Misfolded plant virus proteins: elicitors and targets of ubiquitylation

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Abstract Mutant tobacco mosaic virus (TMV) coat proteins (CPs) with known amino acid replacements provide well defined examples of destabilized tertiary structures. Here we show that misfolded TMV CPs, but not functional wild-type CPs, induce massive ubiquitylation in tobacco cells and that denatured, insoluble CP subunits are the main substrates of ubiquitin conjugation. As TMV CPs can be easily manipulated they are unique tools to study the molecular basis of the plant cell's response to aberrant protein structures and the associated intracellular stress reactions.

Key words: Tobacco mosaic virus; Ubiquitin; Coat protein; Temperature-sensitive mutation; Denaturation; Insoluble protein

1. Introduction

Conjugation with the highly conserved polypeptide ubiquitin is a general principle in eukaryote cells to label proteins for intracellular degradation [1]. In higher plants, differentiation and hormonal responses have been associated with ubiquitylation [2–4], and a few functional plant virus proteins have been observed to become ubiquitylated [5–7]. Whereas the relevant enzymatic machinery involved in ubiquitylation and some of the sequence motifs that target short-lived proteins for ubiquitin-dependent degradation have been characterized, less is known on the molecular features by which misfolded or denatured proteins are recognized. Here we propose to exploit genetic variants of a viral coat protein (CP) to study this aspect of ubiquitylation. Plant viruses have evolved to ensure a maximum production of virus progeny over the temperature range to which their host plant may be exposed, and to minimize premature host cell damage and defense reactions. These adaptations must specifically apply to the virus CP, which often is produced in enormous concentrations. Susceptible tobacco leaves infected with wild-type tobacco mosaic virus (TMV) produce, over a temperature range from 18 to over 30°C, paracrystalline masses of TMV particles so that TMV CP may constitute $>20\%$ of the total protein. The leaf cells survive for weeks and, in contrast to those of hypersensitive cultivars, do not mount an evident defense reaction. The replacement of one or two of the 158 amino acid residues may render the CP temperature-sensitive (ts), so that at moderately elevated ('non-permissive') temperatures (>27°C) most of the CP denatures and low yields of virus particles result (Fig. 1) [8–10]. As TMV CP has no function in the host cell and native CP is dispensable for the progress of infection within leaf tissue, mutant TMV CPs appear as ideal probes to study the plant cell's handling of misfolded proteins.

Previously, we have discovered that misfolded ts TMV CPs induce heat shock proteins and thus act as 'mis-calibrated' molecular thermometers [11]. Here we show that misfolded TMV CPs, but not functional TMV CPs, induce massive ubiquitylation in tobacco cells and that denatured, insoluble CP subunits are the main substrates of ubiquitin conjugation.

2. Materials and methods

2.1. Host plants, virus strains, and conditions of virus multiplication

Standard experiments were performed with the susceptible tobacco cultivar *Nicotiana tabacum Samsun n/n*. The hypersensitive host *N. tabacum Xanthi N/N* was used for virus titration and control purposes. The wild strain *vulgare* of TMV and its mutants were from the collection of the former Max-Planck-Institute for Biology in Tübingen (cf. [12–15]). Fully expanded leaves of 5–6 week-old *Samsun* plants were dusted with glass beads of 40–70 μm diameter and gently rubbed with purified virus solutions (0.5–1.0 mg/ml) using glass spatula. This inoculation condition gave a maximum density of chloroses, or necroses on the hypersensitive host *N. tabacum Xanthi*. The inoculated plants were kept under continuous illumination, for 24 h at 23°C, then for several days at the experimental temperature, 23 and 30°C (or 35°C). Leaf tissue without midribs was harvested, immediately frozen in liquid nitrogen and stored at −70°C.

2.2. Sample preparation and analysis

For the extraction of leaf tissue and separation in soluble and insoluble fractions as well as the conditions for 1D sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, see [11].

2D gels were run according to [16], with a pH gradient from 3.5 to 10. To calibrate 2D gels, rabbit actin was added to plant extracts and localized by Ponceau staining prior to immunostaining of blots. Ubiquitin was detected on immunoblots by a polyclonal rabbit antibody directed against bovine ubiquitin and its protein conjugates (dilution 1:100; Sigma, U5359); identical results were obtained with a monoclonal antibody directed against bovine ubiquitin conjugates (Biotrend, Cologne, Germany, UG 9510). TMV CP was detected with several monoclonal and a polyclonal anti-TMV CP antibodies [11]. Scanning and evaluation of Coomassie blue-stained bands and enhanced chemiluminescence (ECL)-exposed films was done with an Epson 1640 SU scanner using the Phoretix software from non-linear dynamics (Newcastle, UK).

