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Biochemistry

Sindbis virus RNA polymerase is degraded by the N-end rule pathway

(alphavirus/polyprotein processing/ubiquitin-dependent proteolysis)

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ABSTRACT **Upon infection of animal cells by Sindbis virus, four nonstructural (ns) proteins, termed nsP1–4 in order from 5′ to 3′ in the genome, are produced by posttranslational cleavage of a polyprotein. nsP4 is believed to function as the viral RNA polymerase and is short-lived in infected cells. We show here that nsP4 produced in reticulocyte lysates is degraded by the N-end rule pathway, one ubiquitin-dependent proteolytic pathway. When the N-terminal residue of nsP4 is changed by mutagenesis, the metabolic stabilities of the mutant nsP4s follow the N-end rule, in that the half-life of nsP4 bearing different N-terminal residues decreases in the order Met > Ala > Tyr ≥ Phe > Arg. Addition of dipeptides Tyr-Ala, Trp-Ala, or Phe-Ala to the translation mixture inhibits degradation of Tyr-nsP4 and Phe-nsP4, but not of Arg-nsP4. Conversely, dipeptides His-Ala, Arg-Ala, and Lys-Ala inhibit the degradation of Arg-nsP4 but not of Tyr-nsP4 or Phe-nsP4. We found that there is no lysine in the first 43 residues of nsP4 that is required for its degradation, indicating that a more distal lysine functions as the ubiquitin acceptor. Strict control of nsP4 concentration appears to be an important aspect of the virus life cycle, since the concentration of nsP4 in infected cells is regulated at three levels: translation of nsP4 requires read-through of an opal termination codon such that it is underproduced; differential processing by the virus-encoded proteinase results in temporal regulation of nsP4; and nsP4 itself is a short-lived protein degraded by the ubiquitin-dependent N-end rule pathway.**

Sindbis virus, the prototype alphavirus, is an RNA animal virus whose genome is 11,703 nucleotides long. Four nonstructural (ns) proteins, called nsP1–4 from their order in the genome, are required for replication and transcription of viral RNAs and are translated as polyprotein precursors from the genomic RNA (1). We have previously reported that the synthesis of nsP4, which is thought to be the viral RNA polymerase (2–4), is regulated by at least two mechanisms in Sindbis virus. First, nsP4 is produced only upon read-through of an opal codon located at the 3′ end of the nsP3 gene (5). As a consequence, nsP4 and nsP4-containing polyproteins are underproduced compared with nsP1, -2, and -3. The second regulatory mechanism involves differential processing of Sindbis polyprotein precursors by a set of virus-encoded proteinases whose active site is located in the C-terminal half of nsP2 (6, 7). Polyprotein precursors containing nsP2 are all proteolytically active but differ in their cleavage-site specificities: those containing nsP1 are unable to cleave between nsP2 and nsP3, whereas only those containing nsP3 cleave between nsP3 and nsP4 (8, 9). Apparently, these differences lead to a temporal regulation of processing such that very early in infection (0–2 hr after infection) nsP4 is produced (9), whereas later in infection polyprotein P34 is produced rather than nsP4 (9–11). We have proposed that this temporal regulation of the relative amounts of nsP4 and P34 is responsible for the cessation of minus-strand RNA synthesis that occurs 3–4 hr after infection (9, 12, 13).

There are indications that a third regulatory mechanism acts on nsP4. Many studies have found that Sindbis nsP4 is metabolically unstable in infected cells (10, 11, 13), as is nsP4 of Semliki forest virus (14). Moreover, Sindbis nsP4 has also been reported to be short-lived in rabbit reticulocyte lysates (6, 8, 9). Wellink and van Kammen (15) suggested that nsP4 could be a target for the ubiquitin proteolytic pathway, a major system for selective protein degradation (for reviews, see refs. 16 and 17). Proteins to be eliminated by this pathway are first conjugated to ubiquitin and subsequently degraded by a large ATP-dependent protease. While a variety of distinct degradation signals are thought to target proteins for ubiquitin-dependent degradation, only one such signal has been defined thus far. The presence of this signal, the N-end rule-based degradation signal or N-degron, is manifested as the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (18, 19). This degradation signal comprises at least two distinct determinants: a destabilizing N-terminal residue (18, 19) and a specific internal lysine residue (or residues) that serves as a multiubiquitination site (20–22). N-terminal residues can be divided into stabilizing (e.g., methionine, glycine, valine) and destabilizing (e.g., tyrosine, phenylalanine, leucine, isoleucine, aspartic acid, glutamic acid, glutamine, arginine, lysine), depending on whether they confer a short half-life onto a protein that contains the second (lysine) determinant of the N-degron (18–22). Although the substrate selection process for this pathway has been studied extensively, natural substrates for this system have proven elusive. Recently, ubiquitin-dependent degradation via unknown degradation signals has been reported for a plant phytochrome (23), for several nuclear oncoproteins (24), and for cyclin (25).

