Regulated nuclear targeting of cauliflower mosaic virus

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The mature cauliflower mosaic virus (CaMV) capsid protein (CP), if expressed in the absence of other viral proteins, is transported into the plant cell nucleus by the action of a nuclear localization signal (NLS) close to the N terminus. In contrast, virus particles do not enter the nucleus, but dock at the nuclear membrane, a process inhibited by anti-NLS antibodies or by GTPγS, and apparently mediated by interaction of CP with host importin α. The very acidic N-terminal extension of the viral CP precursor inhibits nuclear targeting of the protein and hence the precursor is localized in the cytoplasm. We hypothesize that this provides a control mechanism which ensures that the CP precursor is used for virus assembly in the cytoplasm and that only mature virus particles reach the nuclear pore.

Introduction

Many viruses replicate or transcribe their genome in the nucleus of their hosts, requiring a mechanism by which the genome can enter the nucleus. Generally, the nuclear membrane acts as a barrier to virus entry, and specific viral proteins are required to mediate transport of the viral genome into the nucleus (Whittaker & Helenius, 1998). Most retroviruses circumvent the transport problem by establishing infection during mitosis, when nuclear membranes are broken down (Roe et al., 1993). However other retroviruses, e.g. human immunodeficiency virus type 1 (HIV-1), and pararetroviruses, i.e. animal hepadnaviruses and plant caulimoviruses, can infect resting cells, inferring active transport of the viral genome across the nuclear membrane.

The type member of caulimoviruses is cauliflower mosaic virus (CaMV; Rothnie et al., 1994). A pool of CaMV particles containing open circular DNA is established in the cytoplasm of an infected plant cell via aphid transmission, cell-to-cell transport and de novo synthesis. Another pool of viral genomes, in the order of 10–100 copies of minichromosomes comprising supercoiled circular viral DNA and host histones, accumulates in the nucleus. From these minichromosomes, viral RNAs are produced and transported to the cytoplasm for translation, packaging and reverse transcription. Viral proteins together with virus particles accumulate in inclusion bodies composed of the product of CaMV ORF VI. Virus particles are then transported to other cells, taken up by aphids or used to re-infect the nucleus (see Fig. 5).

The CaMV virion is an icosahedron, with a diameter of 54 nm, composed of 420 subunits of the viral capsid protein (CP) (Cheng et al., 1992). CP exists in three major forms, called p44, p39 and p37, that are processed by removal of the acidic N and C termini of the CP precursor. In p44, the first 76 amino acids (aa) and about 40 aa from the C terminus are removed by the viral aspartic proteinase (Torruella et al., 1989; A. Karsies and others, unpublished). The N termini of p39 and p37 are blocked, precluding exact mapping by protein sequencing (Martinez-Izquierdo & Hohn, 1987). Mature CPs are glycosylated (Du Plessis & Smith, 1981), and p44 (but not p39 or p37) is phosphorylated (Martinez-Izquierdo & Hohn, 1987).

The CaMV CP might be responsible for the nuclear transport of viral DNA since it harbours nucleic acid binding domains close to its C terminus and, close to its N terminus, a simian virus-40 (SV40)-like nuclear localization signal (NLS) that is conserved throughout the caulimovirus family (Leclerc et al., 1999). The NLS is responsible for nuclear import of the mature CP outwith the viral context. In mature viruses, the NLS is exposed at the surface of the virions (Leclerc et al., 1999).

Here we show that purified virions do not enter the nucleus, but dock to the nuclear membrane. This interaction is inhibited by anti-NLS antibodies or by GTPγS, an inhibitor of
nuclear import. The regular pattern of interaction suggests that specific receptors on the nuclear membrane are involved, probably associated with the nuclear pores. Moreover, CP interacts specifically with importin α, indicating that virus docking is mediated by this import receptor.

Methods

Plasmids. The different CaMV CP constructs were cloned downstream of the haemagglutinin (Ha11) epitope in pTARGNLS as described (Leclerc et al., 1999). Clones starting at codon 77 have a fused methionine initiation codon. The three Glu → Ala mutations in positions 83, 84 and 85 to generate p(1–489)*, p(1–332)* and p(77–332)* (* represents the EEE83AAA designations for the mutant sequence) were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) using the complementary oligonucleotide pair 5′-GCAATAGGAGAACATCT-GCAGCACTTACCGATTCAGAGAAACCTG-3′ and 5′-CAGG-TTCTTCTCCTGAAATCGCTAGCCTGACAGATGTCTCCTA- TTGC-3′.