3. Results

3.1. Distribution of TMV CPs and ubiquitin antigen

We have used a collection of natural and chemically induced TMV CP mutants with known amino acid replacements (Fig. 1) [13–15] to test the relevance of destabilized protein tertiary structure for a ubiquitylation response in plant cells.

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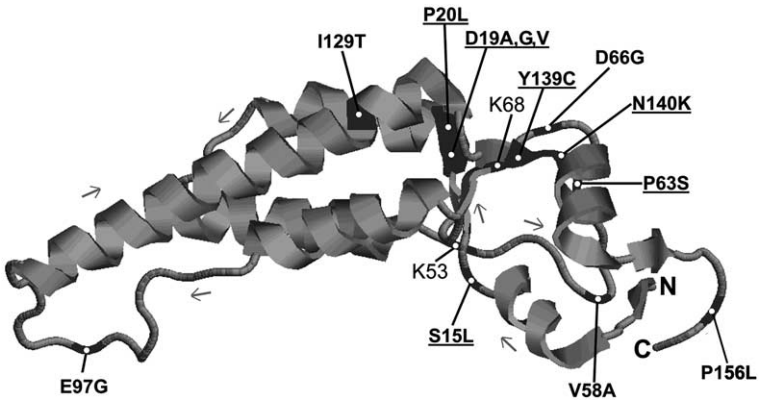

Fig. 1. Schematic representation of the TMV CP mutations used in this work. Shown is the 3D structure of the CP, subunit A, based on four-ring structure (pdbId = 1EI7 [17]), extending from about 20 Å from the central axis (left) to about 95 Å (right). Arrows indicate N to C direction of polypeptide. The subunit is tilted out of the ring's plane so that positions of amino acid replacements (cf. [12–14]) are not obscured. The two lysines (K) indicate possible ubiquitylation sites. Underlined amino acid replacements cause temperature sensitivity of the subunit, non-underlined ones do not ([8–10,12]; cf. [18]).

Leaves of a susceptible tobacco cultivar were inoculated with purified TMV at saturating concentrations and infection was allowed to proceed for 5–7 days, at either the permissive temperature, 23°C, or at a non-permissive temperature, usually 30°C. Soluble and insoluble fractions of homogenates were subjected to SDS gel electrophoresis. The distribution of native (soluble) and denatured (insoluble) TMV CP was scored by Coomassie blue staining and that of ubiquitylated polypeptides by immunoblotting. Ubiquitin signals were only detected in the low-speed pellets of ts mutants, whereas all supernatants and pellets of wild-type *vulgare* TMV and all temperature-resistant (tr) mutants were negative for ubiquitin immunoreactivity, both at 23 and at 30°C (Fig. 2). Of the ts mutants, three showed strong and two a weak ubiquitylation already at 23°C, and all ts mutants showed strong signals at 30°C. Whenever there were considerable amounts of insoluble CP present in the pellets strong ubiquitin signals were found (Fig. 2). For a number of strains these results have been reproduced over a period of 2 years. By mixing wild-type *vulgare* and ts mutant *flavum* in varying proportions it was found that the elicitation of ubiquitylation is dominant, indicating that is a gain of function rather than the loss of a wild-type suppressor function (data not shown).

By semiquantitative evaluation of the signals for ubiquitin and for TMV CP a positive correlation between the amount of insoluble TMV CP and the strength of the ubiquitin signal was obtained (Fig. 3). The defective mutant Ni 2204, which produces mostly non-functional CP, both at low and at high temperature (Fig. 4A and data not shown), gave a strong ubiquitin signal at both temperatures. The ts mutants Ni

