Presentation of a foreign peptide on the surface of tomato bushy stunt virus

Thorleif Joelsson, Lennart Åkerblom, Per Oxelfelt, Bror Strandberg, Karin Tomenius and T. Jack Morris

1 Department of Molecular Biology, Uppsala University, Biomedical Centre, PO Box 590, S-751 24 Uppsala, Sweden
2 Department of Virology, National Veterinary Institute, Biomedical Centre, PO Box 585, S-751 23 Uppsala, Sweden
3 Department of Plant Pathology, Swedish University of Agricultural Sciences, PO Box 7044, S-750 07 Uppsala, Sweden
4 School of Biological Sciences, University of Nebraska, 348 Manter Hall, PO Box 880118, Lincoln, NE 68588-0118, USA

A 13-amino-acid peptide derived from the V3 loop of human immunodeficiency virus (HIV-1) glycoprotein 120 (gp120) was attached as a C-terminal gene fusion to the coat protein of tomato bushy stunt virus (TBSV). The architecture of this plant virus permitted external display of the foreign sequence 180 times on the surface of the chimaeric virus particle. The chimaera replicated to a level similar to wild-type TBSV and the foreign sequence was retained through six sequential passages in plants. The HIV epitope was detected on the surface of the virus capsid by a V3-specific monoclonal antibody and by human sera from HIV-1-positive patients, demonstrating the potential of using plant-derived chimaeric particles for diagnostic purposes. Chimaeric virus also induced a specific immune response to the foreign HIV epitope when injected into NMRI mice.

Epitope presentation systems based on linking an epitope of interest to a carrier molecule capable of assembly into a macromolecular structure have been developed using the coat proteins of bacterial viruses (e.g. Smith, 1985), animal viruses (e.g. Arnold et al., 1994), and the plant viruses cowpea mosaic virus (CPMV) (Usha et al., 1993; Porta et al., 1994; McLain et al., 1995) and tobacco mosaic virus (TMV) (Fitchen et al., 1995; Sugiyama et al., 1995; Turpen et al., 1995). CPMV chimaeric particles containing a peptide from human immunodeficiency virus type 1 (HIV-1) glycoprotein 41 stimulated the production of HIV-1-neutralizing antibodies when injected into mice (McLain et al., 1995).

In this study we describe the use of another icosahedral plant virus, tomato bushy stunt virus (TBSV), for epitope presentation on the surface of the virion. TBSV was chosen because it is very stable, easily purified from host plants, and is non-infectious for animal cells. Furthermore, the known three-dimensional structure of the TBSV particle provides an advantage not available for most other icosahedral viruses: the C-terminal portion of the TBSV capsid protein consists of a protruding domain (P-domain) which is displayed as 180 identical copies on the surface of the virus particle (Harrison et al., 1978; Olson et al., 1983). The location of the P-domain provides a unique opportunity to construct chimaeric virus particles with peptides exposed on the surface as fusions to the free carboxyl end (Olson et al., 1983).

TBSV, the type member of the family Tombusviridae (Russo et al., 1994), has a single positive-sense RNA genome of 4776 nucleotides packaged inside an icosahedral protein shell composed of 180 capsid protein subunits, each of 41 kDa. The genome has been sequenced and highly infectious RNA can be transcribed from full-length cDNA clones (Hearne et al., 1990). Previous work by Scholthof et al. (1993) showed that insertion of reporter genes, e.g. β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT), into the coat protein gene could be tolerated without appreciably affecting the ability of viral RNA to accumulate to near wild-type levels in plants. They also demonstrated that coat protein was not necessary for either cell-to-cell spread or long-distance movement of the TBSV genome.