Plants and viruses. Brassica rapa plants (turnip ‘Just Right’) were grown at 22 °C with a 16 h photoperiod as described (Blanc et al., 1993). The Strasbourg strain of CaMV cloned in the Sfil site of pBR322 (pCa37) was used for the infection of 4-week-old turnip plants. Virus was purified as described (Lebeurier et al., 1980).

Plant protoplasts and protein analysis by indirect immunofluorescence. Culture conditions for Orchiophagus violaceus suspension cultures, protoplast preparation and transfection have been described previously (Fütterer et al., 1989, 1990). Nicotiana plumbaginifolia mesophyll protoplasts (3 × 10⁶) were transfected with 20 μg of plasmid using the polyethylene glycol method (Goodall et al., 1990). B. rapa protoplasts were prepared according to the same protocol with modifications as described (Leclerc et al., 1999). Optimal expression of protein was found to occur 8 h after transfection. Protoplasts were collected and treated for indirect immunofluorescence (Mieszcak et al., 1992).

The rabbit polyclonal anti-Ha11 antiserum was obtained from Babco (Richmond, CA, USA), and the goat anti-rabbit antibody conjugated with IgG (Himmelbach et al., 1996). These antibodies were used for Western blotting to stain nuclear and cytoplasmic proteins and for indirect immunofluorescence. Immunoblotting was performed as described (Richmond, CA, USA), and the goat anti-rabbit antibody conjugated with IgG (Himmelbach et al., 1996). Antibody inhibition tests were performed by pre-treating the labelled virus with anti-NLS immunoglobulin G (NLS-IgG) (Leclerc et al., 1999).

Extraction of proteins, SDS–PAGE and immunoblotting. Proteins were extracted from transfected protoplasts with phenol and precipitated by methanol (Hurkman & Tanaka, 1986). Proteins were separated by SDS–PAGE (Schagger & von Jagow, 1987) and electroblotted overnight to nitrocellulose membranes at 30 V (constant voltage). The membrane was treated and probed with anti-CaMV CP precursor IgG (Himmelbach et al., 1996). These antibodies were used for Western blotting because they gave a stronger signal than the anti-tag antibody.

Yeast two-hybrid system. Interaction between CP and importin α was shown with the DupLEX-A yeast two-hybrid system (Origene Technologies, Rockville, MD, USA). CP fragments 125–332 and 77–332, containing an Ha11 tag at the N terminus (Leclerc et al., 1999), were cloned in-frame with the lexA gene into the BandH–S61 restriction sites of the pEG202 yeast expression plasmid. The pPG4-5 galactose-inducible yeast plasmid was used to express importin α from Capsicum annuum (Szurek et al., 2001), fused in-frame to the B42 activation domain (AD). Yeast strain EGY48 containing the pSH18-34 high sensitivity lacZ reporter plasmid was used. The interaction was quantified in liquid assay using chlorophenol red β-galactosidase (CPRG; Roche Biochemicals) as described in the Clontech yeast protocols handbook.

Results

To analyse nuclear targeting mediated by the NLS of the CaMV CP and its regulation at different stages of the replication cycle, we analysed the intracellular localization of precursor and mature forms of the CP in transfected protoplasts, and of whole virus particles in permeabilized cells. Proteins were localized by immunofluorescence using the Ha11 tag. Whole virus particles were localized by virtue of their labelling with a fluorescent dye.

The acidic N-terminal domain of the CP precursor inhibits nuclear targeting

CaMV p44 starts with ORF IV aa 77 and ends around aa 454 (Martinez-Izquierdo & Hohn, 1987). CP derivatives starting at aa 77 and ending with aa 480, 411 or 332 were all localized in the nucleus after expression, confirming earlier results (Leclerc et al., 1999). This localization is dependent on an NLS motif close to the N terminus (Fig. 1A); mutation of this motif abolished nuclear localization. On the other hand, nuclear localization is independent of the basic C-terminal region and the Zn-finger (Leclerc et al., 1999).

