Detection of beet yellows closterovirus methyltransferase-like and helicase-like proteins in vivo using monoclonal antibodies

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In the positive-stranded RNA genome of beet yellows closterovirus (BYV), the 5′-terminal ORF 1a encodes a 295 kDa polyprotein with the domains of papain-like cysteine proteinase, methyltransferase (MT) and helicase (HEL), whereas ORF 1b encodes an RNA-dependent RNA polymerase. Eleven and five hybridoma cell lines secreting monoclonal antibodies (MAbs) were derived from mice injected with the bacterially expressed fragments of the BYV 1a product encompassing the MT and HEL domains, respectively. On immunoblots of protein from BYV-infected Tetragonia expansa plants, four MAbs against the MT recognized a ~63 kDa protein, and two MAbs against the HEL recognized a ~100 kDa protein. Both the methyltransferase-like protein and the helicase-like protein were found mainly in the fractions of large organelles (P1) and membranes (P30) of the infected plants. These data clearly indicate that (i) the BYV methyltransferase-like and helicase-like proteins, like other related viral enzymes, are associated with membrane compartments in cells, and (ii) the 1a protein, apart from the cleavage by the leader papain-like proteinase that is expected to produce the 66 kDa and 229 kDa fragments, undergoes additional processing by a virus-encoded or cellular proteinase.

Introduction

RNA replicases, being universal and well-conserved enzymes of all positive-stranded RNA viruses, are expressed by a variety of strategies. In some virus groups replicases are single products, whereas in others they represent sets of distinct components whose expression is controlled by proteolytic processing, translational readthrough or frameshifting, and genome partitioning. In the superfamilly of alphalike viruses (Goldbach et al., 1991), the replicase is encoded in the 5′-proximal genomic ORF(s) and encompasses an invariant array of the conserved domains of methyltransferase (MT) (Rozanov et al., 1992), NTPase/helicase (HEL) (Gorbalya et al., 1988) and RNA polymerase (POL) (Kamer & Argos, 1984; Koonin, 1991). In alphaviruses, the papain-like cysteine proteinase (PCP) domain embedded in the replicative core is responsible for multiple in cis and in trans cleavages producing the mature proteins with MT, HEL and POL domains (reviewed in Strauss & Strauss, 1994). In the polyprotein of beet necrotic yellow vein benyvirus, the PCP is located between the HEL and POL domains (Hehn et al., 1997), whereas in those of tymoviruses and carlaviruses it is found between the MT and HEL domains (Bransom & Dreher, 1994; Lawrence et al., 1995). Regardless of its position, the PCP of these alpha-like viruses mediates the splitting of the MT–HEL-containing and POL-containing fragments.

In the genomes of closteroviruses, a group of insect-transmitted filamentous viruses of plants (Agranovsky, 1996; Agranovsky et al., 1994a), the arrangement of replicative and protease domains is distinct. The MT and HEL domains and the POL domain are encoded in overlapping 5′-terminal ORFs 1a and 1b, respectively, and the polymerase expression is likely to require +1 ribosomal frameshifting (reviewed in Agranovsky, 1996). The PCP is located at the N terminus of the beet yellows closterovirus (BYV) ORF 1a product, and its
cleavage, leading to the release of a 66 kDa leader protein from the rest of the polyprotein, has been confirmed experimentally by T7 transcription/translation and point mutagenesis in vitro (Agranovsky et al., 1994a). A similar location of the PCP has been reported in the genomes of other closteroviruses, including citrus tristeza virus (CTV; Karasev et al., 1995), lettuce infectious yellows virus (LIYV; Klaassen et al., 1995), little cherry virus (Jelkmann et al., 1997) and grapevine leafroll-associated virus-2 (Zhu et al., 1998). Recently, the full-length cDNA clones of LIYV (Klaassen et al., 1996) and BYV (Peremyslov et al., 1998) were produced; it has been found that ORFs 1a and 1b are necessary and sufficient for closterovirus RNA replication, whereas the ORFs located in the 3'-terminal part of the monopartite BYV genome and in RNA-2 of the bipartite LIYV genome are dispensable for replication. Interestingly, the leader PCP of BYV, apart from its proteolytic activity, had a function in enhancing viral RNA accumulation (Peremyslov et al., 1998).