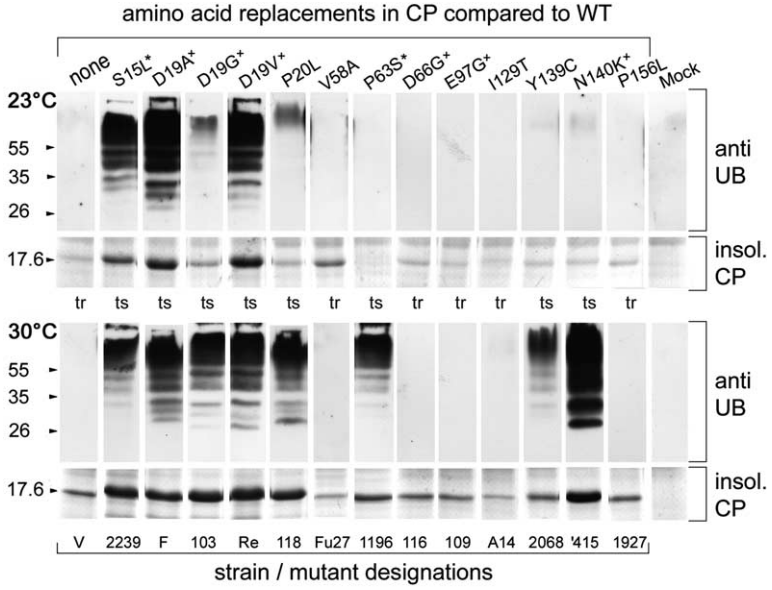

Fig. 2. Ubiquitylation induced by TMV strains and mutants at permissive (23°C) and non-permissive (30°C) temperatures. TMV wild-type and CP mutants were used to infect susceptible tobacco leaves. Soluble and insoluble fractions were separated by 1D SDS gel electrophoresis. Shown are ECL-developed immunoblots from insoluble fractions, using anti-ubiquitin and Coomassie staining of TMV CP. +, Amino acid replacements leading to a net gain of a positive charge; *, mutant carries the additional replacement I129T (as in A14), which by itself does not lead to temperature sensitivity. tr, Temperature-resistant; ts, temperature-sensitive. Strain numbers without letters designate nitrous acid (Ni)-induced mutants, ′415 is CP 415.

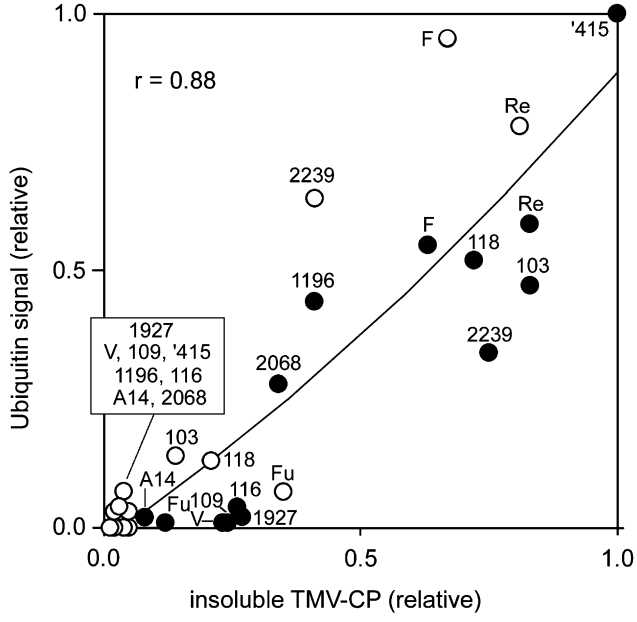

Fig. 3. Correlation between amount of misfolded (insoluble) CP and ubiquitylation. Data derived from experiments as shown in Fig. 2. Signal amplitudes for ubiquitin reactivity and for Coomassie staining of TMV CP bands in insoluble fractions were quantified by densitometry and the values relative to the maximal amplitudes plotted against each other. A quadratic fit with its correlation coefficient *r* is given. TMV strain designations as in Fig. 2. The fact that ubiquitin signals for Ni 2239, *flavum* (F), and *reflavescens* (Re) are lower at 30°C than at 23°C is probably due to the aggressiveness of these 'yellow strains' to the leaf tissue (cf. [19,20]), which is aggravated at elevated temperatures. Ni 116 had been scored ts in [8] but has a stable CP [10].

2239, *flavum*, *reflavescens* also induced high levels of ubiquitylation at low temperature, which correlated with a high amount of insoluble CP. The marginally ts mutant Ni 2068 [10] induces a low level of ubiquitylation at 30°C (Fig. 2).

