable to cleave the 2/3 site *in vitro*, whereas the proteinases lacking nsP1 cleaved the 2/3 site very efficiently. Furthermore, the presence of nsP3 in the proteinase appeared to be required for cleavage of the 3/4 site. An analogous situation exists for poliovirus in that the P2 polyprotein is cleaved *in trans* by the 3C proteinase (Ympa-Wong and Semler, 1987) but the P1 polyprotein can only be cleaved by the 3CD proteinase precursor (Jore *et al.*, 1988; Ympa-Wong *et al.*, 1988). The mechanism by which the SIN proteinases change their cleavage site preference is unknown. It is possible that after nsP1 is removed, the proteinase refolds and assumes an altered conformation that then enables it to cleave the 2/3 site. Alternatively, the nsP1 sequences may simply prevent cleavage of the 2/3 site by steric hindrance. The sequence of the cleavage site could also be important: the 3/4 cleavage site is Gly-Gly-Tyr, while the 1/2 and 2/3 sites are both Gly-Ala-Ala.

Processing of P123 *in vitro* is dilution sensitive, indicating that at least the initial cleavage between nsP1 and nsP2 occurs in a bimolecular reaction (Hardy and Strauss, 1989; Shirako and Strauss, 1990). Apparently, it is this dependence on *trans*-cleavage combined with the fact that the proteinases differ in their cleavage site preference that allows the virus to regulate post-translationally the synthesis of the non-structural proteins and their precursors. Our present view on the early events in SIN infection is as follows (Figure 6). After its release in the cytoplasm, the genomic RNA is translated into P123 and P1234. These proteinases preferentially cut the 3/4 site, resulting in the accumulation of P123 and nsP4 (Figure 6A). The 1/2 site is also cut but with lower efficiency than the 3/4 site (Figure 6B). Cleavage of the 1/2 site, however, unleashes proteinases with a strong preference for the 2/3 site. As these latter proteinases accumulate in the cytoplasm, the cleavage of the polyproteins is gradually redirected from the 3/4 site to the 2/3 site, generating P34 and large amounts of nsP3 and P12. The P12 precursor cleaves *in cis* to generate free nsP1 and nsP2 (Hardy and Strauss, 1989) and thereby adds to the proteolytic activity directed against the 2/3 site. Finally, at 3−4 h after infection a situation is reached in which nsP2 is present in such high concentrations that cleavage of the 2/3 site occurs either cotranslationally or immediately after termination of translation, thereby simultaneously preventing accumulation of P123, eliminating the enzymes that can cleave the 3/4 site, and generating P34 rather than nsP4 (Figure 6C). The fact that at 3−4 h after infection P34 is abundant and nsP4 is present in only low amounts (if at all), had previously led to the hypothesis that P34 is the active protein species rather than nsP4 (Hardy and Strauss, 1988; Strauss *et al.*, 1988). Our present results suggest that both protein species are functional, but at different times in infection.

It seems highly likely that the temporal regulation of the non-structural proteins is important for the development of the virus life cycle. For one, polyproteins like P123 could in principle perform functions required early in infection that cannot be performed by the final endproducts. But even more intriguing is the temporal regulation of the ratio of nsP4 to P34. Several observations indicate that nsP4 is the actual RNA polymerase. It contains the Gly-Asp-Asp motif found in the polymerases of various plant and animal viruses (Kamer and Argos, 1984). Moreover, certain *ts* mutations in nsP4 result in a total termination of RNA elongation at the non-permissive temperature (Barton *et al.*, 1988; Hahn *et al.*, 1989a). It is therefore tempting to speculate that the regulation of the nsP4 to P34 ratio is correlated with a temporal regulation of RNA synthesis, e.g. regulation of minus-strand synthesis, which normally ceases at 3−4 h after infection (Sawicki and Sawicki, 1980; Sawicki *et al.*, 1981a, b), of subgenomic RNA synthesis, or both. Since nsP4 is predominantly made early in infection, this protein could be a polymerase required for minus-strand synthesis, while P34 could be required for the synthesis of the subgenomic RNA species. In this case, competition between nsP4 and P34 for association with replicase complexes could provide a plausible explanation for the shut-off of minus-strand synthesis at 3−4 h after infection. This hypothesis is supported by results obtained for mutant *ts*17. At the restrictive temperature, this mutant shows aberrant processing of the 2/3 site, decreased synthesis of subgenomic RNA, and continuous minus-strand synthesis (Hardy *et al.*,