Sindbis nsP4 bears N-terminal tyrosine, a destabilizing residue. Here, we provide evidence that in rabbit reticulocyte lysates nsP4 is degraded by the ubiquitin-dependent N-end rule pathway. The Sindbis virus RNA polymerase is thus the first natural substrate of the N-end rule pathway to be identified. The use of polyproteins is widespread among RNA viruses. Targeting for degradation by a ubiquitin-dependent pathway adds to the list of strategies by which these viruses can regulate the intracellular levels of mature cleavage products.

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Abbreviation: ns, nonstructural.

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MATERIALS AND METHODS

Plasmids. Plasmids pToto1101 and pToto50 contain full-length cDNA copies of the Sindbis virus genome (26). pToto1101.S (8) is a derivative of pToto1101 in which the opal stop codon at the 3′ end of the nsP3 gene was replaced by a serine codon (11). pToto57 is a derivative of pToto50 in which a unique *Xba* I site has been introduced at position 54 of the SIN genome (restriction sites are referred to by the position in the Sindbis genome of the first nucleotide of the recognition sequence) (27). pToto.234 and pToto.123 from which uncleaved proteinases can be produced by cell-free transcription/translation, have been described (9). Cloning procedures and standard molecular biological techniques were performed as described (28).

Cassette Mutagenesis. To construct clone pToto.34, the region spanning nucleotides 4101–5757 of the Sindbis genome was amplified by PCR using the oligonucleotides CATGGCGCCGTCATACCGCACCAAAAG and GTCGACTATCAGTATTCAGTCCTCCTGCTCCTG. The resulting fragment was cut with *Spe* I (position 5262) and cloned into pToto57, which had been cut with *Xba* I (blunt-ended with Klenow) and *Spe* I. To eliminate the opal stop codon at the 3′ end of the nsP3 gene, the *Spe* I (position 5262)/*Hin*dIII (position 6267) fragment was replaced by the corresponding fragment from pToto1101.S.

To construct pToto.34X, a fragment corresponding to positions 5206–5739 of the Sindbis genome was produced by PCR using the oligonucleotides ATGACAGTAGCGAAGGCTCACTTT and CCTTCTAGACCTGCGTCTACGTCTCTG (the introduced *Xba* I site is underlined) and digested with *Spe* I and *Xba* I. A second fragment corresponding to positions 5733–6280 was made by PCR using the oligonucleotides AGGTCTAGAAGGACTGAATACTGACTA and AACTTCTAAGCTTAGCGGGG and digested with *Xba* I and *Hin*dIII (the restriction sites are underlined). The two PCR fragments were ligated to pToto.34 digested with *Spe* I and *Hin*dIII in a three-piece ligation. The sequence of the *Spe* I/*Xba* I region was confirmed by nucleotide sequencing.

For construction of M13mp19.Ssp4, a PCR fragment spanning the region 5760–6280 of the Sindbis genome was produced using the oligonucleotides GTAGGTGGGAATATTTTTTCGACGG and AACTTCTAAGCTTAGCGGGG, creating a new *Ssp* I site (underlined) at position 5769. This fragment was digested with *Hin*dIII and cloned into M13mp19 that had been prepared by digestion with *Sma* I and *Hin*dIII, and the cloned fragment was sequenced in its entirety.

Double-stranded oligonucleotides were synthesized that spanned the region between the *Xba* I (residue 5730) and *Ssp* I (residue 5769) sites of the Sindbis genome and contained an *Xba* I overhang at the 5′ end, a blunt 3′ end, and substitutions in the Tyr-1 codon of nsP4. These synthetic oligonucleotides (the cassette) were joined to the *Hin*dIII/*Xba* I fragment of pToto.34X and the *Ssp* I/*Hin*dIII fragment from M13mp19.Ssp4 in a three-piece ligation.