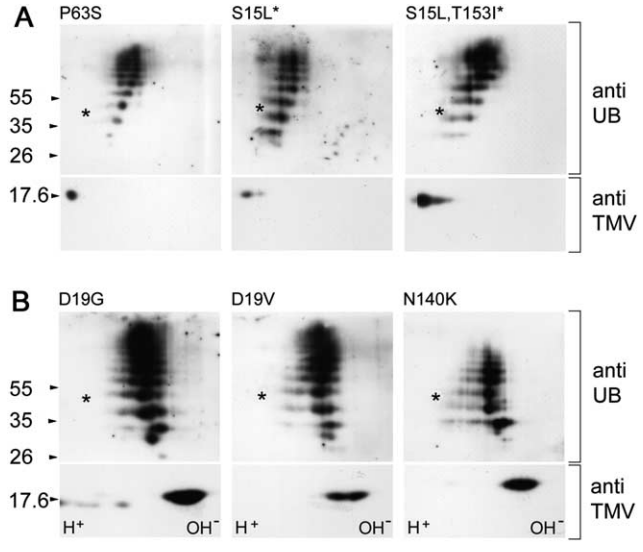

Fig. 4. 2D separation of ubiquitylation products using ts CP mutants (A) without (Ni 1196; Ni 2239 (see Fig. 2); Ni 2204, replacements S15L, T153I) and (B) with a net charge difference to wild-type CP (Ni 103, *reflavescens*, CP 415). The positions of ubiquitylation products of <60 kDa are dependent on the net charge of TMV CPs. Asterisk, position of added actin as a reference.

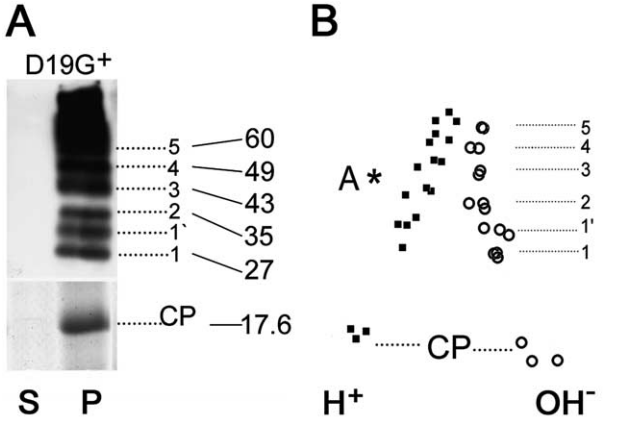

Fig. 5. Synopsis of positions of ubiquitylation products and unmodified CPs in 1D and 2D gels. A: Extracts of TMV *flavum*-infected leaves incubated at 30°C show a molecular mass ladder of ubiquitin conjugates in the insoluble pellet (P) but no conjugates in the soluble supernatant (S). Six bands are numbered 1–5 and 1′, and their estimated molecular masses from 27 to 60 kDa are given. B: Synopsis of positions of ubiquitylation products and TMV CPs taken from Fig. 4, using the actin spot as a common reference. Bands designated with numbers 1–5 are interpreted as ubiquitylated TMV CPs with one to five ubiquityl residues attached. 1′ is probably an isoform of either the mono- or the di-ubiquitylated TMV CP.

Thus, there was a strong overall correlation between the presence and amount of insoluble TMV CP and ubiquitylation, suggesting that the thermally denatured TMV CP contains the signal for, and is the substrate of, ubiquitylation.

