Short communication

Features on the surface of the tobacco rattle tobravirus particle that are antigenic and sensitive to proteolytic digestion

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The particle proteins of tobraviruses and tobamoviruses share six sequence motifs, two of which are also present in furoviruses and hordeiviruses. Analyses of four different polyclonal antisera to tobacco rattle tobravirus by Pepscan revealed that the C-terminal region of the particle protein was immunodominant. The N-terminal region and a central region (residues 110–121) were more weakly immunogenic. These results suggest that these regions are exposed externally on the assembled virus particle. Papain digestion showed that the C terminus can be removed without apparent structural damage to the particle. The external location of the C-terminal region along the sides of the particle could explain some transmission properties of the rod-shaped viruses.

Tobacco rattle tobravirus (TRV) is the type member of its group (Robinson, 1995). Although it has rod-shaped particles, their detailed structure has been less well studied than that of tobacco mosaic tobamovirus (TMV) particles. Difficulties with sample preparation have hampered high resolution X-ray diffraction studies and direct information on particle structure has come mainly from electron microscopy (Roberts & Mayo, 1980). Recently, different indirect approaches have been taken: protein sequence comparisons (Goulden et al., 1992), monoclonal antibody (MAb) studies (Legorburu et al., 1995) and nuclear magnetic resonance spectroscopy (NMR; Mayo et al., 1993).

To compare the particle protein sequences of tobraviruses and tobamoviruses, the alignment of tobravirus sequences given by Goulden et al. (1992) was used. For tobamovirus sequences, the alignment by Gibbs (1986) was modified by neglecting the sequences for tomato mosaic and tobacco mild green mottle viruses, which are too similar to that of tobacco mosaic virus, and by correcting the odontoglossum ringspot virus sequence to that given by Isomura et al. (1991). Each alignment was scored for conservation at each amino acid position. Pairs of amino acids for which the value in the mutation matrix of Gonnet et al. (1992) was greater than 2·2 (the value for self-substitution of serine) were counted as conserved. Consensus sequences for each genus were derived, including positions at which all or all but one of the aligned sequences contained a conserved residue or at which two sequences had one conserved residue and two (for tobraviruses) or three (for tobamoviruses) had another. Inspection of the distribution of conservation scores along the length of the alignments showed that each alignment consisted of conserved, variable and intermediate regions (Fig. 1). Using these as a guide, it was easy to align the two consensus sequences manually (Legorburu, 1993) and to recognize six shared sequence motifs. These motifs, which are located in the same order along the two consensus sequences, represent sequence elements that are conserved in the particle proteins of tobraviruses and tobamoviruses. They are denoted as motifs I to VI in Fig. 1. Many of the individual conserved residues in these motifs have been pointed out by others (Dolja et al., 1991; Goulden et al., 1992), but our approach of comparing consensus sequences leads to recognition of conserved stretches of three to seven residues rather than single residues.

The comparison of consensus sequences also provided a way of comparing the variable regions in the protein sequences. Proteins of both genera have at their C termini a region of variable sequence, which is 21 residues longer in tobraviruses than in tobamoviruses. Another region of variable sequence, at the N terminus of tobravirus proteins, has no counterpart in tobamoviruses. The sequence between motifs II and III is more variable in tobamoviruses than in tobraviruses, but the distance between these motifs is similar in the two

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genera. Tobraviruses, however, have a long region of variable sequence between motifs IV and V, which represents a 27 residue insertion relative to tobamoviruses. In the model for tobavirus particle structure proposed by Goulden *et al.* (1992), the variable regions of the particle protein would be located either on the outer surface (C and N termini) or in the inner channel (central variable region) of the tubular particle. We report results obtained with two techniques, Pepscan analysis (Geysen *et al.*, 1984) of polyclonal antisera and proteolytic digestion of virus particles (Mayo & Cooper, 1973), that confirm these predictions.

Groups of contiguous amino acid residues in the primary sequence of proteins, which represent sites that can react with antibodies to the whole protein, are known as continuous epitopes; their positions have been correlated with certain structural features of the proteins, including hydrophilicity, segmental mobility, surface accessibility, chain termini and turns in the secondary structure (van Regenmortel *et al.*, 1988; Pellequer *et al.*, 1993). In particular, it has been shown for several globular proteins that most continuous epitopes protrude from the surface (Thornton *et al.*, 1986). Thus, it is reasonable to expect that most of the continuous epitopes on virus particles, identified in Pepscan analysis by allowing antisera to intact virus particles to react with a series of synthetic overlapping peptides that represent the whole amino acid sequence of the coat protein, are likely to be on the surface.

A rabbit was injected three times intramuscularly with 8.7 mg purified particles of TRV strain PLB (Angenent *et al.*, 1989) on weeks 1, 15 and 79 and bleeds were taken on weeks 30 (antiserum PLB1), 51 (antiserum PLB2) and 103 (antiserum PLB3). Pepscan analysis of the three antisera, done as described by Legorburu *et al.* (1995) with a set of peptides representing the coat protein of strain PLB (Angenent *et al.*, 1989), showed that they all reacted with the same three groups of contiguous peptides (110–114, 190–193 and 199–202) and the single peptide 196 (Fig. 2). These all correspond to variable regions of the protein, as defined above, and seem to be the dominant continuous epitopes on the virus particle. Other groups of peptides, located in variable (1–5, 12–15, 107–109, 185–188), intermediate (70–74) or conserved regions (96–97, 155–157), reacted increasingly with later bleeds. One pair of contiguous peptides (194–195; variable region) decreased in reactivity. Overall, there was a slight shift towards more conserved regions being involved in the immune response at later times.

