Stability in vitro of the 69K movement protein of Turnip yellow mosaic virus is regulated by the ubiquitin-mediated proteasome pathway

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Plant viruses move to adjacent cells with the use of virus-encoded cell-to-cell movement proteins. Using proteins produced by in vitro translation, we present evidence that the ‘69K’ movement protein of Turnip yellow mosaic virus (TYMV) is recognized as a substrate for the attachment of polyubiquitin chains and for subsequent rapid and selective proteolysis by the proteasome, the ATP-dependent proteolytic system present in reticulocyte lysate. Truncation of the 69K protein suggests the existence of two degradation signals within its sequence. We propose that selective degradation of virus movement proteins may contribute to the previously reported transient nature of their accumulation during infection.

Introduction

Following replication within the initially infected cells, plant viruses move to adjacent cells through intercellular connections, the plasmodesmata, with the use of virus-encoded cell-to-cell movement proteins (MPs) (reviewed in Maule, 1991; Lazarowitz & Beachy, 1999). The MPs of many different plant viruses have been widely studied, and despite the lack of conserved protein motifs among the MPs from different virus families (Mushegian & Koonin, 1993), they possess a number of common properties, and can often complement each other (reviewed in Hull, 1989). To date, the best studied MP is the 30K protein of Tobacco mosaic virus (TMV) (Deom et al., 1987; reviewed in Rhee et al., 2000).

Characteristics commonly found in MPs are their ability to bind single-stranded nucleic acids in vitro (Citovsky et al., 1990) and to increase the size exclusion limit of plasmodesmata (Wolf et al., 1989). In the case of TMV, the MP was demonstrated to colocalize with the cytoskeleton both in vitro and in the context of virus infection (McLean et al., 1995; Heinlein et al., 1995). More recently, its interaction with plant cell wall pectin methylesterases has also been reported (Chen et al., 2000). Based on these observations, the following sequence of events has been suggested: the MP is thought to form complexes with the transported viral RNA, move these complexes throughout the cell using the cytoskeletal network, associate with the cell wall, increase plasmodesmatal permeability and transverse the enlarged plasmodesmatal channels (reviewed in Rhee et al., 2000). The exact mechanism of action is, however, still hypothetical.

It has been proposed that MP activity within infected cells may be negatively regulated in order to prevent continuous interference with host plant intercellular communication. Indeed, the MPs of a number of viruses were found to accumulate only transiently during early and mid-stages of virus infection (Lehto et al., 1990a; reviewed in Maule, 1991). In the case of TMV, the timing of 30K expression, rather than its level of accumulation, was found to be critical in determining the efficiency of virus movement (Lehto et al., 1990b). Because the selective degradation of proteins is a recurrent theme in regulatory mechanisms involving timing control (Hershko, 1996; Hochstrasser, 1996), it is possible that rapid degradation of the virus MP may contribute to the transient nature of its accumulation during viral infection. In this respect, it has recently been reported that the TMV MP is degraded in vivo through a proteasome-dependent degradation pathway (Reichel & Beachy, 2000). Apart from the latter protein, however, no information has been reported to date concerning the processes by which the transient accumulation of MPs operates.

Here, we address this question by studying the MP of Turnip yellow mosaic virus (TYMV), the type member of the tymovirus group. TYMV is a small spherical plant virus that...
infected members of the Cruciferae. It possesses a monopartite positive-strand RNA genome of 6318 nucleotides, which directs the expression from two extensively overlapping open reading frames (ORFs) of nonstructural proteins of 69 and 206 kDa (Morch et al., 1988). A third ORF encodes the 20 kDa coat protein which is expressed from a subgenomic RNA. The 69 kDa (‘69K’) protein is necessary for cell-to-cell movement and systemic spread of the virus within the plant (Bozarth et al., 1992). It shares several common characteristics with MPs of other viruses, and the level of 69K protein in infected plants was found to decline as infected leaves expand and mature (Bozarth et al., 1992).

We have examined the turnover of the TYMV 69K movement protein in rabbit reticulocyte lysate (RRL) with the goal of obtaining information that will facilitate future studies with infected cells. This in vitro system was selected because it has been employed as a model system for examination of cellular proteins which are rapidly degraded (reviewed in Hershko, 1996) and it has been used extensively in studies of the synthesis of plant viral proteins, including those of TYMV (Morch et al., 1989; Rozanov et al., 1995).