Analysis of constructs expressing full-length CP precursor [p(1–489); Fig. 1A], or the C-terminal truncated proteins [p(1–362) and p(1–265)], is complicated by the presence of protein instability determinants at the N terminus and, for the full-length precursor, at the C terminus also (Karsies et al., 2001). However, mutation of a cluster of acidic amino acids to
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Fig. 1. Transport of CP derivatives into the nucleus. (A) Schematic representation of CaMV CP precursor and derivatives. The light grey regions are rich in acidic amino acids and the striped region is rich in basic amino acids. The central white region is believed to be involved in protein–protein interactions and virus assembly. The dark grey bars represent the NLS and the Zn-finger motifs, and a black bar the EEE84AAA mutation. The amino acid sequence of the N terminus of p44, one of the major processed forms of the CP present in the purified virus, is shown below the scheme. The mutation of glutamic acids 83, 84 and 85 to alanine, increasing the stability of the CP precursor, is indicated. (B) Detection of CP derivatives, by Western blotting using anti-CP-precursor antibodies, in extracts of *N. plumbaginifolia* protoplasts transfected with different forms of CaMV CP. (C) Indirect immunofluorescence of *N. plumbaginifolia* protoplasts transfected with different forms of CaMV CP. (a) p(77–332)* with the three Glu residues at positions 83, 84 and 85 mutated to Ala residues. (b) p(1–332) containing wt sequence. (c) p(1–332)* with the same mutation as in (a). (d) Negative control, protoplasts transfected with the empty vector.

yield p(1–332)*, p(77–332)*, etc. (* represents the EEE83AAA exchange) in the N-terminal region stabilizes certain constructs sufficiently to allow detection by Western blotting or immunofluorescence (Fig. 1). Note that the Western signal produced by p(77–332)* is considerably weaker than that of p(1–332)* because the antibody recognizes the acidic N-terminal domain of the latter protein more efficiently. This stabilizing mutation was not sufficient to allow detection of the full-length protein [p(1–489)*], because the acidic C terminus also constitutes an instability element (Karsies et al., 2001).

Indirect immunofluorescence revealed that the protein derived from p(1–332)* remained in the cytoplasm, where it appeared as ring-like structures, probably because of displacement by, or a non-specific interaction with, the chloroplasts (Fig. 1C). In contrast, the protein derived from p(77–332)* (Fig. 1C) or p(77–332) (not shown) was found in
CaMV particles dock to the nuclear membrane via the NLS of their CP

Mature CaMV virions were isolated and tested for their intracellular localization. For this purpose, permeabilized *N. tabacum* BY-2 protoplasts (Merkle et al., 1996) were incubated with labelled virions and analysed. BY-2 cells were disrupted with 0.01% Triton X-100 by passing the cells several times through a 1 ml syringe. The cells were collected by low-speed centrifugation and washed twice with the nuclear import buffer. The resulting permeabilized cells were incubated with the fluorescent CaMV particles, washed once with the nuclear import buffer and mounted on a glass slide. The fluorescence was localized to the nucleus and confocal scanning microscopy revealed that virions were actually located at the surface of the nuclear membrane (Fig. 2A, B), rather than being in the interior of the nucleus. Possibly the size of the virions precluded their migration through the nuclear pore. The interaction was inhibited by the addition of anti-NLS antibodies (Fig. 2C), but not by pre-immune serum (not shown), indicating that the NLS located at the surface of the virion is responsible for the interaction. Inhibition of virion–nucleus interaction was also observed when GTP\(_{\gamma}\)S, an efficient inhibitor of nuclear import (Merkle et al., 1996), was included in the reaction mixture (Fig. 2E).

Tobacco is not a natural host of CaMV. We therefore repeated the experiments described above using isolated nuclei (Merkle et al., 1996) from cells of *O. violaceus* (Fig. 3A–C) and *B. rapa* (Fig. 3D, E), which are host plants for CaMV. Again we observed an interaction between the labelled virus and the surface of the nuclear membrane (Fig. 3A). In this case, the fluorescence appeared as discrete foci when the surface of the nucleus was scanned with the confocal microscope (Fig. 3C).
Based on this observation we suggest that the labelled virions interact with the membrane at specific sites, probably the nuclear pore. In future studies we aim to confirm this hypothesis by electron microscopy.

