Probing Plasmodesmal Transport with Plant Viruses

Vitaly Citovsky*

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794

Plant intercellular connections, the plasmodesmata, effectively link individual cells into one symplastic continuum and function as conduits for transport and cell-to-cell communication processes. Although plasmodesmal ultrastructure has been extensively studied (see below), very little is known about the biochemistry, molecular biology, and regulation of these intercellular channels. This lack of knowledge stems mainly from technical difficulties in isolation and purification of plasmodesmata structures that are embedded in the plant cell-wall matrix. Thus, plasmodesmata remain essentially a biological "black box." Recently, however, a potentially powerful approach with which to study plasmodesmal function has been developed. This approach is based on the general observation that complex biological pathways can be functionally dissected using specific biologically active compounds (e.g. inhibitors or inducers) or mutations that perturb the pathway. In the case of plasmodesmata, a unique class of biological molecules is known to specifically and dramatically alter plasmodesmal function: the cell-to-cell movement proteins of plant viruses.

In this review, I focus on the use of plant virus cell-to-cell movement proteins as molecular tools to study plasmodesmata. For detailed descriptions of the role of movement proteins in cell-to-cell spread of plant viruses, the reader is referred to recent comprehensive reviews (Atabekov and Taliansky, 1990; Citovsky and Zambryski, 1991; Hull, 1991; Maule, 1991; Deom et al., 1992; Citovsky and Zambryski, 1993; McLean et al., 1993).

PLASMODESMATA

Structure

A simple plasmodesma is a pore that is lined with plasma membranes of the connecting cells (Fig. 1A). The center of this pore is occupied by an appressed strand of ER connected to the ER of the adjacent cells. In the center of the appressed ER is a vertical row of globular proteinaceous particles (3 nm in diameter) associated with the inner leaflet of the appressed ER membrane (Fig. 2). Most of the space between the plasma membrane surface of the pore and the appressed ER is also thought to be occluded by 3-nm protein particles. One layer of particles is embedded in the inner leaflet of the plasma membrane; a second layer is embedded in the outer leaflet of the appressed ER and is connected by filamentous structures to protein particles in the central part of the appressed ER structure (Fig. 2). The small (2.5 nm in diameter) space between the two layers of protein particles (Fig. 2) forms aqueous microchannels (usually 7–10 per single plasmodesma) through which plasmodesmal transport occurs (Ding et al., 1992b). The function of these protein particles is unknown; possibly they regulate the size of permeable microchannels by changing their positions within the plasmodesmal pore (see below).

Structurally and developmentally, plasmodesmata can be divided into two groups. (a) Single plasmodesmata are found predominantly in young immature tissue; they are thought to represent the primary plasmodesmata, i.e. the connections formed between cells during cell division (Fig. 1A). (b) Branched plasmodesmata consist of several single plasmodesmata interconnected by a central cavity (Fig. 1B). These plasmodesmata are found only in mature tissue and are considered to represent the secondary connections inserted into the existing cell walls after cell division (Ding et al., 1992a). Both primary and secondary plasmodesmata exhibit similar size exclusion limits (Ding et al., 1992a), implying that these two types of connections are physiologically similar. Recently, however, a specific protein kinase activity was suggested to associate with secondary but not primary plasmodesmata (Citovsky et al., 1993) (see below). This finding may reflect differences in mechanisms by which these two classes of plasmodesmata are regulated.

Permeability

In higher plants, permeability of plasmodesmata has been extensively studied by microinjecting dyes of increasing molecular mass. These studies defined the size exclusion limit of plasmodesmal microchannels as 1.5 to 2 nm in diameter (800–1000 D) depending on the plant species (Terry and Robards, 1987; Wolf et al., 1989). Considering the physical dimensions of plasmodesmal channels (2.5 nm as estimated from electron micrographs [Ding et al., 1992b]), it appears that, for efficient transport, the transported molecule must be smaller than the actual diameter of the channel.