These results suggested that the TBSV coat protein gene might be amenable to direct manipulation without detrimental effects on virus replication and virion accumulation. To test this, we constructed a chimaeric TBSV genome with a foreign sequence inserted in-frame at the 3’ end of the coat protein gene such that there would be a 16-amino-acid peptide fused to the C terminus of the capsid protein subunit. The foreign peptide consisted of 13 residues derived from the V3 loop of HIV-1 (LaRosa et al., 1990) and a three residue linker sequence. We describe the construction of this chimaeric virus (STV3) in this communication, demonstrate that the yield and stability of chimaeric virus particles in infected plants are comparable to
Fig. 1. Diagram showing the genomic organization of tomato bushy stunt virus (TBSV) and the derived mutant pTBSV-STV3. The open boxes represent the genes identified in Hearne et al. (1990). The capsid protein gene is designated CP. The positions of the two Asp-718 restriction sites used in this study and the newly introduced sites (BamHI, ApaI and SalI; included to facilitate future cloning experiments) at the end of the CP gene are identified. The nucleotide sequences derived from HIV-1, and those corresponding to the three linker amino acids (GSP), respectively, cloned at the end of the coat protein gene are in bold with the adjacent TBSV nucleotide sequences in lower-case. The amino acid sequence of the insert is in bold above the nucleotide sequence.

wild-type TBSV, and show that the foreign peptide is sufficiently exposed on the surface of the STV3 particles to react with HIV-1-specific antibodies. We also demonstrate the utility of the STV3 particles for diagnostic applications and provide preliminary results for potential use of the chimaeric virus in vaccine development.

Construction of the chimaeric virus with the V3-derived sequence at the coat protein C terminus is illustrated as pTBSV-STV3 in Fig. 1. Standard methodologies including site-directed mutagenesis and cloning of oligonucleotides were used. RNA transcripts for inoculations were derived from the plasmids pTBSV-100 (wild-type) and pTBSV-STV3 (chimaera) as previously described (Hearne et al., 1990). STV3 produced typical local lesions on Chenopodium amaranticolor between 48–72 h post-inoculation, and symptoms in inoculated and systemically infected leaves of Nicotiana benthamiana that were identical to those produced by wild-type TBSV.

Clarified extracts of N. benthamiana leaves inoculated with wild-type or STV3 transcripts contained numerous virus particles when examined by immunosorbtent electron microscopy (ISEM). The leaf extracts were highly infectious when tested on new hosts, and double antibody sandwich (DAS) ELISA using rabbit anti-TBSV serum showed similar virus titres in plant sap (data not shown). Virus was readily purified from infected leaf tissue (Hearne et al., 1990) with average virus yields of 0.9 mg/g of leaf tissue for STV3 and 1.1 mg/g for wild-type virus. Any sedimentation difference between wild-type and STV3 virus was not evident in the 10–50% sucrose density gradients used to purify the virus. We did not attempt to confirm the slight sedimentation difference expected for STV3 by performing additional analytical sedimentation analysis. No discernible size and/or shape differences were evident when wild-type and STV3 density-gradient purified virus were examined at 80 kV in a Zeiss-902 electron microscope on hydrophilic, carbon-coated grids, stained with ammonium molybdate, pH 7±0 (Fig. 2a, b).

Evidence for external display of the foreign epitope on the surface of the STV3 particle was provided when the electrophoretic mobilities of the purified, intact viruses were examined in 1–2% non-denaturing agarose gels (Fig. 2c) as described by Heaton (1992). The distinctively slower electrophoretic mobility displayed by STV3, compared with wild-type virus, was expected because of the combined effects of a predicted altered basic charge on the surface of the virion and an expected slight increase in the hydrodynamic diameter of the particle.

The genetic stability of STV3 was examined by RT–PCR after two and six passages in plants. Sequence analysis of the gel-purified RT–PCR products confirmed that STV3 was identical to the original construct after two passages. However, after six passages of the same clone of STV3, the inserted sequence had acquired a mutation that resulted in replacement of the underlined isoleucine residue with methionine (KS\IHIGPGRAFYT). The significance of this apparently random mutation was not examined further.