**Interaction of the CP with importin α**

Since importin α is involved in most cases of nuclear transport (Whittaker & Helenius, 1998), we used the LexA two-hybrid system to test for a specific interaction between CP and plant importin α. Interactions were quantified using a β-galactosidase liquid assay (Fig. 4). CP fragments p(77–332) and p(125–332) could be used for this experiment. However, p(1–332) could not be used, since it showed non-specific interaction with the B42 activation domain. Results showed that p(77–332) interacts with importin α in this system whereas p(125–332), which lacks the NLS sequence, does not. Although further studies will be needed to address the role of importin α, this result correlates interaction between the CP and importin α with CP nuclear localization.

**Discussion**

Isolated mature CaMV CP is transported into the nucleus via an NLS close to its N terminus (Leclerc et al., 1999). Here we show that this NLS also functions in the context of assembled virions. However, in this case the (fluorescently labelled) particles bind to the nuclear membrane and remain there. The NLS-mediated specific binding of the virus to the nuclear membrane can be interpreted as being mediated by importin α, a protein involved in nuclear import of NLS-containing proteins, which in plants is found mainly at the nuclear membrane surface (Smith et al., 1997). Support for this interpretation is given by the weak, but specific, interaction of the CP with a plant importin α in the LexA two-hybrid system (Fig. 4). This interaction may be stronger for the virus particle, since it is built from 420 capsid subunits and hence presents 420 NLSs. In contrast to animal cells, importin α is closely associated with the nuclear pore of plant cells (Merkle et al., 1996). This is the reason, as shown by Merkle et al. (1996), that there is no requirement for cytosolic components for the interaction of the virus with the cellular membrane. The inhibition of binding of the virus to the nuclear membrane by GTP/S could be the result of the inactivation of RAN, a GDP-binding protein involved in nuclear import, since this analogue of GTP is known to maintain RAN in a GTP-bound form that cannot be hydrolysed (Dickmanns et al., 1996). As a consequence, the interaction of the virion with the surface of the nucleus is inhibited because the RAN-GTP form dissociates the importin αβ heterodimer by displacing α from an overlapping binding site on β (Moroianu et al., 1996).

The interaction of the virus with the nuclear membrane is not uniform and discrete foci can be seen at the surface of the membrane (Fig. 3C). This discontinuity of the signal indicates that the virus interacts with discrete structural units of the nuclear membrane that are probably the nuclear pores. Very similar images were obtained for constituents of the nuclear pores (e.g. Nup84, Nup49p or Nup133p) (Bastos et al., 1997; Belgareh & Doyle, 1997; Bucci & Wente, 1997). Importin α associated with an NLS-containing protein has been shown to interact strongly with the cytoplasmic filaments of the nuclear pore (Panté & Aebi, 1996). These filaments could thus represent the specific sites of the interaction between virus and nucleus.

CaMV now joins a collection of different DNA viruses (Izaurralde et al., 1999), e.g. papovaviruses (Yamada & Kasamatsu, 1993), geminiviruses (Kunik et al., 1998) and hepadnaviruses (Kann et al., 1997), that target their genomic DNA to the nucleus via an NLS located on the CP. In contrast, most retroviruses do not need an NLS, since they are restricted to dividing cells (Roe et al., 1993). Exceptions are the lentiviruses; their capsids disassemble prior to nuclear targeting releasing a preintegration complex (PICs). Several constituents of the PIC [integrase (Gallay et al., 1997), Vpr (Freed et al., 1997) and perhaps matrix protein (Gallay et al., 1995)] have been implicated in nuclear targeting.

