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Regulation of Nuclear Transport of a Plant Potyvirus Protein by Autoproteolysis

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The NIa proteinase encoded by tobacco etch potyvirus catalyzes six processing events, three of which occur by an autoproteolytic mechanism. Autoproteolysis is necessary to cleave the boundaries of both NIa and the 6-kDa protein, which is located adjacent to the N terminus of NIa in the viral polyprotein. As a consequence, NIa may exist in a free form or in a transient polyprotein form containing the 6-kDa protein. While the majority of NIa molecules localize to the nuclei of infected cells, a fraction of the NIa pool is attached covalently to the 5′ terminus of genomic RNA in the cytoplasm. To determine whether the presence of the 6-kDa protein affects the nuclear transport properties of NIa, we have generated transgenic plants that express genes encoding a reporter enzyme, β-glucuronidase (GUS), fused to NIa or NIa-containing polyproteins. The NIa/GUS fusion protein was detected by histochemical analysis in the nucleus. Similarly, an NIa/GUS fusion protein that arose by autoproteolysis of a 6-kDa/NIa/GUS polyprotein was found in the nucleus. In contrast, fusion protein consisting of 6-kDa/NIa/GUS, which failed to undergo proteolysis because of the presence of a Cys-to-Ala substitution in the proteolytic domain of NIa, was detected in the cytoplasm. The inhibition of NIa-mediated nuclear transport was not due to the Cys-to-Ala substitution, since this alteration had no effect on translocation in the absence of the 6-kDa protein. These results indicate that the 6-kDa protein impedes nuclear localization of NIa and suggest that subcellular transport of NIa may be regulated by autoproteolysis.

The synthesis of viral RNA in well-characterized positive-stranded RNA virus systems has been shown to occur in the cytoplasm (18). However, several of these viruses encode one or more RNA replication-associated proteins that accumulate in the nuclear compartment. For example, the Semliki Forest virus (an alphavirus) nsP2 protein, which is necessary for viral RNA synthesis (20) and proteolytic processing of the viral nonstructural polyprotein (11, 21), localizes to the nuclei of cells infected by Semliki Forest virus and cells transfected by plasmids containing the nsP2 coding sequence (27). The NIa and NIb proteins encoded by some plant potyviruses localize primarily to the nucleus (3, 15, 24), and each has been shown to contain a nuclear localization signal (NLS) (28). The multifunctional NIa protein serves as the genome-linked protein, or VPg, which has been postulated to be necessary for initiation of RNA synthesis or for processing of RNA intermediates (26). This protein also functions as a viral polyprotein-specific proteinase (7). On the basis of sequence similarities with RNA-dependent RNA polymerases of other positive-stranded RNA viruses, the NIb protein appears to be the catalytic subunit of the viral replicase (1, 13). The role of these replication-associated proteins in the nucleus is not clear.

As members of the picornavirus superfamily of positive-stranded RNA viruses, the potyviruses contain a genome encoding a single polyprotein that is processed by three viral proteinases (NIa, HC-Pro, and the 35-kDa N-terminal protein) (5, 7, 14, 31). Cleavage at the N and C termini of NIa is mediated by an autoproteolytic, or *cis*, reaction (6). Similarly, cleavage at the N terminus of the 6-kDa protein, which is located adjacent to the N terminus of NIa in the polyprotein, occurs preferentially by an autoproteolytic mechanism (4, 6). Processing of synthetic polyproteins at these three autocatalytic sites is extremely inefficient when the only active NIa proteinase is supplied in *trans*. If proteolysis occurs first at the N terminus of the 6-kDa protein, it is predicted that the 6-kDa protein/NIa polyprotein would accumulate transiently and then cleave to form mature products (Fig. 1).