Using proteins produced by in vitro translation, we present evidence that the TYMV 69K protein is recognized as a substrate for the attachment of polyubiquitin chains and for subsequent rapid and selective proteolysis by the proteasome, the ATP-dependent proteolytic system present in reticulocyte lysate.

Methods

Construction of plasmids. All DNA manipulations were performed using standard techniques (Ausubel et al., 1987; Sambrook et al., 1989). Plasmids TYFL7, C7835 and TYFL814, from which genomic length transcripts corresponding to the entire genome of TYMV can be obtained, were described previously (Rozanov et al., 1995; Boyer et al., 1993). Plasmid E17 was constructed by digesting pTYFL7 with SmaI and EcoRI and inserting the corresponding 263 nucleotide SmaI–EcoRI fragment of TYFL814, therefore removing the 75 non-viral nucleotides present at the 3’ terminus of the TYMV cDNA copy. To obtain E17-206K-stop, a DNA fragment was amplified by PCR using Pfu DNA polymerase (Promega) and primers 5’ GGAATGGACCATGGGTAGGTCTGTATCTAGGAGCGA-3’ and 5’ GAATTGACCATGGGTAGGTCTGTATCTAGGAGCGA-ATTG 3’, which hybridize to E17. After digestion with HindIII and NcoI, the 230 nucleotide fragment was cloned in the similarly restricted E17 to create E17-206K-stop, in which a stop codon truncates the 206K ORF at amino acid 33 without modification of the 69K ORF.

Plasmid E17-AN was obtained by digestion of E17 with MfeI and Psfl, followed by insertion of the adaptor 5’ AATTCGAAAAATCCTAG ACC3’. Plasmid E17-206K-stop-AN was constructed by digesting E17-206K-stop with MfeI and NcoI followed by Klenow filling-in and religation. Constructs were verified by sequencing with an ABI PRISM 377 DNA sequencer (Applied Biosystem) using a BigDye Terminator Sequencing kit and specific primers.

In vitro transcription. Capped in vitro transcripts were obtained from linearized DNA templates using the Message Machine Transcription system (Ambion) according to the supplier’s instructions.

Preparation of in vitro translation products. In vitro translation reactions of viral RNA or in vitro transcripts were carried out in micrococcal nuclelease-treated reticulocyte lysate with conditions based upon those previously described (Morch et al., 1989). A typical reaction mixture (10 µl) contained 250 ng of TYMV RNA or 200 ng of in vitro transcript in 50% (v/v) reticulocyte lysate supplemented with 25 µM of each amino acid except methionine and cysteine. Labelled proteins were prepared by including 530 kBq of a mixture of t-labeled methionine and t-labeled cysteine (Pro-Mix, Amersham; 37 TBq/mmol) in the reaction mixture. Alternatively, an amino acid mixture without leucine was used together with 2.8 kBq t-labeled leucine (Amersham, 11 GBq/mmol). Incubations were performed at 30 °C for 1 h and terminated by the addition of Laemmli sample buffer (Laemmli, 1970). Translation products were analysed by 10, 12.5 or 15% SDS–PAGE. Gels were fixed, dried and exposed to film or analysed by Storm Phosphorimager (Molecular Dynamics).

Assays for protein degradation. The stability of the proteins synthesized in reticulocyte lysate was evaluated using previously described methods (Oberst et al., 1993; Orian et al., 1995) with minor modifications. Following incubation for 1 h at 30 °C, the translation reactions were terminated by addition of 1/10 vol. of mix stop (20 mM methionine, 20 mM cysteine, 1 mg/ml cycloheximide, 500 µg/ml RNase A), followed by a further 15 min incubation at 37 °C. The stability of the proteins was then monitored by incubating 2.2 µl of terminated translation mixture in 30 µl reaction mixtures containing 40 mM Tris–HCl pH 7.5, 5 mM KCl, 5 mM MgCl2, 2 mM DTT, 0.5 mM ATP, 10 mM creatine phosphate, 200 µg/ml creatine kinase, 40 µg/ml (5 µM) ubiquitin (Ub; Sigma) in 30–50% (v/v) untreated reticulocyte lysate or 2 µg/ml reticulocyte fraction II (Affiniti). Control samples were processed similarly, except that untreated reticulocyte lysate was omitted from the reaction mixture. The mixtures were incubated further at 37 °C for the indicated period of time and, after addition of Laemmli sample buffer, the samples were resolved by SDS–PAGE and analysed as described above. The loss of labelled proteins during the incubations was quantified with a Phosphorimager and ImageQuant software (Molecular Dynamics). The amount of radioactivity present in the zero-time samples after addition of untreated lysate was taken to represent 100% of the labelled protein present at the beginning of the incubation.