Transcleavage Assays. Transcleavage assays were performed as described (9). For inhibition experiments, bestatin (to inhibit aminopeptidase activity) and 2 or 8 mM dipeptides were included during the cleavage assay (19, 29).

RESULTS

Mutant Constructs. To test whether the metabolic stability of nsP4 was a function of its N-terminal residue, we constructed plasmid pToto.34X, a derivative of the full-length cDNA clone pToto1101 of Sindbis virus (26) in which the nsP1 and nsP2 genes (nucleotides 60–4101) have been deleted, the opal stop codon near the 3′ end of the nsP3 gene has been replaced with a serine codon, and an initiation codon was inserted at the start of nsP3. To facilitate mutagenesis of the 3/4 cleavage site, a unique *Xba* I site was created upstream of the region encoding the cleavage site (note that the introduction of this site did not alter the amino acid

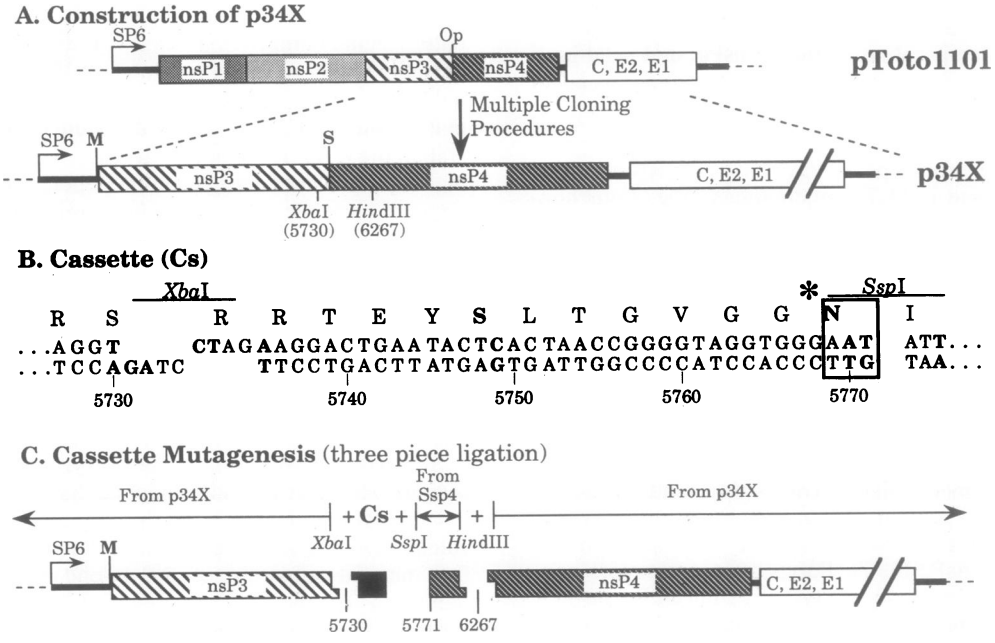

FIG. 1. Cassette mutagenesis. (*A*) Schematic of the Sindbis virus genome as it is present in plasmid pToto1101, together with a schematic of plasmid pToto.34X (p34X) derived from it. The in-frame opal termination codon present in wild-type virus (Op) was replaced by serine (S) in p34X. (*B*) Sequence of the synthetic double-stranded cassette used to mutagenize the first position of nsP4 in p34X. The cassette spans the region between newly created *Xba* I and *Ssp* I sites. The boxed AAT, encoding asparagine, that resulted in creation of the *Ssp* I site, is substituted in the various cassettes by codons for tyrosine (the wild-type amino acid), methionine, phenylalanine, alanine, or arginine. The site of cleavage between nsP3 and nsP4 is indicated by the asterisk. Nucleotides or amino acids altered during the constructions are shown in boldface. (*C*) Three-fragment ligation between the cassette, the *Ssp* I/*Hin*dIII fragment from M13mp19.Ssp4 (Ssp4) and the *Hin*dIII/*Xba* I fragment of p34X, which was used to produce the mutant p34X derivatives.

sequence). The resulting nsP3/nsP4 genes are downstream of the 5′ noncoding region of the Sindbis genome (nucleotides 1–59) and an SP6 promoter (Fig. 1*A*). Transcription of this plasmid with SP6 RNA polymerase and translation of the transcribed RNA in reticulocyte lysates leads to production of the P34 precursor. The N-terminal residue of nsP4 in P34 was changed by cassette mutagenesis, using cassettes illustrated in Fig. 1*B* and the three-piece ligation scheme depicted in Fig. 1*C*. The double-stranded synthetic cassettes span the region between two newly constructed restriction sites and have a 5′ *Xba* I overhang for directional insertion and a blunt 3′ end. The three terminal nucleotides of the cassette (boxed) encode the first amino acid of nsP4 (illustrated as AAT encoding asparagine in Fig. 1*B*, resulting in the creation of an *Ssp* I site). These three nucleotides were changed in the various cassettes such that the N terminus of nsP4 was either tyrosine (the wild-type amino acid), phenylalanine, arginine, methionine, or alanine.