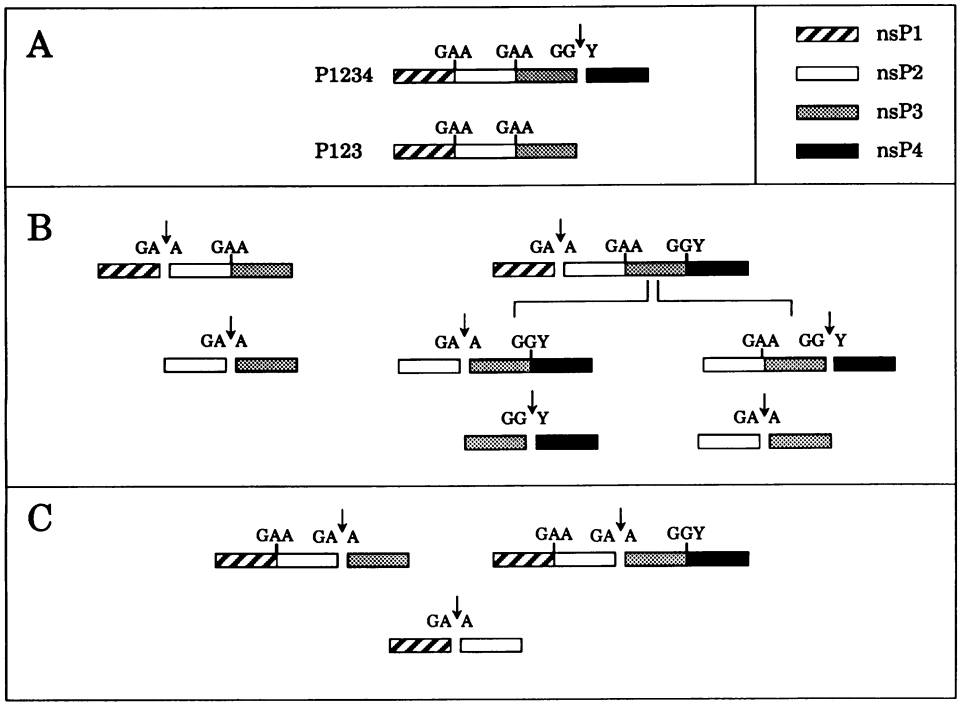

Fig. 6. Cleavage pathways of the SIN non-structural polyproteins: a model for the temporal regulation of cleavage. (**A**) Very early in infection cleavage of the 3/4 site is presumed to be the major cleavage pathway resulting in accumulation of P123 and nsP4. (**B**) P123 and P1234 are also cut at the 1/2 site early in infection but with lower efficiency. Cleavage of the 1/2 site results in the production of proteinases (nsP2, P23 and P234) that can cleave the 2/3 site. The mixture of proteinases eventually leads to the cleavage of all three bonds and thus to the production of nsP1, nsP2, nsP3 and nsP4. (**C**) As infection proceeds proteinase active on the 2/3 site accumulates in the cytoplasm and pathway C becomes increasingly important until at 4 h p.i. it is predominant: the polyproteins are cut at the 2/3 site cotranslationally or immediately after translation termination, preventing accumulation of P123. Proteinases that can cleave the 3/4 site (P123, P1234, P23 and P234) are eliminated and P34 is produced rather than nsP4.

1990; Hahn *et al.*, 1989b; Sawicki and Sawicki, 1985). In accordance with our hypothesis, the *ts* lesion also results in an increased production of nsP4 at the restrictive temperature, whereas P34 is turned over or processed rapidly (Hardy *et al.*, 1990). Sawicki and Sawicki (1985) showed that at the restrictive temperature, *ts*17-infected cells can resume minus-strand synthesis after complete shut-off and this resumption occurs even when protein synthesis is inhibited by cycloheximide. On the basis of these results it was concluded that proteolytic cleavage could not explain the shut-off of minus-strand synthesis. However, it seems quite possible that after the shift to the non-permissive temperature, nsP4 could be generated from pools of P34 already present in the cell, i.e. nsP4 could be produced even without *de novo* protein synthesis and lead to the resumption of minus-strand synthesis.

The question remains open as to the function of the opal termination codon between nsP3 and nsP4, especially in view of the fact that in at least two alphaviruses this codon is replaced with a sense codon (Strauss *et al.*, 1988; Takkinen, 1986). Readthrough of the SIN opal codon occurs at a frequency of ~20% *in vitro* (R.J.de Groot, unpublished data). If one assumes that readthrough occurs with similar efficiency *in vivo*, the synthesis of P34 and nsP4 would be reduced at least 5-fold as compared with nsP1, nsP2 and nsP3. Apparently, this down-regulation is either not necessary or accomplished in a different fashion in those viruses lacking the termination codon.

In summary, we have shown that the SIN non-structural proteinases differ in their cleavage site preferences and we have provided evidence that these differences result in a temporal regulation of protein processing during infection. Several observations link the temporal regulation of the nsP4 to P34 ratio to the regulation of RNA synthesis, but direct evidence for this has not yet been obtained. We hope to address this point by studying the kinetics of viral RNA synthesis for SIN mutants in which the 3/4 cleavage site has been rendered uncleavable or more efficient by site directed mutagenesis.