MG132 ([Cbz-L-L-L]-carbobenzoxy-leucyl-leucyl-leucinal) (Calbiochem) was dissolved in DMSO and proteasomal degradation was determined by adding either 1% DMSO or 1% DMSO plus 100 µM MG132 to the degradation assay.

Measurement of the effects of ATP analogues on degradation. ATP-dependent processing was performed in the presence of 0.5 or 2 mM ATP, 10 mM phosphocreatine and 0.2 mg/ml creatine kinase. Mixtures were incubated for the indicated period of time on ice or at 37 °C. Enzymatic ATP depletion was accomplished by adding 10 mM deoxyglucose and 20 µg/ml of hexokinase to the degradation assays. The effect of ATP analogue was assayed by substituting 10 mM ATPγS (adenosine 5’-O-(3-thiotriophosphate)) (Roche) for ATP and the ATP-regenerating system. Following incubation, aliquots from the reaction mixtures were resolved by SDS–PAGE and analysed as described above.

Detection of Ub conjugates. Translation was carried out in micrococcal nuclease-treated rabbit reticulocyte lysate at 30 °C in the presence of t-labeled leucine and 1 µM Ub aldehyde (Affiniti), either alone or with added 20 µM Ub (Sigma) or 200 µM methylated Ub (Affiniti). Aliquots of 8 µl were removed at the indicated times and subjected to SDS–PAGE as described above.

For immunoprecipitation, the translation mixture (20 µl) was mixed
with Laemmli sample buffer and boiled for 10 min. The sample was then diluted to 300 μl with IP1 buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% sodium deoxycholate, 0.5% NP40) and 4 μl of an antiserum raised against the 69K protein (Séron et al., 1996). After overnight incubation at 4 °C, the antigen–antibody complexes were precipitated with Pansorbin (Calbiochem) according to the supplier’s instructions. After washes in IP2 buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS) and IP3 buffer (20 mM Tris–HCl pH 7.5, 0.1% NP40) the immunoprecipitates were separated by SDS–PAGE as described above.

### Results

#### Stability of TYMV-encoded proteins in the RRL system

The stability of the TYMV-encoded proteins was studied in the RRL system. This in vitro system was selected because it has been employed as a model system for examination of cellular proteins which are rapidly degraded (Hershko et al., 1983; reviewed in Hershko, 1996) and the reticulocyte Ub-mediated degradation process is therefore one of the most thoroughly characterized. In addition, RRL has been used extensively in studies of the synthesis and processing of TYMV-encoded proteins (Morch et al., 1989; Bransom et al., 1991; Rozanov et al., 1995). In particular, it has been shown that the 206K protein undergoes co-translational proteolytic processing that gives rise to two protein products of 140 kDa (140K) and 66 kDa (66K) (Morch et al., 1989; Bransom et al., 1991).

35S-Labelled TYMV-encoded proteins were synthesized in RRL, followed by SDS–PAGE analysis and autoradiography. As expected, proteins corresponding to the 69K MP, to the 206K polyprotein and its self-processed products 140K and 66K, were produced by translation of TYMV RNA (Fig. 1a, lane 1). In addition to the major viral proteins, translation of TYMV RNA typically gives rise to the synthesis of several minor bands that are most likely incomplete in vitro translation products (Morch et al., 1989).

The stability of the viral proteins was then monitored by incubating the terminated translation mixtures for various times in the presence of either added buffer or untreated RRL. As shown in Fig. 1(a), lanes 11–13, incubation in the presence of added buffer resulted in little degradation of the viral proteins. This is consistent with the fact that the nuclease-treated RRL used for in vitro translation contains haemin, which is an inhibitor of protein degradation (Etlinger & Goldberg, 1980; Haas & Rose, 1981). However, protein degradation can be restored by addition of untreated RRL, which provides an active proteolytic system. In these conditions, and with percentages of added untreated RRL varying from 30 to 50% of the final reaction mixture volume, the TYMV 69K protein rapidly disappeared from the reaction mixture (Fig. 1a, lanes 2–10).