Cleavage of P34 *in Vitro*. SP6 transcripts from constructs encoding different N-terminal residues of nsP4 were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. The P34 species produced were incubated with Sindbis P234 proteinase for 2 hr at 30°C (9) and examined for cleavage at the 3/4 cleavage site. Cleavage was expected to yield nsP4 derivatives differing only in their N-terminal residue.

As shown in Fig. 2, no detectable cleavage occurred in the absence of added P234 enzyme. There was a small amount of nsP4 (but no nsP3) produced in the case of the Met^1 mutant, which we assume arose by initiation at the N-terminal methionine of nsP4. Quantitation of the results with a Molecular Dynamics computing densitometer showed that initiation at the 5′ methionine to produce P34 occurred ≈50-fold more frequently than initiation at the internal methionine.

Addition of P234 led to cleavage of all five P34 derivatives, as shown by the production of nsP3 and nsP4 (Fig. 2). To demonstrate that the site of cleavage was the same in each

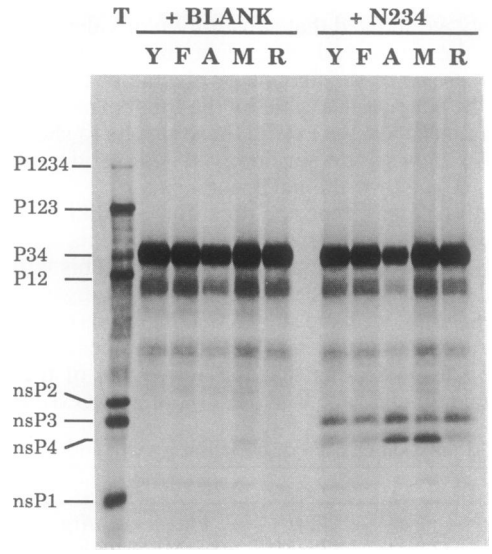

FIG. 2. Metabolic stability of nsP4 is a function of its N-terminal residue. SP6 transcripts of p34X modified to contain different amino acids in the first position of nsP4 were translated for 1 hr at 30°C in rabbit reticulocytes in the presence of [^{35}S]methionine. Translations were terminated by addition of cycloheximide (6 mg/ml) and 1 mM unlabeled methionine. Translates were then incubated with blank reticulocyte lysate for 2 hr at 30°C (*Left*) or with translates of SP6 transcripts of plasmid pToto.234 as a source of P234 enzyme (9) (*Right*). Lane T, translate of Sindbis RNA to mark the positions of the various products. The N terminus of nsP4 in each construct is indicated above the corresponding lane.