Using several antibodies directed against TMV or TMV CP we were unable to unambiguously demonstrate the presence of TMV CP in the ubiquitin-positive bands. In order to test whether these represent ubiquitylation products of TMV CP, we made use of the charge differences caused by some amino acid replacements. By immunoblotting two-dimensional gels with anti-ubiquitin, it was found that with charge mutants of TMV CP the ubiquitin-positive bands were shifted, as were the mutant CPs, to more positive positions in the pH gradient relative to the pattern of charge-neutral mutants (Fig. 4). We conclude that ubiquitin-positive bands between molecular masses 28 and 60 kDa represent ubiquitylated TMV CPs, that TMV CP is the main substrate for ubiquitylation, and that the 'ladder' of bands represents a stepwise addition of ubiquitin moieties (Fig. 5). The width of the steps from unmodified CP and the first ubiquitin-positive band and between those bands (numbered from 1 to 5 in Fig. 5) corresponds, on average, to a molecular mass of 8.4 kDa or about 76 amino acid residues (Fig. 5A), suggesting that single ubiquitin moieties are added at a time to the substrate. Band 1′, which is localized between 1 and 2, must also represent a ubiquitin conjugate of TMV CP as it is subject to charge shift with mutant CPs. It may be an isomer of either mono- or di-ubiquitylated CPs due to different conjugation states at the two available lysine residues, K53 and K68. Ubiquitylation may mask important TMV CP epitopes, thus preventing their detection by our anti-TMV-CP antibodies. Despite the massive ubiquitin signal, only a small fraction of the insoluble TMV CP seems to be conjugated since the amount of CP found at the position of unconjugated CP is not dramatically reduced in most ts mutants as compared to wild-type *vulgare*.

4. Discussion

Temperature sensitivity is a property of the nascent or free TMV CP subunit. Once incorporated into virus particles and stabilized by their interaction with RNA, even the most thermolabile CPs attain a remarkable stability: They resist temperatures of up to 50°C [8–10]. It is known that about one CP subunit in TMV particles (i.e. one in approx. 3000), though native and functional, carries a single ubiquitin residue [5]. Yet, the underlying recognition and following modification reaction must differ from the massive and multiple ubiquitylation of denatured TMV CP, as shown here. The observation that a large proportion of the denatured CP is spared from ubiquitylation may be due to either the exhaustion of the ubiquitylation capacity of the host cell or, more likely, to the inaccessibility of CP molecules in insoluble aggregates.

At temperatures up to 30°C, wild-type TMV neither induces ubiquitylation in susceptible nor in the hypersensitive tobacco cultivars (data not shown). Thus, ubiquitylation induced by ts CPs is unrelated to host defense and probably reflects the activation of the machinery normally targeted to misfolded cellular proteins.

Due to the ease with which the TMV genome can be manipulated, the TMV mutant–host plant system is a unique model to study the recognition signals required for ubiquitylation and the plant cell's stress response. This model may also be relevant for protein misfolding diseases affecting animal cells including neurons.

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References

[1] Hershko, A. and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479.
[2] Bachmair, A., Novatchkova, M., Potuschak, T. and Eisenhaber, F. (2001) Trends Plant Sci. 6, 463–470.
[3] Hellmann, H. and Estelle, M. (2002) Science 297, 793–797.
[4] Frugis, G. and Chua, N.H. (2002) Trends Cell Biol. 12, 308–311.
[5] Dunigan, D.D., Dietzgen, R.G., Schoelz, J.E. and Zaitlin, M. (1988) Virology 165, 310–312.
[6] Reichel, C. and Beachy, R.N. (2000) J. Virol. 74, 3330–3337.
[7] Hazelwood, D. and Zaitlin, M. (1990) Virology 177, 352–356.
[8] Jockusch, H. (1966) Z. Vererbungsl. 98, 320–343.
[9] Jockusch, H. (1966) Biochem. Biophys. Res. Commun. 24, 577–583.
[10] Jockusch, H. (1968) Naturwissenschaften 55, 514–518.
[11] Jockusch, H., Wiegand, C., Mersch, B. and Rajes, D. (2001) Mol. Plant Microbe Interact. 14, 914–917.
[12] Jockusch, H. (1964) Z. Vererbungsl. 95, 379–382.
[13] Wittmann, H.G. (1962) Z. Vererbungsl. 93, 491–530.
[14] Wittmann, H.G. (1964) Z. Vererbungsl. 95, 333–344.
[15] Wittmann, H.G. and Wittmann-Liebold, B. (1966) Cold Spring Harb. Symp. Quant. Biol. 31, 163–172.
[16] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[17] Bhyravbhatla, B., Watowich, S.J. and Caspar, D.L. (1998) Biophys. J. 74, 604–615.
[18] Dobrov, E.N., Abu-Eid, M.M., Solovyev, A.G., Kust, S.V. and Novikov, V.K. (1997) J. Protein Chem. 16, 27–36.
[19] Jockusch, H. and Jockusch, B. (1968) Mol. Gen. Genet. 102, 204–209.
[20] Banerjee, N., Wang, J.Y. and Zaitlin, M. (1995) Virology 207, 234–239.