SDS–PAGE analysis of wild-type and STV3 virus purified from inoculated and systemically infected leaves identified coat protein subunit monomers and dimers that had very similar electrophoretic mobilities in the 12% acrylamide gels used. Western blot analyses (Fig. 2d) of wild-type and STV3 coat proteins demonstrated that both the monomer and dimer bands reacted with an antiserum to SDS-disrupted TBSV. As expected, a monoclonal antibody directed against the peptide KSIHIGPGRAFYT, which is the central motif of the V3 region in HIV-1 gp120 (MAb SP.BAL.114 described by Arendrup et al., 1993) reacted with the STV3 but not the wild-type coat.
Fig. 2. Characterization of chimaeric TBSV-STV3 by electron microscopy, electrophoresis and Western blot analyses. High resolution electron microscopy of purified wild-type and chimaeric TBSV-STV3. (a) Wild-type TBSV; (b) TBSV-STV3. Scale bar, 100 nm. (c) Electrophoresis in a 1.2% Tris–glycine agarose gel of purified virus particles derived from transcripts of pTBSV-100 (wild-type) and pTBSV-STV3 (STV3). Virus was isolated from *N. benthamiana* leaves 4 days after inoculation. P1 identifies the virus isolated after the initial inoculation and P2 virus isolated after passage of the P1 virus to a second host plant. Stained with ethidium bromide. (d) Western blot analyses of SDS-disrupted wild-type TBSV particles (lanes 1 and 2), and chimaeric TBSV-STV3 particles (lanes 3 and 4). A hyperimmune rabbit antiserum against SDS-treated TBSV was used in lanes 1 and 4. A monoclonal antibody specific for an epitope in the V3 sequence of gp120 was used in lanes 2 and 3. Lane S, prestained molecular mass markers (Bio-Rad) as follows: 140, 87, 48, 33, 29 and 21 kDa. The positions of the coat protein monomer (m) and dimer (d) are indicated on the right side of the figure.
The efficacy of the TBSV epitope presentation system for eliciting an antibody response to the foreign peptide was tested by injecting STV3 virus into mice. Two groups of ten NMRI mice were injected subcutaneously with 5 µg of either wild-type TBSV or STV3 virus in PBS with an ISCOM-matrix (a saponin adjuvant; Sjölander et al., 1996). Sera were tested in ELISA 3 weeks after the primary injection for the presence of antibodies to TBSV and to a 24 residue synthetic peptide containing the sequence KSIHGPGRAFYT. All 20 sera had a high antibody response against TBSV after one injection (mean dilution end-point of $1 \times 10^5$; SD $\pm 1.5 \times 10^4$). Although a specific primary antibody response to the synthetic peptide was detected in the group injected with STV3, it was relatively low (dilution end-points in the range $1 \times 10^4$ to $1 \times 10^5$) compared with the controls (dilution end-point < 50). A second injection of 5 µg of STV3 virus 4 weeks after the first injection failed to boost the antibody titre against either the TBSV coat protein or the V3 epitope.

Our results demonstrate that the genome of a plant tombusvirus can be engineered to express a foreign peptide as a C-terminal extension of the viral coat protein subunit without appreciably disturbing assembly, disassembly and stability of the virus particle. Specifically, we have shown that a 16-amino-acid peptide containing an HIV-1-specific V3 epitope (13 residues) could be readily detected by specific antibodies directed against the epitope. These results confirm structural predictions that the foreign epitope would be well exposed on the surface of the chimaeric particles because it would be at the very end of the P-domain of the subunit.

The location of the foreign sequence did not seem to destabilize the virus as evidenced by the simplicity with which it was purified, its functionality in serological tests and its ability to induce a clear primary antibody response when injected into mice. Although the C termini of the small coat protein subunits of CPMV are exposed on the surface of the particle, similar C-terminal fusions are not feasible in CPMV because of the proteolytic C-terminal trimming that occurs \textit{in vivo} and during storage (McLain et al., 1995). A potential advantage in making fusions of foreign peptides at the C terminus of a viral coat protein, as described here, is that there is less likelihood of the insert placing structural constraints on assembly. We are in the process of studying the immunogenicity of additional viable TBSV chimaeras in the hope of improving the use of recombinant TBSV particles for diagnostic applications.

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**Fig. 3.** Detection of human antibodies reactive to chimaeric TBSV-STV3 particles by indirect ELISA. Plates were coated with TBSV-STV3 at 1 µg/ml, then incubated with dilutions of sera from patients at different stages of HIV-1 infection (according to Mildvan & Solomon, 1987): stage I serum (●); stage II serum (■); stage III serum (▲); stage IV serum (□).
References


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