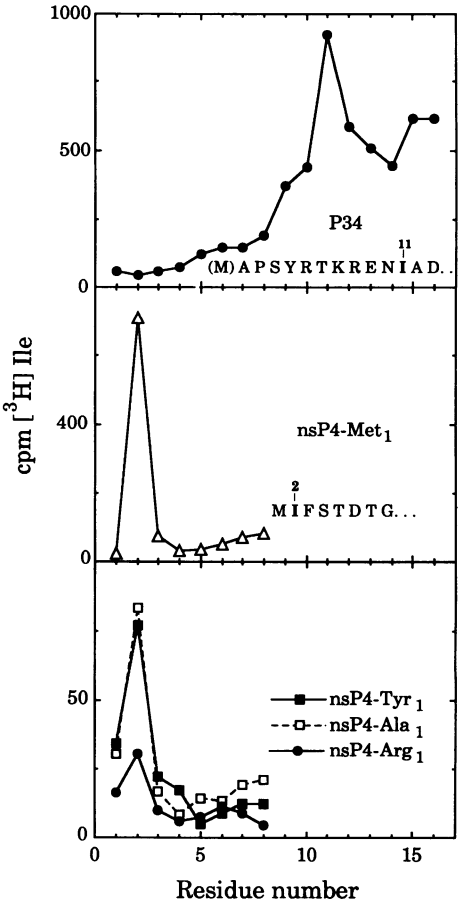

FIG. 3. N-terminal sequence analysis of nsP4 and P34. P34 was made and cleaved as described in the legend to Fig. 2, but the 100-μl translation reaction mixture contained 150 μCi of [^{3}H]isoleucine (1 Ci = 37 GBq) and cleavage was with P123 as enzyme, which appeared to be more efficient than P234. Cleavage of Tyr^1-nsP4 was conducted in the presence of 20 mM Tyr-Ala to stabilize the nsP4 produced (29), but the recovery of Tyr^1-nsP4 was nonetheless low in comparison to Met^1-nsP4. Products were separated on 10% acrylamide gels and microsequenced as described (30). Predicted sequences of P34 and nsP4 are shown (31, 32).

case, N-terminal microsequencing was performed on preparations of wild-type and three mutant nsP4s, on P34, and on two samples of nsP3 (Fig. 3; data not shown). This analysis showed that the initiating methionine was removed from P34 and that in all cases cleavage of P34 occurred at the site predicted from homology with Semliki forest virus (31).

To estimate the extent of cleavage, the bands in the film shown in Fig. 2 and in lighter exposures of this gel were quantitated and the molar ratio of nsP3 to P34 was determined from the known compositions of the proteins (32). From this, the extent of cleavage of P34 was calculated and is shown in Table 1 for each construct. The site GG*A (where the site of

Table 1. Cleavage and stability of nsP4 as a function of its N terminus

N terminus of nsP4	% cleavage of P34	Molar ratio nsP4/nsP3	nsP4 $t_{1/2}$, hr
Arg	18	0.25	0.3
Phe	12	0.32	0.4
Tyr	16	0.34	0.5
Ala	30	0.60	1.2
Met*	11	0.81	3.2

*Data have been corrected for nsP4 produced in the absence of P234 enzyme.

cleavage is indicated by the asterisk) was cleaved about twice as efficiently as the other sites, of interest since both the nsP1/nsP2 and nsP2/nsP3 sites are GA*A. The other four sites tested—GG*Y (the wild-type site), GG*F, GG*M, and GG*R—were all cleaved with similar efficiency. We conclude that the P1′ residue in the cleavage site (here the N terminus of nsP4) has only modest effects on the efficiency of cleavage.

The Metabolic Stability of nsP4 Is a Function of Its N-Terminal Residue. Fig. 2 shows that the amounts of nsP4 found after 2 hr of cleavage by the P234 proteinase depended on the identity of its N-terminal residue. Small amounts of nsP4 (relative to nsP3) were found when the N terminus was tyrosine, phenylalanine, or arginine, while larger amounts were present when the N terminus was methionine or alanine. These results were quantitated and the molar ratios of nsP4 to nsP3 are given in Table 1.

The half-life of nsP4 in reticulocyte lysates can be estimated from these data. The nsP4-producing proteinase is limiting in this experiment, so that nsP3 and nsP4 should be produced linearly with time. If we assume that nsP3 is stable once produced, and that nsP4 is degraded with a first-order rate constant k, then $[nsP4]/[nsP3] = (1 - e^{-kt})/kt$, where t is the time, and the half-life of nsP4 is $t_{1/2} = \ln 2/k$. Half-lives calculated from these equations are given in Table 1; they are probably underestimated because the rate of nsP4 production appears to decrease after the first hour, presumably because of inactivation of the processing proteinase (data not shown).

Degradation of nsP4 Is Inhibited by Dipeptides Bearing Destabilizing N-Terminal Residues. Reiss *et al.* (29) and Gonda *et al.* (19) have shown that the protein substrates of the N-end rule pathway can be divided into distinct groups. Type I substrates have N-terminal arginine, lysine, or histidine; their ligation to ubiquitin and subsequent degradation can be inhibited by dipeptides whose N terminus is one of these three basic amino acids. Type II substrates have bulky, uncharged N-terminal residues. For these substrates, dipeptides containing N-terminal leucine, tyrosine, phenylalanine, or tryptophan have dramatic inhibitory effects, whereas dipeptides bearing basic N-terminal residues have no effect. Fig. 4 shows that the degradation of wild-type nsP4 bearing an N-terminal tyrosine was inhibited by the dipeptides Tyr-Ala, Phe-Ala, or Trp-Ala, but not by His-, Arg-, or Lys-Ala. Similar results were obtained with nsP4 bearing N-terminal phenylalanine. Conversely, when the N terminus of nsP4 was arginine, its degradation was inhibited by His-, Arg-, or Lys-Ala but not by Tyr-, Trp-, or Phe-Ala.