To determine if the instability of the TYMV 69K protein is a characteristic unique to this protein, the percentages of the viral 206K, 140K, 66K and 69K proteins remaining in the reaction mixtures were measured as a function of time and their rates of degradation were quantitatively compared. As shown in Fig. 1(b), on addition of untreated RRL the 69K protein disappeared with a half-life of about 25 min. The other viral products all appeared to have a half-life of ~ 3 to 4 h. Both the rate and extent of disappearance of the 69K protein were similar in the presence of various batches of RRL (data not shown). This demonstrates that the TYMV 69K protein is selected for rapid degradation by the reticulocyte proteolytic machinery.

#### Degradation of the 69K protein is independent of the TYMV-encoded proteinase domain

The TYMV 206K protein has been shown to contain a papain-like cysteine proteinase domain that is involved in its self-processing (Bransom & Dreher, 1994; Rozanov et al., 1995). Therefore, the question arises whether this proteinase domain might be involved, either directly or indirectly, in degradation of the 69K protein, or whether the proteolytic system responsible resides exclusively in the reticulocyte lysate. To answer this question, transcripts that correspond to the viral genome and encode wild-type proteinase (E17) or mutated transcripts carrying a point mutation in the proteinase active site (C783S) (Rozanov et al., 1995) were translated in vitro. Debilitation of the viral proteinase proteolytic activity of C783S is evident from the lack of processing of 206K protein into the 140K and 66K cleavage products (Fig. 2, lane 7), as previously reported (Rozanov et al., 1995). On addition of untreated RRL to completed translation mixtures, the 69K proteins encoded by both transcripts were degraded at similar rates (Fig. 2, lanes 4–9). This observation was further confirmed by translation of E17-206K-stop transcripts in which synthesis of the 206K protein is abolished by introduction of a nonsense codon at nucleotides 194–196 of the viral genome. Degradation of the 69K protein encoded by such transcripts (Fig. 2, lanes 10–12) was unaffected by the lack of 206K protein, therefore demonstrating that the TYMV-encoded proteinase activity is not required for the rapid degradation of the 69K protein in this system.

#### Degradation of the 69K protein is dependent upon 26S proteasome and ATP hydrolysis

The best characterized mechanism for the selection and degradation of short-lived proteins in eukaryotic cells is the Ub-mediated proteolytic system (reviewed in Hershko & Ciechanover, 1998). This multicomponent system, which has been most extensively studied in RRL and yeast, targets substrate proteins for degradation by covalent attachment of the polypeptide Ub. The proteolytic degradation process of poly-Ub-marked proteins is then accomplished by the 26S proteasome, a very large ATP-requiring multicatalytic cellular protease complex (reviewed in Voges et al., 1999). Experiments...
Fig. 1. Stability of TYMV-encoded proteins in the RRL system. (a) TYMV viral RNA was translated for 1 h in nuclease-treated RRL in the presence of $[^{35}\text{S}]$Met and $[^{35}\text{S}]$Cys. After termination of translation, the reaction mixtures were incubated in the presence of either untreated RRL (30–50% of total volume) or added buffer as described in Methods. Aliquots were removed at various times and the samples were then analysed by 10% SDS–PAGE and autoradiography. Lane 1, TYMV translation products prior to incubation. Incubations were performed in the presence of RRL at 30% (lanes 2–4), 40% (lanes 5–7) or 50% (lanes 8–10) of total volume or in the presence of buffer (lanes 11–13). Aliquots were removed at 0 min (lanes 2, 5, 8 and 11), 45 min (lanes 3, 6, 9 and 12) and 105 min (lanes 4, 7, 10 and 13). The incubation times are indicated at the top of the lanes. Bands corresponding to the viral proteins 206K, 140K, 69K and 66K are indicated. (b) Comparison of the stabilities of the 206K, 140K, 69K and 66K TYMV-encoded proteins in the presence of added untreated RRL (50% of total volume). The reaction mixtures were prepared, analysed by SDS–PAGE and quantified as described in Methods.