Isolation of virus replication-associated enzymes from infected eukaryotic cells (and their detection therein) is not an easy task, as the proteins normally accumulate to only low levels. The use of antibodies specific to recombinant or synthetic peptides has allowed the in vivo identification and functional dissection of several animal and plant viral replicases (Baron & Baltimore, 1982; Hayes et al., 1994; Hills et al., 1987; Manabe et al., 1994; Scholthof et al., 1995). In this study, monoclonal antibodies (MAbs) to the bacterially expressed N- and C-terminal fragments of the BYV 1a were produced and used for detection of the methyltransferase-like and helicase-like proteins in vivo.

Methods

Construction of expression cDNA clones and purification of BYV 1a protein fragments with MT and HEL domains. The cDNAs encoding amino acids 665–813 and 2450–2630 of the BYV ORF 1a product were excised as BamHI–SalI and BglII–PstI fragments from plasmids 156 and 115, respectively (Agranovsky et al., 1994a). The inserts were cloned into pQE-31 and pQE-10 vectors (Diagen) under the control of a bacteriophage T5 promoter and a ribosome-binding site (Fig. 1A). The resulting constructs, pQE-N6H-mt and pQE-N6H-hel, were used to transform E. coli strain M15 (Diagen) containing the repressor pREP4 plasmid. The procedure for bacterial overexpression and nickel-chelate chromatography purification of recombinant proteins with a tag of six histidines has been described (Agranovsky et al., 1994b; Hochuli et al., 1988). The pure soluble recombinant protein N6H-hel was obtained after gradient dialysis of the 8 M urea-containing eluate from a Ni-NTA agarose column against a buffer containing 0.2 M NaCl, 1 mM EDTA and 50 mM HEPES/KOH pH 7.0, at 4 °C (Agranovsky et al., 1994b). The soluble N6H-mt protein was obtained after direct dialysis against distilled water at room temperature.

Monoclonal antibodies. For mouse immunization, 50–100 μg of soluble N6H-mt or N6H-hel mixed with an equal volume of Freund’s complete adjuvant was injected into the peritoneal cavity of 6–8-week-old BALB/c mice, followed by injections with incomplete Freund’s adjuvant repeated twice at 2-week intervals, and the final intraperitoneal injection of 50 μg of the antigen without adjuvant. Immunized spleen cells were isolated 3 days later for cell fusion (Plechko et al., 1991). Splenocytes from immunized mice were fused with mouse myeloma cell lines P3X63.Ag.6.653 or Sp2/0 using 45 % polyethylene glycol 1500 (Torrance, 1992). Cells were cultured under selective conditions on Dulbecco’s HAT MEM supplemented with 10 % foetal calf serum (Gibco BRL) in the presence of mouse peritoneal macrophages as feeder cells (Torrance, 1992). Ten to twelve days after fusion the culture fluids were screened for the presence of specific antibodies by indirect ELISA. Specific antibody-secreting hybridomas were cloned twice under conditions of limiting dilutions. The MAbs were purified from the ascitic fluid by affinity chromatography on Avidin AL Gel (BioPresto International) as recommended by the manufacturer. MAb isotypes were determined with a MAb Mouse Isotyping kit (Gibco BRL). MAb 1470, specific to the BYV p22 coat protein (CP), was kindly made available by F. Rabenstein (Federal Center of Breeding Research, Aschersleben, Germany).

Serological tests. Total protein was isolated by phenol extraction (Van Etten et al., 1979) from healthy and BYV-infected Tetragnia expansa leaf samples collected at 2, 4, 7, 11, 15, 20, 32 and 49 days post-inoculation (p.i.). Subcellular fractions S30 (cytoplasm), P30 (membranes), P1 (nuclei and large organelles) and CW (cell walls) were obtained from healthy and infected plants (12–20 days p.i.) by filtering the sap through Miracloth followed by differential centrifugation at 1000 g and 30000 g (Niesbach-Klosgen et al., 1990). The protein samples were separated on gradient (6–15 % polyacylamide) SDS gels (Laemmli, 1970) and transferred to nitrocellulose membranes (Hybond-C extra, Amersham). Immediately after the transfer, the blots were UV-irradiated in Stratalinker-1800 (Stratagene); this treatment was found to greatly increase the sensitivity of protein detection (R. Zinovkin, unpublished observation). The blots were incubated with a purified MAb (3 μg/ml) or diluted ascitic fluid (1:1250), followed by goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma). Visualization was by treatment with BCIP/NBT substrate (Promega).