The results from several experiments using dipeptide inhibitors were quantitated, and the data are presented in Table 2. Inhibition is a function of both the concentration and the identity of the N-terminal residue in a dipeptide relative to the N-terminal residue of nsP4. Taken together, these results (Figs. 2 and 4; Tables 1 and 2) indicate that the degradation of nsP4 is carried out by the N-end rule pathway.

Lys^{14} and Lys^{15} of nsP4 Are Not Required for Its Degradation. The N-terminal residue is one essential determinant of the N-degron, the other being a specific internal lysine residue (or residues) (20–22). For example, dihydrofolate reductase is metabolically stable irrespective of whether it bears a destabilizing N-terminal residue (20), but a chimeric protein consisting of dihydrofolate reductase coupled to a 43-residue N-terminal leader derived from an internal sequence of the *lac* repressor is metabolically unstable if it bears a destabilizing N-terminal residue (20). Two specific lysine residues within the leader segment (positions 15 and 17 from the N terminus) act as alternative acceptors for a multiubiquitin chain; the presence of at least one of these lysines is essential for degradation of dihydrofolate reductase bearing a destabilizing N-terminal residue (20, 21). It was proposed that a lysine residue in spatial proximity to the N terminus is required for degradation of a protein by the N-end rule pathway. Interestingly, nsP4 contains two lysine residues in close proximity to the N-terminal tyrosine, at positions 14 and 15. Lys^{15} is conserved in all alphaviruses studied except Middelburg virus. To test whether Lys^{14}-Lys^{15} in Sindbis nsP4 are required for degradation, they were substituted by Gln^{14}-Arg^{15}, the sequence found in Middelburg virus. These mutations did not influence the metabolic instability of nsP4 (data not shown). It appears that a more distal lysine residue acts as ubiquitin acceptor in nsP4.

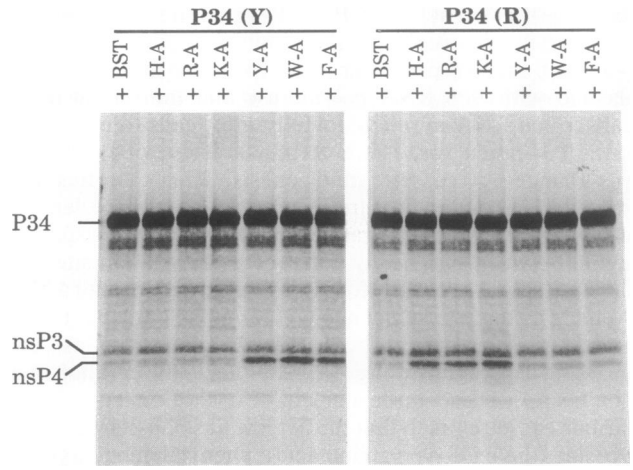

FIG. 4. Stabilization of nsP4 in reticulocyte lysates by dipeptides. nsP4 containing N-terminal tyrosine (*Left*) or arginine (*Right*) was produced by transcleavage as in Fig. 2. Either bestatin (BST) alone or bestatin plus 8 mM dipeptide (indicated at the top of each lane) was present during incubation with P234.

DISCUSSION

Cleavage of P34. We have found that P34 is a substrate for cleavage in trans by the polyprotein proteinase P234 or P123. Previously we showed that substitution of valine or glutamic acid for the conserved glycine at the P2 position of the cleavage site or of the alanine or glycine at the P1 position renders the site noncleavable by the Sindbis nsP2 proteinase (8, 9). In this paper, we extend these results to show that the proteinase shows little sensitivity to the residue at the P1′ position, at least for the nsP3/nsP4 site.