Fig. 2. Degradation of the 69K protein is independent of the TYMV-encoded proteinase domain. TYMV viral RNA or in vitro transcripts were translated for 1 h in nuclease-treated RRL in the presence of $[^{35}\text{S}]$Met and $[^{35}\text{S}]$Cys. After termination of translation, the reaction mixtures were incubated in the presence of untreated RRL (50% of total volume) as described in Methods. Aliquots were removed at various times and the samples were then analysed by 10% SDS–PAGE and autoradiography. Templates were TYMV RNA (lanes 1–3) or in vitro transcripts deriving from plasmids E17 (lanes 4–6), C783S (lanes 7–9) and E17-206K-stop (lanes 10–12). Aliquots were removed at 0 min (lanes 1, 4, 7 and 10), 30 min (lanes 2, 5, 8 and 11) and 60 min (lanes 3, 6, 9 and 12). The incubation times are indicated at the top of the lanes. Bands corresponding to the viral proteins 206K, 140K, 69K and 66K are indicated.

were carried out to assess the involvement of the proteasome in the TYMV 69K protein turnover.

Previous studies on Ub conjugation and proteolysis in vitro have predominantly employed RRL ‘fraction II’ (Ciechanover et al., 1978). Fraction II contains the 26S proteasome complex and supports the ATP-dependent conjugation and degradation of target proteins, as long as ATP and Ub are provided. As shown in Fig. 3 (lanes 2–4), RRL fraction II with added Ub and an energy-generating system readily supported degradation of the TYMV 69K protein. Since the turnover of proteins by the 26S proteasome degradation complex requires energy produced by the hydrolysis of ATP (Hershko et al., 1980),
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Fig. 3. Degradation of the 69K protein requires ATP hydrolysis. TYMV viral RNA was translated in nuclease-treated RRL for 1 h in the presence of [35S]Met and [35S]Cys. After termination of translation, the reaction mixtures were incubated in the presence of RRL fraction II as described in Methods. Aliquots were removed at various times and the samples were then analysed by 10% SDS-PAGE and autoradiography. Lane 1, TYMV translation products prior to incubation. Incubations were performed in the presence of 0–5 mM ATP (lanes 2–4), 2 mM ATP (lanes 5–7), without ATP (lanes 8–10) or with 10 mM ATP (lanes 11–13). Incubations were performed at 37 °C (lanes 2–4 and 8–13) or 0 °C (lanes 5–7). Aliquots were removed at 0 min (lanes 2, 5, 8 and 11), 30 min (lanes 3, 6, 9 and 12) and 60 min (lanes 4, 7, 10 and 13). The incubation times are indicated at the top of the lanes. Bands corresponding to the viral proteins 206K, 140K, 69K and 66K are indicated.

Fig. 4. Degradation of the 69K protein requires proteasome activity. In vitro transcript E17-206K-stop was translated for 1 h in nuclease-treated RRL in the presence of [35S]Met and [35S]Cys. After termination of translation, the reaction mixtures were incubated in the presence of untreated RRL (50% of total volume) with or without added 100 µM MG132 as described in Methods. Aliquots were removed at various times and the samples were then analysed by 10% SDS-PAGE and autoradiography. Incubations were performed in the presence of 1% DMSO (lanes 1–3) or 1% DMSO plus 100 µM MG132 (lanes 4–6). Aliquots were removed at 0 min (lanes 1 and 4), 30 min (lanes 2 and 5) and 60 min (lanes 3 and 6). The incubation times are indicated at the top of the lanes.

degradation of the 69K protein was assayed in the absence or presence of ATP, and incubation was carried out at 37 or 0 °C. As can be seen in Fig. 3, incubation on ice (lanes 5–7) or ATP depletion (lanes 8–10) both considerably reduced the rate at which the 69K protein was degraded. It thus appears that the system which degrades the TYMV 69K protein requires ATP hydrolysis. This conclusion is further supported by degradation assays performed in the presence of ATPγS, an ATP analogue that supports conjugation of Ub to substrate proteins but does not sustain the ATP-dependent functioning of the 26S proteasome complex (Johnston & Cohen, 1991). As shown in Fig. 3 (lanes 11–13) the presence of the analogue inhibited the degradation of 69K protein by RRL fraction II.