In vitro transcription and translation. The cDNA sequence encoding a portion of the BYV 1a product between amino acids 589 and 1120 (60-2 kDa, containing the MT domain; Fig. 1 A) was amplified by PCR using the cDNA clone 515 142 (nt 1–4007 in the complete BYV genome sequence) as template, with the specific positive-sense primer 5′-dTCCGATTCATGGAAGAAGGATTCCT3′ (introduced Ncol site is underlined) and M13 reverse primer (Promega). The Ncol–SalI fragment of the PCR product was cloned between the respective sites of the T7 expression vector pHIES GS (gift of P. Ivanov; Ivanov et al., 1997) to replace the GUS gene. The cDNA encoding the C-proximal 894 amino acids of BYV 1a (997 kDa, containing the HEL domain; Fig. 1A) was amplified by reverse transcriptase PCR on the BYV virion RNA (Agranovsky et al., 1994b) with the specific primers 5′-dAGGGATCCC-TCTCGGACGCAATC 3′ and 5′-daACACATGCGCCCGGTTTGC 3′ (negative-sense and positive-sense, respectively; introduced BamHI and Ncol sites are underlined). The cDNA was digested with Ncol and BamHI and inserted between the respective sites of pHIES GS. The resulting cDNAs, pSK-BYV.MT and pSK-BYV.HEL, were linearized with Sacl. In vitro transcription with the T7 RNA polymerase and translation in the TNT Coupled Wheat Germ Extract system (Promega) were done according to the manufacturer’s protocol. The [35S]methionine-labelled products were separated on denaturing protein gels and visualized by autoradiography, or transferred to nitrocellulose for immunoblot analysis with the specific MAbs.
Results

Monoclonal antibodies

In this study, we purified from E. coli two fragments of the BYV ORF 1a product, the N-terminal fragment of 148 amino acids (19-5 kDa, encompassing the conserved MT domains I–III) and the C-terminal fragment of 180 amino acids (21 kDa, encompassing the HEL domains V and VI) (Fig. 1B; Agranovsky et al., 1994a; Gorbalenya et al., 1988; Rozanov et al., 1992). The purification procedure yielded 5–7 mg of each soluble antigen (N6H-mt or N6H-hel) from 50 ml of the liquid bacterial growth medium. Upon electrophoresis in SDS–polyacrylamide gels, the purified N6H-mt migrated as a single band with the expected mobility (apparent molecular mass of 20 kDa). The purified N6H-hel had a mobility lower than expected (apparent molecular mass of 28 kDa) (Fig. 2B).

Mouse immunization with N6H-mt and the two subsequent fusion experiments resulted in 384 MAb-producing hybrid clones, of which eleven (3B6, 3C5, 2D5, 4A5, 2C10, 2D10, 4B4, 4C5, 2A4, 4A2 and 3H4, all secreting IgG1-type antibodies) were selected based on their strong positive reaction with the homologous immunogen in indirect ELISA and on immunoblots (Fig. 2A and data not shown). The three fusion experiments with mice injected with N6H-hel yielded 288 clones. Of the five MAbs selected from this library by indirect ELISA, four belonged to the IgM class (1A2, 1D1, 2C6 and 2B5) and one to the IgG1 class (1C4). All these MAbs positively reacted with the homologous immunogen on immunoblots (Fig. 2B and data not shown). MAbs 3C5, 3B6, 2D5 and 4A5 (MT MAb panel) and 1C4, 1A2, 1D1, 2C6 and 2B5 (HEL MAb panel) were used in further immunoblot analysis of proteins from the BYV-infected and healthy plants.

In order to obtain recombinant immunogens representing the central portion of the 1a protein, we constructed pQE-based expression vectors with BYV cDNAs overlapping the respective genomic region (nucleotides 3997–4323, 4323–4837, 3997–4837 and 4837–5220). However, none of these constructs provided detectable expression of the BYV-specific products in M15 and SG13009 bacterial strains. In our experience, some other coding regions of BYV (particularly, those for the PCP and POL domains) are also non-expressible in E. coli, presumably because of interference of the virus-specific RNA and/or protein with bacterial growth.