Two rabbit antisera against TRV strain PRN (a member of the same serotype as TRV PLB) were kindly supplied by W.P. Mowat (SCRI): PRNwp, raised against whole purified virus particles, and PRNdiss, raised against particle protein recovered after SDS-PAGE. No major differences were apparent between the Pepscan reaction patterns of these two antisera. Both reacted with groups of peptides located near both termini and in the central variable region, as well as to other peptides scattered throughout the sequence (data not shown). Interestingly, neither of these antisera was more cross-reactive than the other in ELISA against a selection of tobavirus serotypes (Legorburu, 1993), suggesting that immunization with dissociated antigen is not guaranteed to provide cross-reacting antisera.

Two mice were injected twice intraperitoneally with 100 μg purified virus particles on weeks 1 and 3 and bled...
on week 25 (antisera PLBa and PLBb). Both antisera reacted with C-terminal peptides (198–201) and one of them also reacted with N-terminal peptides (2–6) (data not shown).

Two rabbit antisera to other tobravirus isolates, TRV N5 (a natural recombinant isolate with a particle protein typical of pea early-browning virus, Dutch serotype; Robinson et al., 1987) and pepper ringspot virus strain CAM, were also tested. Neither of them gave positive reactions with any of the peptides in the analysis.

Summarizing the results from four animals (two rabbits and two mice) immunized with whole particles of TRV PLB or TRV PRN, antisera from all four reacted with peptides representing the C-terminal region of the particle protein. Antisera PRNwp and PLBb reacted with the N-terminal region and antisera PLB1-3 and PRNwp with the central variable region (residues 110–121). Thus, in TRV particles, the three variable regions, two of which are the termini of the peptide chain, proved to be immunogenic. The C terminus, which is the immunodominant region, was shown by NMR experiments to be mobile (Mayo et al., 1993) and has been predicted to contain several turns (L. Sawyer, P. Tollin & D.J. Robinson, unpublished results). The correlation of these features with antigenicity has been noted previously (van Regenmortel et al., 1988; Pellequer...
The C terminus of the particle protein of several other viruses, in particular that of TMV (van Regenmortel, 1986), is also strongly immunogenic.

Further evidence that the C-terminal region of the TRV particle protein is exposed on the surface of the virus particle was obtained from experiments in which particles were treated with protease. Purified particles of TRV PLB (1 mg/ml in 66 mM-phosphate buffer pH 7.3 containing 0.1% 2-mercaptoethanol) were treated with 20 µg/ml papain at 37°C for 2–8 h. The products were separated by electrophoresis in 12% SDS-polyacrylamide gels, transferred to nitrocellulose and immunostained using MAbs (Legorburu et al., 1995). Under these conditions, digestion was complete in 2 h and no further changes were detected by SDS-PAGE when the incubation was extended up to 8 h. The protein from digested particles migrated as a double band of apparent molecular mass 25–26 kDa, whereas that from untreated particles had an apparent molecular mass of 30 kDa (Fig. 3); however, it should be noted that tobravirus proteins migrate anomalously in SDS-PAGE (Mayo & Robinson, 1975). Digested particles had a normal appearance in the electron microscope and retained infectivity in tobacco (data not shown). Fig. 3 shows that the protein from digested particles did not react in Western blots with MAbs SCR80 and SCR81, which are specific for the C-terminal region of the particle protein, but did react with MAb SCR78, which is specific for conserved motif II (Legorburu et al., 1995), and with mouse polyclonal antiserum. This experiment confirms that the C-terminal part of the TRV particle protein is exposed on the particle surface and shows that it is dispensable for structural stability and infectivity by mechanical inoculation.

Our results are consistent with the predictions that the C- and N-terminal regions of the protein are exposed on the sides of the assembled particle and that the central variable region is exposed in the inner channel and accessible to antibodies at one end. Tobravirus isolates from different serotypes have been shown to be specifically transmitted by different trichodorid nematode species (Ploeg et al., 1992a, b). The three regions of the particle protein which are exposed on the particle surface and variable in sequence are obvious candidates for the determinants of this specificity. Moreover, the protease sensitivity of the C terminus could provide the molecular basis of a mechanism for detachment of the virus from the vector by digestive proteases during transmission.

The sequence elements designated as motifs III and VI in tobraviruses and tobamoviruses are also recognizable in furoviruses and hordeiviruses (Dolja et al., 1991). However, each genus has a different feature at the C terminus of its particle protein: a long mobile peptide in nematode-transmitted tobraviruses, a carbohydrate moiety in seed-transmitted barley stripe mosaic hordeivirus, read-through into a long open reading frame in fungus-transmitted furoviruses and nothing in mechanically-transmitted tobamoviruses. It is tempting to conclude that this region of the protein may be involved in transmission in three of these genera.

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