Processing of the 6-kDa protein/NIa cleavage site. The efficiency of proteolysis at various tobacco etch potyvirus (TEV) cleavage sites has been proposed to be a regulatory mechanism controlling the rates of formation and processing of polyprotein intermediates (16). Neither the rates of NIa-mediated autoproteolytic cleavage at the CI/6-kDa protein, 6-kDa protein/NIa, and NIa/NIb junctions nor the proportions of polyproteins that undergo cleavage at each of these sites have been determined previously. However, when autoprocessing occurs at the CI/6-kDa protein junction, a polyprotein intermediate consisting of the 6-kDa protein and NIa must be formed (Fig. 1). The longevity of this polyprotein, which is referred to here as 6/NIa, should depend on the rate of autoproteolysis between the 6-kDa protein and NIa. To determine the efficiency of autoproteolytic processing at this site, the 6/NIa polyprotein was synthesized in a rabbit reticulocyte lysate with transcripts derived from pTL7SN-6/NIa (containing TEV genome nucleotides 5533 through 6981) (Fig. 2), and autoproteolysis was examined by a pulse-chase analysis. Radiolabeled protein was generated during a 5-min pulse in the presence of [^{35}S]methionine, which was followed by a chase period in the presence of excess nonlabeled methionine. The polyprotein was detected 5 min after initiation of the chase, while the processed NIa product was detected 10 min after initiation of the chase (Fig. 3). Proteolysis of the polyprotein proceeded with a half time of approximately 20 to 30 min postchase. If the rate of processing measured in vitro reflects that occurring in vivo, the 6/NIa polyprotein may have a relatively prolonged half-life in virus-infected cells.

Effect of the 6-kDa protein on NIa-mediated nuclear localization. The NLS within NIa has been mapped to a bipartite

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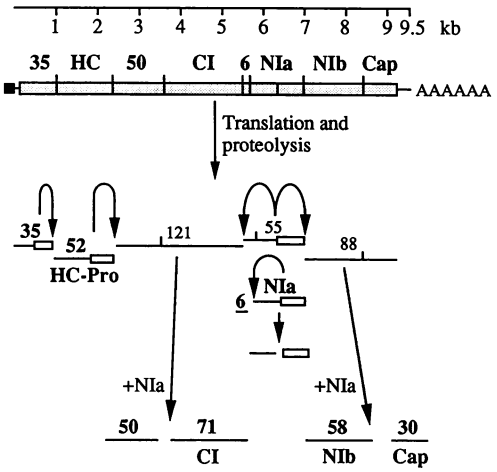

FIG. 1. TEV genome organization and polyprotein processing model. The TEV genome with polyprotein coding region (shaded), genome-linked protein (closed box), and polyadenylate tail is illustrated to scale. The sequences coding for polyprotein cleavage sites are indicated by the vertical dashes. A proposed model for the proteolytic processing steps, as well as the approximate sizes (in kilodaltons) of the polyprotein intermediates and mature proteolytic products, is indicated below the genome map. Cleavage reactions that have been shown or proposed to occur by autoproteolysis are indicated by the curved arrows. The proteolytic domains of the three TEV proteinases are represented by the open boxes. Abbreviations: HC-Pro, helper component-proteinase; CI, cylindrical inclusion protein; NIa and NIb, nuclear inclusion proteins a and b, respectively; Cap, capsid protein.

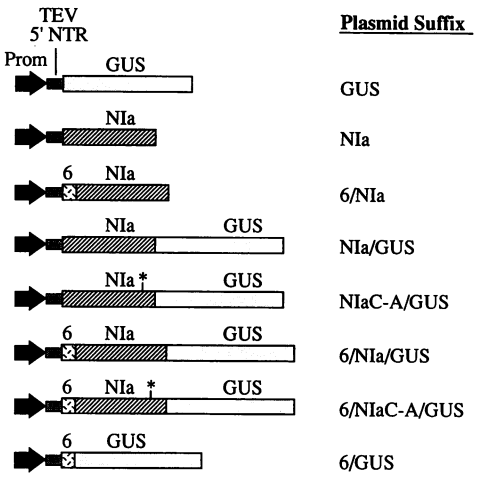

FIG. 2. Diagrammatic representation of relevant portions of GUS fusion plasmids. Each of the coding sequences represented in this diagram was inserted into a vector containing a bacteriophage SP6 promoter (pTL7SN) and a vector containing a cauliflower mosaic virus 35S promoter and terminator-polyadenylation sequence (pRTL2). The expression cassette (transcriptional control sequences and coding regions) from each pRTL2-based plasmid was inserted into the binary vector pGA482. Asterisks indicate the position of a proteinase-inactivating mutation in the NIa sequence at codon 339. The coding sequences in all recombinant plasmids used in this study were linked to the TEV 5′ nontranslated region, which has been shown to enhance the level of translation in vitro and in vivo (8). Plasmids contained cDNA corresponding to TEV genome nucleotides as follows: 6/GUS, 5533 through 5691; NIa, NIa/GUS, and NIaC-A/GUS, 5692 through 6981; 6/NIa, 6/NIa/GUS, and 6/NIaC-A/GUS, 5533 through 6981. As described previously (28), the TEV sequences were fused to the GUS coding region by ligation of introduced *Bgl*II sites. Abbreviations: Prom, promoter; NTR, nontranslated region.