Recently, it was reported that for Semliki forest virus cleavage at the 3/4 site is effected by a proteinase residing in nsP4 (33). This is clearly not the case for Sindbis virus. First, nsP3 and nsP4 were not detected upon mock digestion of P34 (Fig. 2). Second, we have shown that deletions or even single amino acid substitutions in nsP2 abolish processing of the 3/4 cleavage site, indicating that the active site of the enzyme responsible for cleavage is in nsP2 (6, 8, 9; E.G.S., R.J.d.G.,

Table 2. Inhibition of nsP4 degradation by dipeptides

	N terminus				
Inhibitor	Tyr (2 mM)	Tyr (8 mM)	Phe (8 mM)	Arg (8 mM)	Arg (8 mM)
None	0.20	0.22	0.28	0.20	0.19
His-Ala	0.19	0.25	0.27	0.60	0.58
Arg-Ala	0.22	0.25	0.29	0.59	0.63
Lys-Ala	0.18	0.31	0.30	0.76	0.74
Tyr-Ala	0.54	1.05	0.95	0.28	0.26
Trp-Ala	0.48	1.06	1.09	0.29	0.26
Phe-Ala	0.38	0.68	1.01	0.25	0.25

Results are nsP4/nsP3 molar ratio. The two results for arginine are from independent experiments. Numbers in parentheses are inhibitor concentration.

R. Levinson, and J.H.S., unpublished data). It is possible that Semliki forest and Sindbis viruses differ in this respect. However, it is also possible that the nsP4-related polypeptides found upon *in vitro* translation of Semliki forest P34 (33) were generated by internal initiation (e.g., at Met^{35} of Semliki forest nsP4) rather than by proteolytic processing. As described in *Results*, we have observed some synthesis of Sindbis nsP4 by internal initiation in the case of the $Tyr^1 \rightarrow Met^1$ nsP4 mutant. Further characterization of the Semliki forest virus system will be required to resolve this point.

nsP4 Is Degraded by the Ubiquitin-Dependent N-End Rule Pathway. We have found that degradation of Sindbis nsP4 in lysates of rabbit reticulocytes follows the N-end rule: the rate of degradation of the nsP4 derivatives differed, depending on their N-terminal residue. Specifically, the half-life of nsP4 bearing different N-terminal residues was found to decrease in the order Met-nsP4 > Ala > Tyr ≥ Phe > Arg. Using a chimeric polypeptide, Gonda *et al.* (19) obtained the same relative order of metabolic stabilities. Furthermore, the pattern of inhibition of degradation by dipeptides is the same as that previously found (19, 29). It seems clear that nsP4 is degraded by the N-end rule pathway of ubiquitin-mediated proteolysis. We propose that this pathway is also responsible for the instability of nsP4 previously observed in infected cells (10, 11, 13).

Specific internal lysine residues have also been found to be required for degradation by the N-end rule pathway, by functioning as acceptors for multiubiquitination (20, 21). In the case of the chimeric protein studied by Varshavsky and colleagues, the essential lysines were residues 15 and 17 from the N terminus, within an artificial 43-residue leader segment derived from the *Escherichia coli lac* repressor. The Sindbis nsP4 sequence contains two lysine residues at almost the same distance from the N terminus. However, we have shown that these lysines are not essential for degradation; we therefore assume that a more C-terminal lysine acts as ubiquitin acceptor. It is noteworthy that a multisubunit protein was recently described in which the destabilizing N-end was not in the same polypeptide chain as the ubiquitin-accepting lysine (22).

The importance of the metabolic instability of nsP4 for Sindbis virus replication remains to be determined. We presume that the virus has evolved to take advantage of the ubiquitin system to regulate its life cycle. It is noteworthy that nsP4 appears to be long-lived very early in infection (9) and that a fraction of nsP4 produced upon shift to the nonpermissive temperature in cells infected with Sindbis mutant ts17 is also stable (13). One interpretation is that nsP4 is protected against degradation once incorporated in a complex, whereas free nsP4 is rapidly degraded. This view is consistent with recent results that nsP4 associated with replication complexes is stable for several hours (34). A similar phenomenon has been reported for other proteins that form part of multiprotein complexes, such as ribosomal proteins (35), keratin chains (36), and hemoglobin chains (37).

Viruses that express their genetic information as polyproteins have been found to use several strategies to regulate the concentrations of the mature end products. These strategies include synthesizing reduced amounts of the proteins by requiring read-through of leaky stop codons or ribosomal frameshifting (38, 39) and differential processing of polyproteins to produce different products (9, 40, 41). Selective degradation of viral proteins by the ubiquitin-dependent proteolytic pathway provides a regulatory mechanism that may apply to a number of viruses. It is also possible that the mechanism described here in which a viral polypeptide produced by cleavage from a larger protein becomes a target for the N-end rule pathway might be applicable to aspects of cellular metabolism.

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