Detection of the BYV methyltransferase-like and helicase-like proteins in vivo

Upon immunoblot analysis of the subcellular fractions of the BYV-infected and healthy T. expansa plants, MAbs 3B6, 3C5, 2D5 and 4A5 visualized a single band specific to the virus-infected tissue, corresponding to a protein with an apparent molecular mass of 63 kDa (p63), and gave virtually no cross-reaction with healthy plant proteins (Fig. 2A and data not shown). The seven other antibodies in the MT MAb panel did not detect p63 or any other protein specific to the BYV-infected tissue. The bulk of p63 was in fractions P1 (containing nuclei, chloroplasts and associated membranes) and P30 (membranes from the endoplasmic reticulum and dissociated organelles), with only a trace of it found in the CW fraction (Fig. 2A).

In the immunoblot lanes corresponding to the subcellular fractions of the BYV-infected, but not healthy, plants, antibody 1C4 from the HEL MAb panel readily recognized a protein with an apparent molecular mass of 100 kDa (p100) (Fig. 2B). Like p63, p100 was found to be predominantly associated with fractions P1 and P30. MAb 1D1 also detected the p100 band, albeit with lower efficiency compared with 1C4, whereas 1A2, 2C6 and 2B5 showed no detectable reaction with any protein on immunoblots (data not shown). MAb 1C4 did not show a positive reaction to any protein specific to the citrus leaf tissue.
Fig. 2. Western blots of the recombinant MT and HEL fragments and of the proteins from the subcellular fractions of healthy (H) and BYV-infected (I) T. expansa plants. The protein samples were separated in denaturing SDS gels, transferred to nitrocellulose membranes, and incubated with a specific MAb followed by goat anti-mouse IgG–alkaline phosphatase conjugate. (A) Immunoblot developed with MAb 3B6, specific to the MT domain. Lane N6H-mt, 1 pg of the purified recombinant N6H-mt; lanes CW, cell wall debris; lanes P1, 1000 g pellet; lanes P30, 30000 g pellet; and lanes S30, 30000 g supernatant. (B) Immunoblot developed with MAb 1C4, specific to the HEL domain. Lane N6H-hel, 1 pg of the purified recombinant N6H-hel; the designations for other lanes are as in (A). The molecular masses of the 63 kDa and 100 kDa BYV-specific proteins were determined by comparison with the M markers separated on adjacent lanes. The markers, 10 kDa Protein Ladder (Gibco BRL), have M of 10–200 kDa (with a 10 kDa step in the 10–120 kDa range); the sizes in kDa are indicated for each second marker.

infected with CTV, a closterovirus closely related to BYV (M. Bar-Joseph, personal communication), thus suggesting its specificity to a unique epitope(s) in the BYV helicase-like protein.

The total protein from T. expansa leaf samples taken at different times after aphid inoculation was analysed with MAbs 3B6 and 1C4 and MAb 1470 (specific to the major BYV CP). All three BYV proteins – p22 coat protein, p63 and p100 – were detectable from 11 days p.i. (at the onset of leaf symptoms development) to 49 days p.i., although the levels of p63 and p100 declined in older leaves collected after 20 days p.i. (Fig. 3).

**In vitro translation of the T7 transcripts encoding the MT- and HEL-containing domains of BYV**

To assess the electrophoretic properties of the methyltransferase-like and helicase-like proteins, we made in vitro translations of T7 transcripts encoding the 1a protein portions mimicking p63 and p100 in size. The T7 transcript of pSK-BYV.MT encoded a portion of the 1a protein between amino acids 589 (the leader PCP cleavage site) and 1120, with a calculated molecular mass of 60-3 kDa (Fig. 1A). In SDS gels, the respective [35S]S-methionine-labelled product had an apparent molecular mass close to the expected value (Fig. 4). The translation product of pSK-BYV.HEL, corresponding to the C-proximal portion of the 1a protein (calculated molecular mass of 99-7 kDa; Fig. 1A), behaved as a 120 kDa protein (Fig. 4). The fact that the recombinant 21 kDa protein, N6H-hel, had an apparent molecular mass of 28 kDa (Fig. 2B) indicates that this might be the C-terminal part of p100 which was responsible for its anomalously slow migration in gels. Given that the 99-7 kDa fragment had an electrophoretic mobility about 20% lower than expected, p100 might have a true molecular mass of 80–85 kDa. MAbs 3B6 and 1C4 recognized the respective translation products of pSK-BYV.MT and pSK-BYV.HEL on immunoblots (data not shown).