sequence between amino acid residues 1 and 72 near the amino-terminal cleavage site (9). To examine the effect of the 6-kDa protein on NIa-mediated nuclear transport, plasmids encoding the 6/NIa polyprotein fused to the N terminus of the reporter protein, β-glucuronidase (GUS), were constructed (Fig. 2). Polyproteins containing the wild-type NIa sequence should undergo proteolysis between the 6-kDa protein and NIa, as shown above. In contrast, polyproteins containing the proteinase-debilitating substitution of Ala for Cys-339 at the putative active site of the NIa proteinase should not undergo processing (6). Designations for the 6/NIa polyproteins containing this substitution include the term "C-A." In addition, GUS fusion plasmids were assembled by using the 6-kDa protein, NIa, and NIaC-A coding sequences independently. All fusion constructs were inserted into three vectors that were described previously (8, 28). The pTL7SN vector permitted expression in vitro by SP6 transcription and cell-free translation. The pRTL2 vector contains the cauliflower mosaic virus 35S promoter and polyadenylation signal for expression in plant cells. The transcriptional regulatory sequences and polyprotein coding regions of the pRTL2-based plasmids were transferred to a binary vector, pGA482 (2), to facilitate integration in transgenic plants via *Agrobacterium tumefaciens*-mediated transformation, as described previously (8, 10). The names of all plasmids include a designation for the vector (pTL7SN-, pRTL2-, or pGA-) as well as for the coding sequence inserted (e.g., 6/NIa/GUS).

The pTL7SN-based plasmids were used to verify that the TEV and GUS coding sequences were fused in frame and to confirm the presence or absence of proteolytic activities associated with the various polyproteins. Translation of transcripts derived from pTL7SN.3-GUS and pTL7SN-NIa, which encoded nonfused GUS (68-kDa) and NIa (49-kDa) proteins (8, 28), respectively, yielded products of the expected size (Fig. 4, lanes 2 and 3). The apparent sizes of the translation products directed by transcripts from pTL7SN-NIa/GUS (Fig. 4, lane 4) and pTL7SN-NIaC-A/GUS (data not shown) corresponded to that expected of an NIa/GUS fusion protein (117 kDa). A product that comigrated during

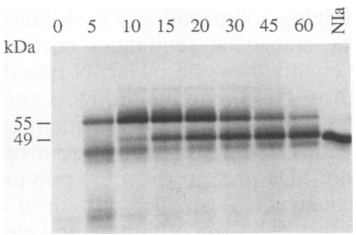

FIG. 3. Pulse-chase analysis of autoproteolytic processing of the 6-kDa/NIa polyprotein in vitro. Translational products encoded by pTL7SN-6/NIa transcripts were synthesized in a rabbit reticulocyte lysate for 5 min in the presence of [^{35}S]methionine, and then excess nonlabeled methionine was added. Samples were withdrawn to protein dissociation buffer after addition of excess cold methionine at the times (in minutes) indicated above the lanes and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The NIa protein was synthesized by translation of transcripts from pTL7SN-NIa. The molecular masses of NIa and the 6/NIa polyprotein are indicated on the left.