Additional evidence for involvement of the proteasome was obtained by use of the peptide analogue MG132, a specific inhibitor of proteasome proteolytic activity (Lee & Goldberg, 1996). As can be seen from Fig. 4 the presence of MG132 throughout the incubation period (lanes 4–6) significantly reduced the extent of degradation of TYMV 69K protein as compared to degradation assays containing solvent only (lanes 1–3). Altogether, these results indicate that degradation of the TYMV 69K protein is an ATP-dependent process that is accomplished by the 26S proteasome complex.

Evidence for conjugation of the 69K protein with Ub

The great majority of proteins that are degraded by the 26S proteasome are targeted for degradation by multiple Ub conjugation (reviewed in Pickart, 1997). Ubiquitination involves the covalent conjugation of Ub, a highly conserved 76 amino acid residue protein, to the target protein via an isopeptide bond between the C terminus of Ub and the ε-amino group of one or more lysine residues of the protein. The linkage of the first Ub moiety is usually followed by the sequential conjugation of additional Ub molecules by isopeptide linkages between the ε-amino group of a specific lysine residue of Ub and the C-terminal carboxylic acid group of the
next molecule in the chain. Such poly-Ub chains appear to be essential for recognition and subsequent degradation by the proteasome of the target protein.

Experiments were therefore designed to evaluate the possibility that 69K–Ub conjugates may be generated in RRL. To improve the detection of the 69K-derived products, in vitro translation of E17-206K-stop transcripts was performed in the presence of [14C]leucine, because the 69K protein encodes many more leucines than methionines and cysteines. Since Ub conjugation occurs concurrently with degradation of the modified proteins and with disassembly of the poly–Ub chains by deubiquinating activities present in cell lysates, it may be necessary to inactivate both proteasomal proteases and Ub-cleaving isopeptidases in order to detect ubiquitinated proteins (Mimnaugh et al., 1999). For this reason, conjugation was analysed in the presence of ubiquitin aldehyde (Ubal), an inhibitor of Ub-cleaving isopeptidases (Hershko & Rose, 1987), using haemin-inhibited RRL as a source of conjugating enzymes. As shown in Fig. 5, translation of E17-206K-stop transcripts revealed the appearance of labelled species with molecular masses greater than that of the TYMV 69K protein. These ‘smears’ of labelled species are characteristic of the formation of a heterogeneous mixture of poly-Ub–69K conjugates as a consequence of progressive addition of single Ub molecules to multiple lysine residues or to poly-Ub chains of various lengths (Mimnaugh et al., 1999). Without Ubal (Fig. 5, lanes 5 and 9), the steady-state level of conjugate accumulation led to products that accumulated in the ~150 kDa region of the gel. The size of Ub–protein conjugate intermediates was increased substantially (> 200 kDa) when Ubal was added to the reaction (Fig. 5, lanes 6 and 10). This effect was further amplified in the presence of supplementary free Ub (Fig. 5, lanes 7 and 11), where the smears of mult ubiquitinated material extended toward the top of the gel. Further evidence for ubiquitination of the 69K protein was obtained by including methylated ubiquitin (MeUb) in the reaction mixtures. The blocked amine groups allow MeUb to become incorporated into conjugates with other proteins, but the subsequent conjugation of additional Ub molecules is prevented (Hershko & Heller, 1985). Under the conditions employed, MeUb may be linked to free amino groups of the native Ub present in the RRL to form mixed conjugates. Indeed, incubation in the presence of MeUb resulted in the generation of discrete higher molecular mass products (Fig. 5, lane 12, indicated by *), whose spacing was consistent with the synthesis of a classical ladder of 69K–Ub conjugates. Those products were readily immunoprecipitated by an antiserum raised against the 69K protein (Fig. 5, lane 13), therefore providing evidence for Ub conjugation of the 69K protein in RRL.