Materials and methods

Plasmids, enzymes and general methods

pToto1101 is a full-length cDNA clone of the HR strain of SIN from which infectious RNA can be transcribed *in vitro* (Rice *et al.*, 1987). pToto1000.S, a derivative of the full-length clone in which the opal codon between nsP3 and nsP4 has been replaced by a serine codon (Li and Rice, 1989), was kindly provided by C.M.Rice. pToto57, containing a unique *Xba*I site at position 54 of the SIN genome, was kindly supplied by R.J.Kuhn. pGEM5-Zf was obtained frm Promega Biotech. The plasmids were introduced in *Escherichia coli* strain MC1061.1 by $CaCl_2$ transformation. *E.coli* strain BW313 was used for the preparation of uracil-containing template DNA used for *in vitro* mutagenesis (Kunkel, 1985). Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, polynucleotide kinase, the Klenow fragment of *E.coli* DNA polymerase I, and SP6 RNA polymerase were from New England Biolabs. S1 nuclease was from BRL. Modified T7 DNA polymerase (Sequenase) was from US Biochemicals. *Taq* DNA polymerase was from Promega. Both single stranded and double stranded DNA were sequenced using the T7 DNA polymerase (sequenase) dideoxy chain termination method using conditions recommended by the manufacturer (US Biochemicals). Standard DNA manipulations and cloning procedures were done according to Maniatis *et al.* (1982).

Preparation of in vitro transcripts

Plasmid DNA for *in vitro* transcription was prepared by alkaline lysis (Maniatis *et al.*, 1982). The DNA templates were linearized with *Xho*I,

purified and used for *in vitro* transcription by SP6 RNA polymerase as described previously (Hardy and Strauss, 1989).

In vitro translation

In vitro translation was carried out in a 10 μl reaction containing nuclease treated, methionine depleted rabbit reticulocyte lysate (Promega) supplemented with 10 μCi [^{35}S]methionine (>1000 Ci/mmol; Amersham), 20 μM of an unlabeled amino acid mixture lacking methionine (Promega) and 10–50 ng RNA. Incubation was for 1 h at 30°C. The reactions were stopped by adding 40 μl sample buffer (Laemmli, 1970).

For *trans*-cleavage experiments, the polyproteins to be tested for proteinase activity were prepared by *in vitro* translation as described but in the absence of [^{35}S]methionine. Instead unlabeled methionine was added to a final concentration of 20 μM. Synthesis of both substrate and enzymes was stopped by adding 6 mg/ml cycloheximide and 20 mM methionine to final concentrations of 0.6 mg/ml and 1 mM, respectively. Subsequently, enzymes and substrate were mixed at a 1:1 (v/v) ratio and allowed to incubate for an additional 2 h at 30°C. The translation products were analyzed by SDS–PAGE (Laemmli, 1970) in 7.5% polyacrylamide gels (acrylamide:*bis*-acrylamide 75:1). The gels were fixed in 50% methanol, 12% acetic acid for 30 min, impregnated with EN3HANCE (Dupont) according to the instructions of the manufacturer, and autoradiographed. Densitometry was performed using a computing densitometer (Molecular Dynamics, model 300A) and the IQuant software. The relative amount of input substrate S1234 (determined for the blank) was compared with that of the cleavage products. A [^{35}S]Met-labeled endogenous protein of the reticulocyte lysate served as an internal control to assure that similar amounts of the undigested and digested S1234 had been loaded on the gel.