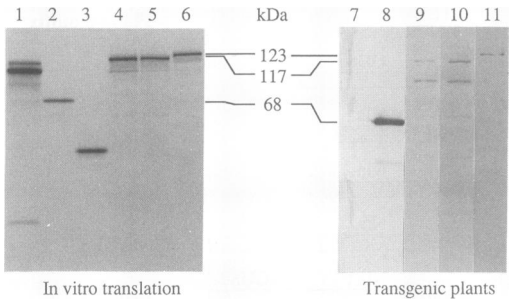

FIG. 4. Synthesis and processing of GUS fusion proteins in vitro and in transgenic plants. Proteins were synthesized in the rabbit reticulocyte lysate system in the presence of [^{35}S]methionine and were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography (lanes 1 through 6). Translation reactions were programmed with brome mosaic virus RNAs (lane 1) and with synthetic mRNA transcripts derived from pTL7SN.3-GUS (lane 2), pTL7SN-NIa (lane 3), pTL7SN-NIa/GUS (lane 4), pTL7SN-6/NIa/GUS (lane 5), and pTL7SN-6/NIaC-A/GUS (lane 6). Total SDS-soluble protein extracts from nontransformed and transgenic *Nicotiana tabacum* cv. Xanthi nc were subjected to immunoblot analysis using anti-GUS serum and alkaline phosphatase-linked rabbit immunoglobulin G (lanes 7 through 11). Immunoreactivity was detected with the colorimetric substrates 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and *p*-nitroblue tetrazolium chloride. Extracts were from plants that were nontransformed (lane 7) or transformed with pGA-GUS (lane 8), pGA-NIa/GUS (lane 9), pGA-6/NIa/GUS (lane 10), or pGA-6/NIaC-A/GUS (lane 11). The approximate molecular masses of GUS, NIa/GUS, and 6/NIa/GUS fusion proteins are given between the panels.

electrophoresis with the NIa/GUS fusion protein accumulated after translation of transcripts from pTL7SN-6/NIa/GUS, which encodes a polyprotein consisting of 6/NIa fused to GUS (Fig. 4, lane 5). This indicates that the 6-kDa protein was proteolytically removed from the rest of the translation product. In contrast, the major product that accumulated after translation of transcripts from pTL7SN-6/NIaC-A/GUS migrated at a position expected of a 123-kDa polyprotein containing the 6-kDa protein, NIa, and GUS, indicating that proteolytic removal of the 6-kDa protein was inhibited (Fig. 4, lane 6).

Expression and processing of fusion proteins in transgenic plants were confirmed by using an immunoblot assay with anti-GUS serum. The sizes of nonfused GUS (Fig. 4, lane 8) and GUS-containing polyproteins (Fig. 4, lanes 9 through 11) encoded by the transgene sequences corresponded to the sizes of those encoded by the pTL7SN-based transcripts in vitro (Fig. 4). Anti-GUS-reactive products encoded by pGA-NIa/GUS and pGA-6/NIa/GUS were identical in size (117 kDa) (Fig. 4, lanes 9 and 10), indicating proper proteolytic removal of the 6-kDa protein from the polyprotein directed by the latter construct. A smaller protein of approximately 95 kDa, which resulted from NIa-mediated cleavage at a site between the VPg and proteinase domains of NIa (17), was also detected in transgenic plants containing these polyproteins. Proteolytic processing between the 6-kDa protein and NIa sequences (and at the internal NIa site) in the pGA-6/NIaC-A/GUS-encoded polyprotein was inhibited, resulting in accumulation of the 123-kDa product (Fig. 4, lane 11). Immunoblot analysis with pGA-6/GUS-transformed plants revealed accumulation of a product with the proper apparent molecular mass (74 kDa) (data not shown).

The subcellular sites of accumulation of GUS activity in epidermal cells of transgenic plants were determined by using a histochemical assay with the colorimetric substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (23, 28). At least five independently transformed plants, one of which was used for the immunoblot assay whose results are shown in Fig. 4, were analyzed for each construct. GUS activity was distributed throughout the cytoplasm of cells containing nonfused GUS (encoded by pGA-GUS) and in the nuclei of cells containing the NIa/GUS fusion protein (pGA-NIa/GUS) (Fig. 5B and C). Since previous studies have shown that the GUS/NIa fusion protein (containing NIa appended to the C terminus of GUS) also localizes to the nuclei of transformed cells (28), we conclude that the NLS functions in a position-independent manner. It should be noted that the enzymatic activity and level of accumulation of nonfused GUS generally were much higher than those of NIa/GUS fusion proteins (28) (Fig. 4), accounting for the widely differing levels of histochemical reactions.