**Discussion**

Our immunoblot analysis identified the BYV methyltransferase-like and helicase-like proteins in planta as 63 kDa and 100 kDa products, respectively, but not as parts of a single 229 kDa product that could have been expected to result from the translation of ORF 1a with concomitant release of the 66 kDa leader proteinase (Agranovsky et al., 1994a). This indicates that the closterovirus 1a protein undergoes multiple proteolytic cleavages in vivo, in a pattern reminiscent of the processing of a precursor of the nonstructural proteins of alphaviruses, and of the 1a/1b polyprotein of corona-like viruses (reviewed in Dougherty & Semler, 1993; Snijder & Horzinek, 1993; Strauss & Strauss, 1994). The central region of
MAb detection of RNA virus replicative proteins

Fig. 3. Immunoblot analysis of total protein extracted from the BYV-infected *T. expansa* leaves collected at 2, 4, 7, 11, 15, 20, 32 and 49 days p.i. Lane H, total protein from a healthy plant. Immunoblots were developed with MAbs 3B6 (A), 1C4 (B) and 1470, specific to the BYV CP (C). The detected proteins are designated and marked by arrows.

Fig. 4. *In vitro* translation of T7 transcripts encoding portions of the BYV 1a protein that mimic in size the in vivo proteins p63 and p100. The transcripts from pSK-BYV.MT (lane 2) and pSK-BYV.HEL (lane 3) were translated in the wheat germ system in the presence of [35S]methionine and the products were separated in denaturing gels. Lane 1 is an RNA-free control. Arrows indicate the major products.

the BYV 1a protein contains a domain distantly resembling the aspartic proteinase of retroviruses (Agranovsky *et al.*, 1994a). However, the activity of this domain in splitting of the closterovirus polyprotein may be questioned, as it is not conserved (or is conserved only partially) in the 1a products of the related closteroviruses (Karasev *et al.*, 1995; Klaassen *et al.*, 1995). Alternatively, the cleavage may be mediated in *trans* by the BYV leader PCP, or by a host enzyme. Notably, the sum of the apparent molecular masses of the detected MT and HEL fragments gives a value considerably less than expected (163 versus 229 kDa). This discrepancy could hardly be explained by anomalously fast migration of the MT- and HEL-containing fragments in SDS gels; our experiments with the T7 transcripts proved that the 60 kDa MT-containing portion of the 1a had an electrophoretic mobility close to the expected value, whereas the 99 kDa HEL-containing product migrated more slowly than expected. Hence, the sizes of the p63 and p100 proteins suggest more than one cleavage site in the central domain of the BYV 1a protein.

Formally, it cannot be excluded that p100 contained both the HEL and POL domains in a fusion produced by the ORF1a/1b ribosomal frameshifting (Agranovsky *et al.*, 1994a). However, the fact that the MAbs specific to the C-terminal part of the BYV 1a protein detected a single protein band makes this possibility very unlikely, unless nearly all the translating ribosomes negotiated the stop codon in ORF 1a to produce the fusion. In fact, the efficiency of the closterovirus frameshifting signals *in vitro* was found to be less than 2% (Agranovsky, 1996; ten Dam, 1995; B. Renecke & W. Jelkmann, unpublished data).

The observed distribution of the BYV methyltransferase-like and helicase-like proteins in the subcellular fractions agrees well with that of other RNA virus replication-associated enzymes, which are most often found in the membrane compartments (Hills *et al.*, 1987; Scholthof *et al.*, 1995; reviewed in David *et al.*, 1992). Closterovirus infection is accompanied by the induction of characteristic membranous ultrastructures, the so-called BYV-type vesicles, which have

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been implicated in virus replication (reviewed in Coffin & Coutts, 1993). It would be interesting to see if these structures do accumulate the viral replicative proteins and are indeed the specific sites of closterovirus replication. Conceivably, p63 and p100 of BYV should have membrane-binding domain(s), unless they are retained in a membrane by protein–protein interactions. It should be noted, however, that the 2a protein of barley stripe mosaic hordeivirus (carrying the related MT and HEL domains) is found predominantly in the soluble protein fraction (Donald et al., 1993). This implies that the variable amino acid contexts of the MT and HEL, rather than the conserved domains per se, may influence the intracellular destination of the viral proteins.

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