Plasmid constructs

The SIN nsP2 protein is generated by proteolytic cleavage of a polyprotein precursor and therefore does not have an initiation or a termination codon. To construct a transcription/translation vector that could be used to express the nsP2 gene, the 714 bp *Pst*I–*Bam*HI fragment derived from pToto1101 (nucleotides 3949–4663 of the SIN genome), was subcloned into M13mp19. The Ala1 codon of nsP3 was converted into an amber stop codon by site directed mutagenesis (Kunkel, 1985) using the oligonucleotide primer 5′-GACGCCTAGGCTCCAACTCCATCTCT-3′. The introduced nucleotide substitutions (underlined) also resulted in the creation of an *Avr*II site. The mutated *Pst*I–*Bam*HI fragment was then excised from RF DNA and inserted into an intermediate vector pSCV23, which contains the 2974 bp *Bgl*II-*Spe*I fragment of pToto1101 (nucleotides 2288–5262 of the SIN genome) (Shirako and Strauss, 1990), and which had been prepared by digestion with *Pst*I and *Bam*HI. The resulting plasmid was called p6-8-2. To generate an initiation codon at the 5′ end of nsP2, the 1146 bp *Fnu*4HI fragment from pToto1101 (nucleotides 1677–2823 of the SIN genome) was treated with S1 nuclease followed by digestion with *Cla*I. This *Fnu*4HI(blunt-ended)-*Cla*I fragment (nucleotides 1680–2712), was then joined to the *Cla*I–*Avr*II fragment (nucleotides 2712–4099 of the SIN genome) from p6-8-2 and to the *Nco*I (blunt-ended with Klenow)–*Spe*I fragment from pGEM5-Zf in a three piece ligation. The resulting plasmid pGEM 8-2 contains a complete nsP2 gene, with an initiation codon (supplied by the blunt-ended *Nco*I site) preceding Ala1 and an opal stop codon at its 3′ end. The reconstructed nsP2 gene was inserted downstream of the SP6 promoter and the SIN 5′ non-coding leader sequence in a four piece ligation involving the 2849 bp *Xba*I (blunt-ended with Klenow)–*Sal*I (position 11087) fragment of pToto57 and the following fragments of pGEM 8-2: *Nco*I–*Ban*I (nucleotides 1675–1902), *Ban*I–*Cla*I (nucleotides 1902–2712) and *Cla*I–*Sal*I (nucleotides 2712–4104; the *Sal*I site was provided by the polylinker of pGEM 5-Zf). The nsP2 moiety of the resulting construct, pToto.2, was sequenced to ensure that no additional mutations had arisen during the manipulations. The sequence obtained was identical to that of the parental clone pToto1101.

pP1-539E/P2-806E is a derivative of pToto1101 in which the nsP1/2 and the nsP2/3 cleavage sites have been eliminated by changing them from Gly-Ala-Ala to Glu-Ala-Ala (Shirako and Strauss, 1990) (P1-539E signifies that amino acid 539 of nsP1 has been changed to Glu, etc.). pP1-539E/P2-806E was cut with *Cla*I (position 2712) and *Sal*I (position 11087) and ligated to the *Cla*I–*Sal*I fragment from pToto.2 to give plasmid pToto.12, which encodes a non-cleavable P12 polyprotein.

pToto.SA3 is a pToto1101 derivative in which the nsP3/4 cleavage site has been destroyed by changing it to Gly-Val-Tyr (R.J.De Groot, unpublished results) and in which the opal to Ser change following nsP3 has been transferred from pToto1000.S. To create plasmid pToto.1234 encoding a non-cleavable P1234 polyprotein, the *Spe*I-*Bss*HII fragment from pTotoSA3 (nucleotides 5262–9804 of the SIN genome) was cloned into pP1-539E/P2-806E. Plasmid pToto.234, encoding non-cleavable P234, was constructed by cloning the 9028 bp *Cla*I–*Xho*I fragment of pToto.1234 into pToto.2.

To construct transcription/translation plasmids for P123 and P23, an additional stop codon was created immediately downstream from the opal stop codon following nsP3 (position 5750) in order to eliminate the partial readthrough that occurs. A 458 bp fragment was produced by PCR amplification (Saiki *et al.*, 1988) using the oligonucleotides 5′-ATGACAGTAGCAAGGCTCACTTT-3′ (SIN nucleotides 5207–5230) and 5′-GTCGACTATCAGTATTCAGTCCTCCTGCTCCTG-3′ (nucleotides 5633–5665; nucleotide substitutions are underlined) as primers and pToto1101 as a template. The resulting fragment was cut with *Spe*I and cloned into pToto.1234 or pToto.234 which had been cut with *Spe*I (position 5262) and *Stu*I (position 10768), to produce pToto.123 or pToto.23, respectively.

In vivo labeling of non-structural proteins

Confluent monolayers of chicken embryo fibroblasts were infected at a multiplicity of 50 p.f.u./cell with Sindbis virus strain HR as described previously (Hardy and Strauss, 1988). After 60 min at 37°C [1 h post infection (p.i.)] the inoculum was removed and the monolayers were washed with phosphate buffered saline lacking divalent cations (PBS) to remove unabsorbed virus. Eagle's Minimal Essential Medium, pre-warmed to 37°C, containing 3% dialyzed fetal calf serum, 1 μg/ml actinomycin D, and 5 μM methionine (1/20 the normal concentration) was then added and incubation was continued at 37°C. At either 1 h 45 min p.i. or 4 h p.i. the medium was removed and the cells were washed with PBS, prewarmed to 37°C, to remove any residual methionine. The cells were then labeled for 5 min in Eagle's methionine free medium supplemented with 80 μCi/ml [^{35}S]methionine (>1000 Ci/mM, Amersham Corp.). After the pulse the cells were lysed, either immediately or following a chase for various times in Eagle's medium containing 2 mM methionine. The preparation of whole cell lysates and the immunoprecipitations were performed as described previously (Hardy and Strauss, 1988). The immunoprecipitated products were analyzed by electrophoresis on 7.5% SDS–polyacrylamide gels.

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