The GUS activity in cells transformed with pGA-6/NIa/GUS sequences was localized to the nucleus (Fig. 5E). Since these cells produce a 6/NIa/GUS fusion protein that undergoes autoproteolysis to remove the 6-kDa protein from NIa/GUS, it is concluded that free 6-kDa protein has no effect on NIa-directed nuclear translocation. Strikingly, GUS activity in cells transformed with pGA-6/NIaC-A/GUS was confined to the cytoplasm (Fig. 5F). In fact, the subcellular distribution of GUS activity in pGA-6/NIaC-A/GUS-transformed cells was similar to that observed in cells producing the 6/GUS fusion protein (pGA-6/GUS), which lacks the NIa sequence (Fig. 5G). As the sequences required for nuclear translocation are located between amino acid residues 1 and 72 of NIa, the Cys-to-Ala substitution at position 339 in the processing-defective polyprotein was predicted to have no effect on translocation. Indeed, GUS activity was detected only in the nuclei of cells transformed with pGA-NIaC-A/GUS (Fig. 5D), indicating that the substituted amino acid residue had no detectable effect on the nuclear transport activity of NIa. These results suggest that the 6-kDa protein impedes NIa-mediated nuclear translocation and that this effect requires linkage of the 6-kDa protein and NIa within a polyprotein.

Regulation of nuclear transport of NIa. The movement of proteins into the nucleus is an active and regulated process that depends on the presence of one or more NLSs (12, 19, 29). Several studies have indicated that translocation, and therefore nuclear function, of proteins can be controlled by posttranslational modifications or noncovalent interactions with cytoplasmic "anchor" proteins (19, 22, 30). Translocation of NIa may be regulated by the differential utilization of proteolytic processing sites. Cleavage at the N terminus of the 6-kDa protein would generate the cytoplasmic 6/NIa polyprotein; acquisition of nuclear transport competence would then be dependent on the rate of NIa self-processing. An autoproteolytic regulatory mechanism may result in transient localization of a proportion of NIa molecules within the cytoplasm of TEV-infected cells.

The NIa protein functions as both a viral polyprotein-specific proteinase and as a protein that attaches covalently to the 5′ terminus of viral RNA (26). Since most TEV-encoded proteins accumulate in the cytoplasm, the proteolytic functions of NIa presumably are carried out prior to nuclear translocation. Considering that replication complexes have been identified in association with a nonnuclear fraction from plants infected by another potyvirus, plum pox virus (25), linkage of NIa to viral RNA during synthesis most likely occurs in the cytoplasm. A transient cytoplasmic