**Mapping the region of the 69K protein that governs degradation**

Degradation signals (degrons) usually comprise two essential and separable determinants: an amino acid or conformational primary determinant that functions as a binding site for a substrate recognition factor, and one or more internal lysine residues that are the sites of ubiquitylation (reviewed in Kornitzer & Ciechanover, 2000). Experiments were carried out to identify degrons present within the TYMV 69K protein. We initially examined the behaviour of an N-terminal deletion of the 69K protein (69KΔ1) encoded by the plasmid E17-ΔN, as...
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Fig. 6. Localization of sequences governing degradation of the 69K protein. (a) Diagram summarizing the primary structures of the proteins used in (b). Positions of lysine residues are indicated by dots. The expected molecular mass of each translation product is indicated on the right. The expected molecular mass of each translation product is indicated on the right. (b) The plasmids E17-ΔN, E17-206K-stop and E17-206K-stop-ΔN were linearized at various restriction sites and the corresponding in vitro transcripts were translated for 1 h in nuclease-treated RRL in the presence of [35S]Met and [35S]Cys. After termination of translation, the reaction mixtures were incubated in the presence of untreated RRL (50% of total volume) as described in Methods. Aliquots were removed at various times and the samples were then analysed by 12–15% SDS–PAGE and autoradiography. Templates were in vitro transcripts obtained after digestion of the plasmid E17-ΔN with AgeI (lanes 10–12), plasmid E17-206K-stop with AgeI (lanes 1–3), PstI (lanes 4–6), AccI (lanes 7–9), BsrGI (lanes 13–15), AvaI (lanes 16–18) and BsmFI (lanes 19–21) and plasmid E17-206K-stop-ΔN digested with AciI (lanes 22–24). Aliquots were removed at 0 min (lanes 1, 4, 7, 10, 13, 16, 19 and 22), 30 min (lanes 2, 5, 8, 11, 14, 17, 20 and 23) and 60 min (lanes 3, 6, 9, 12, 15, 18, 21 and 24). The incubation times are indicated at the top of the lanes.

Discussion

Research into cellular protein turnover has shown that proteins are degraded both in vitro and in vivo, with half-lives...
ranging from a few minutes to several weeks (Hershko & Ciechanover, 1992; Vierstra, 1996). The results shown here demonstrate that the TYMV 69K protein is selectively degraded in terminated in vitro translation reaction mixtures with a half-life of about 25 min (Fig. 1). The TYMV 69K protein can therefore be categorized as a very unstable protein. This degradation process was found to be independent of other virus-encoded proteins (Fig. 2) but dependent on ATP hydrolysis (Fig. 3), a feature that is a hallmark of degradation by the Ub–proteasome pathway (reviewed in Hershko & Ciechanover, 1992; Hershko & Ciechanover, 1998). Accordingly, degradation of the 69K protein was readily supported by RRL fraction II (Fig. 3), while inhibitors of proteasomal function stabilized the protein (Figs 3 and 4). Reconstitution of a cell-free conjugation system (Fig. 5) further corroborated the notion that the 69K protein is multi-ubiquitinated, a process that typically precedes proteasomal degradation.

These findings show that the rapid degradation of the TYMV 69K protein in vitro can be accomplished by the Ub-mediated proteasome proteolytic system. Taking into account the correlation between in vitro and in vivo degradation that has been reported by different laboratories (Rote et al., 1989; Gonda et al., 1989; Ciechanover et al., 1991), we suggest that the previous report on the transient nature of accumulation of TYMV 69K protein in the course of infection (Bozarth et al., 1992) may be caused by its rapid and selective degradation through a Ub-dependent proteasome degradation pathway. Further studies in vivo are required to elucidate whether this is indeed the case. Interestingly, evidence for the involvement of such a degradation pathway in control of stability of another virus MP, that of TMV, has recently been presented (Reichel & Beachy, 2000). While it may be coincidental that the TYMV 69K and TMV 30K proteins are both substrates for proteasomal proteolysis, it may also be regarded as an emerging pattern of metabolic instability among virus movement proteins. It should be emphasized that these two proteins do not share sequence similarity and belong to two different classes of virus MPs (Mushegian & Koonin, 1993). Since transient accumulation of MP during early and mid-stages of virus infection has been described for a number of virus families (reviewed in Maule, 1991), it will be interesting to determine whether control of MP accumulation in infected cells also involves rapid proteasomal degradation or whether additional regulatory mechanisms involving RNA synthesis or translational control are involved.