It is still unclear how CaMV DNA enters the nucleus. In our assay the bulk of CP remains at the surface of the nuclear membrane and hence we consider it unlikely that intact CaMV particles pass into the interior of the nucleus as has been suggested for SV40 (Yamada & Kasamatsu, 1993). Although we cannot exclude the possibility that an essential element that would mediate this process is missing, the addition of cytoplasmic extracts to our in vitro assay did not change the interaction of the labelled virus with the nucleus (data not shown). In any case, entry of the complete capsid into the nucleus would have to involve major distortions of the particle. With a diameter of more than 50 nm, the capsid is much larger than the size limit for NLS-coated gold particles (26 nm; Feldherr et al., 1984), and also still larger than cargo-receptor–gold complexes (39 nm; Panté & Kann, 2002), which

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**Fig. 4. CaMV CP interacts with importin α.** Two fragments of CP were individually fused to the LexA DNA-binding protein, and cotransformed in yeast with *Capsicum annuum* importin α fused to the B42 activation domain. The strength of the interaction was measured by liquid assay using CPRG as a substrate.
can enter through a nuclear pore. More likely, after docking of the virion to the nuclear pore, the capsids would fully or partially disassemble and the DNA would be released into the nucleus, as has been suggested for hepatitis B virus (HBV) (Kann et al., 1997, 1999) and herpes simplex virus (Izaurralde et al., 1999). In CaMV, transport through the pore could then be mediated by a few CP subunits or by another protein such as reverse transcriptase. In HBV, not only the CP but also the reverse transcriptase carries an NLS (Kann et al., 1997).

The CaMV CP precursor is very unstable in plant cells, probably because the N and C termini removed during maturation contain sequences conferring instability (Karsies et al., 2001). However, removal of the acidic C-terminal region and mutation of three adjacent glutamic acids within the acidic N-terminal region created a more stable CP precursor variant, p(1–332)*. The acidic N terminus of this variant appears to be a cis-active inhibitor of nuclear targeting. The fact that CP precursor protein is excluded from the nucleus despite the presence of a NLS may point to an interesting regulation of intracellular localization: the NLS might be masked by the acidic N terminus of the precursor protein in order to retain it in the cytoplasm to interact with RNA, form previrions and allow reverse transcription. This would assure that only mature, DNA-containing, virions are transported to the nucleus.

It could be that the N-terminal extension of the CP precursor competes with the NLS by binding to a cytoplasmic receptor, e.g. to mediate assembly. In HIV-1, for instance, myristylation of the matrix domain directs the Gag precursor protein to the plasma membrane, where assembly and budding occurs (Gelderblom et al., 1989). Alternatively, the N-terminal polypeptide could mask the NLS directly by modifying the protein structure. A masking of the NLS in immature capsid (core) protein was suggested for HBV. In this case, however, and in contrast to CaMV (Leclerc et al., 1999), activation of the NLS occurred upon phosphorylation of a specific site (Kann et al., 1999). We hypothesize that for both viruses, HBV and CaMV, regulation of the CP NLS is important since it ensures virus assembly in the cytoplasm and minimizes the damage...
that CP could cause in the nucleus. Interestingly, the CaMV CP is highly toxic to E. coli cells, where the DNA replication- and RNA production-machineries are not shielded by a nuclear membrane (Fütterer et al., 1988).

To refine the model of the virus replication cycle we propose that one or more of the NLSs located on the CP molecules interact with the nuclear pore (Fig. 5). Subsequently, the virus disassembles and the DNA passes through the pore, either as such or complexed with some CP molecules and/or other proteins, but not with the complete capsid. Once inside the nucleus, the viral genome is repaired and the now supercoiled form associates with histones to form a minichromosome, which is transcribed. The acidic N-terminal region of the CP precursor (1–489) inhibits nuclear targeting and therefore this form of protein remains in the cytoplasm, where it is readily degraded (Karsies et al., 2001). Gradually, as viral infection progresses, inclusion bodies appear that protect the CP precursor from degradation and in which virus particles accumulate. During or after assembly and packaging, the viral protease will remove the first 76 amino acids from the CP precursor, and this proteolysis will expose the NLSs at the surface of the virions, allowing their transport to the nucleus, interaction with the nuclear pore and DNA release into the nucleus to increase the number of nuclear viral minichromosomes. There are indications that the C-terminal acidic region of the CP precursor also has masking functions. In this case the nucleic acid-binding motif of the CP precursor is affected (Guerra-Peraza et al., 2000).

The mature virus particles can also remain in the inclusion body, be transported to a neighbouring cell via plasmodesmata or be captured by a feeding aphid that will transmit the virus to another plant. The mechanism of partition between these fates remains an open question.

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References


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