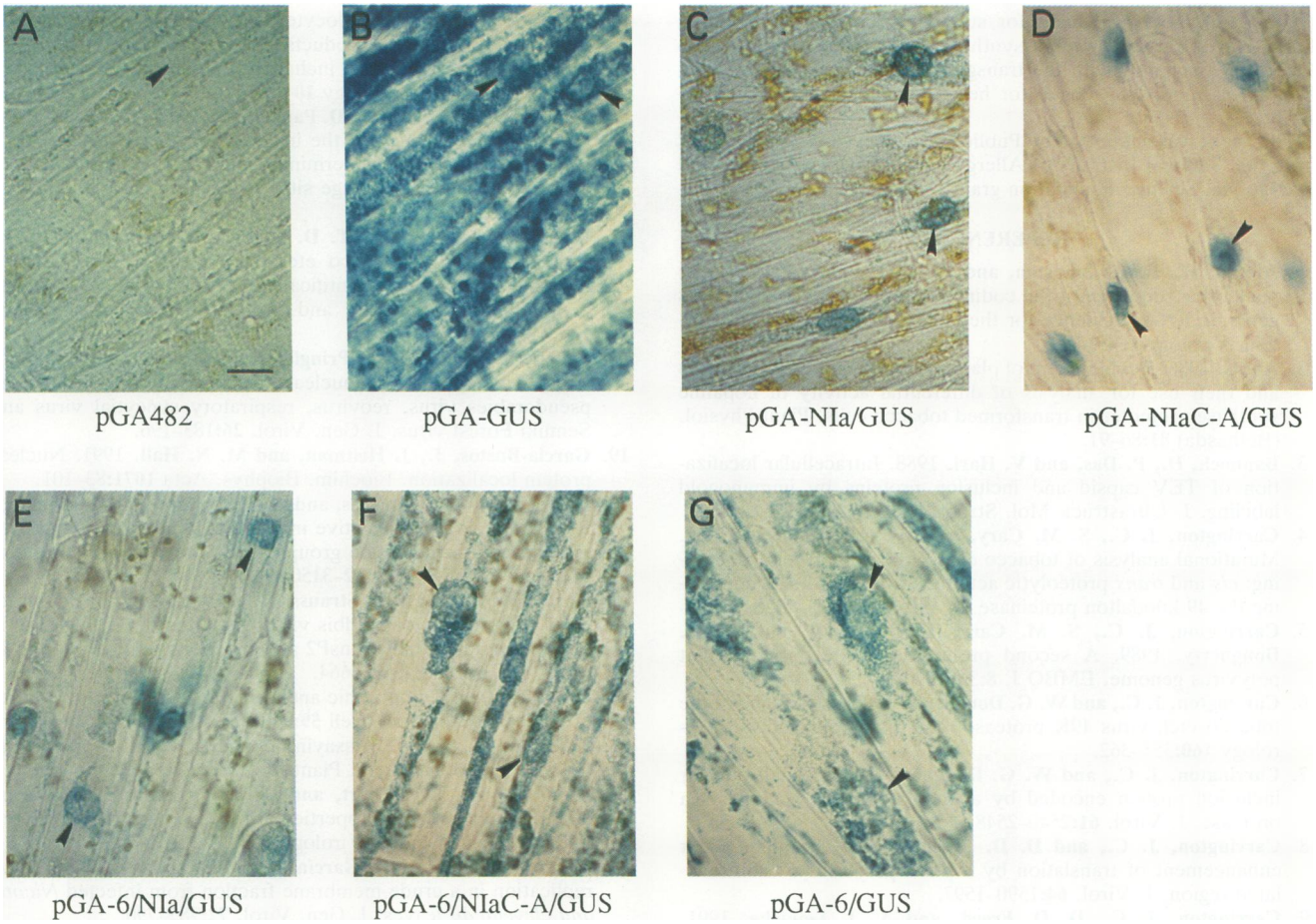

FIG. 5. In situ localization of GUS activity in transgenic plants. Epidermal strips containing single layers of cells from leaves of transgenic plants were incubated in the colorimetric GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. The presence of GUS activity is indicated by the formation of an insoluble indigo precipitate. pGA482 (A) and pGA482-based plasmids (B through G) were used to transform *N. tabacum* plants via *A. tumefaciens*-mediated DNA transfer. Note that GUS activity is localized to the nucleus only in plants transformed with pGA-NIa/GUS (C), pGA-NIaC-A/GUS (D), and pGA-6/NIa/GUS (E). The broad distribution of GUS activity in plants transformed with pGA-GUS (B), pGA-6/NIaC-A/GUS (F), and pGA-6/GUS (G) is indicative of cytoplasmic localization, although some activity appears concentrated immediately outside the nucleus (F and G). Arrowheads, locations of some of the nuclei. Bar = 20 μm.

phase may enable NIa to perform these or other activities. The ca. 20- to 30-min half-life of the 6/NIa polyprotein observed in this study suggests that this putative cytoplasmic form may have ample time to function.

The role(s) of NIa (and NIb) in the nucleus is not known. It is conceivable that NIa (and/or NIb) affects host gene expression, ribosome assembly, or some other nuclear event, although there is little published evidence to suggest that TEV affects host nuclear functions. Many other positive-stranded RNA viruses, however, induce rapid shutoff of host cell transcription and translation. Alternatively, transport of the TEV proteins to the nucleus may be a means of reducing their cytoplasmic concentrations to levels that permit completion of the virus replicative cycle. All TEV-encoded proteins are synthesized in equimolar ratios, which is a consequence of the polyprotein strategy of gene expression. Transport of NIa to the nucleus after a transient period in the cytoplasm would provide a means of limiting the functional concentration of this protein in the absence of transcriptional or translational regulation. It is reasonable to propose that high levels of NIa and/or NIb in the cytoplasm may be inhibitory. For example, accumulation of NIa or NIb in the cytoplasm to the level of capsid protein might interfere with virus assembly.

Two hypotheses concerning the mechanism whereby the 6-kDa protein interferes with the nuclear transport activity of NIa should be considered. First, the 6-kDa protein may mask the NLS of NIa, either by physically interacting with the NLS or by inducing a conformational change in NIa. Such an interaction would be disrupted by autoprocessing, which would expose the NLS and allow access to the nuclear translocation apparatus. Second, the 6-kDa protein may bind to a cytoplasmic anchor which functions to override the nuclear transport capacity of NIa. The putative anchor may be a cytoplasmic protein or a nondiffusible cellular structure. As discussed above for the masking hypothesis, release of NIa from the 6-kDa protein and subsequent translocation would be triggered by autoproteolysis. In either scenario, cellular or viral factors that influence NIa-mediated proteolysis would indirectly affect nuclear translocation of NIa.

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