The selective Ub-mediated proteolytic system is known to be involved in a variety of basic cellular processes, for instance development, apoptosis and cell cycle regulation (reviewed in Hershko & Ciechanover, 1998). Several viral pathogens have also been reported to exploit this cellular pathway. In a few cases, viral proteins were found to take advantage of the Ub system by targeting cellular substrates which could interfere with propagation of the virus (Scheffner et al., 1990; Schubert et al., 1998). In other cases, viral proteins themselves were the targets of the Ub-dependent proteasome pathway (Ciechanover et al., 1991; de Groot et al., 1991; Oberst et al., 1993), but whether these degradation events are obligatory steps in the virus life-cycle is not presently known. The mechanism of using disposable proteins may be essential to ensure irreversibility of temporally controlled processes. The importance of such a timing control in the efficiency of virus movement has been reported in the case of TMV (Lehto et al., 1990b). It will be interesting in the future to investigate whether the lability and the temporally controlled expression of the 69K protein is a crucial event in the infectious cycle of TYMV as well. Alternatively, removal of MP from infected cells may primarily be a host defence mechanism. A number of virus MPs functionally modify plasmodesmal permeability and promote the intercellular movement of viral nucleic acids (reviewed in Lazaro & Beachy, 1999). Such functions may be deleterious for long-term survival of the infected cells and removal of the MP by the proteasome pathway could prevent continuous interference with the intercellular flow of substances. Use of the Ub-mediated proteasome proteolytic system may also correspond to a cellular attempt to interfere with virus multiplication. Interestingly, perturbation of the Ub system was found to alter the plant response to pathogen infection (Becker et al., 1993; reviewed in von Kampen et al., 1996). Additional data are required to permit a better understanding of the role of Ub and proteasome in the development or control of plant virus diseases.

The selective and rapid degradation of TYMV 69K protein is of interest from another standpoint, because it can serve as a model for the study of cellular processes which function to degrade proteins. An increasing number of proteins are known to be rapidly degraded and complete understanding of the mechanism involved will require identification and characterization of the components of the proteolytic system responsible. Many components of the ubiquitination and 26S proteasome degradation pathways have already been identified in plants (reviewed in Vierstra, 1996; Callis & Vierstra, 2000) and an examination of the recently completed Arabidopsis genome sequence revealed that an extremely large number of genes are related to ubiquitination and proteolysis (Bachmair et al., 2001). However, the list of identified proteins which serve as substrates for this system in plants is rather short and little is known about the exact functions of the different plant Ub-conjugating enzymes and Ub–protein ligases (E3) (reviewed in Vierstra, 1996; Estelle, 2001; Bachmair et al., 2001). The identification of other potential substrates for these E3 enzymes may contribute to our understanding of their biological functions.

Of equal importance is the identification of signals in proteins that target them for ubiquitination and degradation. It is now clear that the essential determinants are often contained within small, sometimes conserved domains (Varshavsky, 1997). In eukaryotes, several motifs have been correlated with instability, including the cell cycle-related destruction box
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(Grünz et al., 1991), sequences enriched in the amino acids proline, glutamic acid, serine and threonine (PEST sequences) (Rechsteiner & Rogers, 1996) or the N-end rule, a relationship observed between the identity of the N-terminal residue of a protein and its metabolic stability (Varshavsky, 1996). Using deletion studies (Fig. 6), we have shown that the 69K protein is likely to contain two degradation signals. The N-terminal degron does not appear to conform to the N-end rule, because the N-terminal residues of both the wild-type protein and the N-terminally deleted versions 69KΔ1 and 69KΔ7 belong to the highly stabilizing category (Met-Ser, Met-Pro and Met-Val respectively). The 69K protein also lacks PEST sequences, or any other obvious primary structure motif which might represent a recognition signal. Despite its high proline content, proline-rich (PY) motifs recently identified as interaction domains with particular Ub ligases (Ikeda et al., 2000; Harty et al., 2000) were not identified in the 69K protein sequence. Some degradation signals can also be active conditionally, for instance through phosphorylation (reviewed in Kornitzer & Ciechanover, 2000). Interestingly, the TMV MP was shown to be phosphorylated in planta (Watanabe et al., 1992) and phosphorylation was proposed to regulate its intracellular localization and stability (Kawakami et al., 1999). The TYMV 69K protein has been shown to be phosphorylated when expressed in insect cells (Séron et al., 1996), but its phosphorylation status in plants has not been specifically addressed. Further studies are required to identify more precisely the 69K protein features that correlate with its instability and to determine the role, if any, that phosphorylation of the MP plays in the degradation process. The results of the study described here will provide a foundation for the exploration of these questions.

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