Control of plasmodesmal permeability is largely an unexplored subject. Several factors (e.g. Ca2+ and phosphoinositides) have been shown to reduce plasmodesmal flow (Eppel et al., 1988; Tucker, 1988), but these regulatory factors do not specifically affect plasmodesmata, but instead act as second messengers for many cellular processes. Although

Abbreviations: NBP, nuclear localization signal binding protein; NLS, nuclear localization signal; SSB, single-strand DNA binding protein; TMV, tobacco mosaic virus.
Figure 1. Ultrastructure of primary (single) (A) and secondary (branched) (B) plasmodesmata. Electron micrographs of plasmodesmata between tobacco mesophyll cells (magnification x131,500) are from Ding et al. (1992a) and they were provided by B. Ding and W. Lucas (University of California, Davis). ER, Cellular endoplasmic reticulum; aER, appressed ER; CC, central cavity.

Figure 2. Schematic structure of a simple plasmodesma. Diagrams of longitudinal view and of transverse view of a closed plasmodesma are adapted from Ding et al. (1992b). A model for plasmodesmal opening (transverse view) is explained in the text.
these secondary messengers can decrease plasmodesmal permeability, no known plant factors increase the permeability. Still, recent evidence suggests that the plasmodesmal size exclusion limit can increase significantly to allow transport of proteins and even larger nucleic acid molecules. For example, cellular proteins appear to be transported through the plasmodesmata linking companion and sieve cells in the vascular bundle (Fisher et al., 1992). A striking example of plasmodesmal expansion is transport of plant virus nucleic acids from infected into healthy adjacent cells, a process caused not by plant proteins but by viral proteins (Wolf et al., 1989; Derrick et al., 1992).

Specifically, the virus-encoded movement proteins mediate cell-to-cell spread of plant viruses through plasmodesmata (reviewed by Atabekov and Taliansky, 1990; Citovsky and Zambryski, 1991; Hull, 1991; Maule, 1991; Deom et al., 1992; Citovsky and Zambryski, 1993; McLean et al., 1993). Although these proteins originally were studied in the context of plant virus pathology and plant protection, they now receive attention as molecular tools to probe plasmodesmal transport.

**PROTEIN MACHINERY OF PLASMODESMAL TRANSPORT**

**Movement Proteins**

Movement proteins specifically alter plasmodesmal function and thus can be used to characterize transport pathways through plasmodesmata. All cell-to-cell movement proteins known to date are virus encoded. Evolutionary studies suggest that invading viruses insinuate into the existing cellular processes and adapt them for their own life cycle. It is possible, then, that viral movement proteins are functionally analogous to unidentified plant cellular proteins that mediate transport of macromolecules through plasmodesmata. Indeed, recent amino acid sequence analyses indicate that most viral cell-to-cell movement proteins appear to have evolved from a single ancestor (Melcher, 1990), possibly a captured cellular protein (Koonin et al., 1991). Thus, study of viral movement proteins may directly reflect mechanisms of plasmodesmal transport in normal, uninfected plants rather than a plant virus-specific phenomenon.

One of the best-studied cell-to-cell movement proteins is the P30 protein of TMV. Two lines of evidence implicate P30 in cell-to-cell movement of TMV: (a) P30 mutations specifically restrict the cell-to-cell spread of TMV (Jockusch, 1968; Peters and Murphy, 1975; Nishiguchi et al., 1978; Meshi et al., 1987); and (b) P30 expressed in transgenic plants restores cell-to-cell spread of movement-deficient TMV strains (Deom et al., 1987). How does P30 mediate plasmodesmal transport? Recent evidence suggests that P30 (as well as movement proteins of many other plant viruses [Citovsky et al., 1991; Osman et al., 1992; Schumacher et al., 1992]) may interact with the transported viral nucleic acid molecule and function as its molecular chaperone during transport (Citovsky et al., 1990; Citovsky and Zambryski, 1991). P30 is a single-strand nucleic acid binding protein with two independently active binding domains at its carboxyl terminus (Citovsky et al., 1992a). Similar to all known SSBS (Chase and Williams, 1986), P30 binding to single-strand nucleic acids is cooperative and sequence nonspecific; unlike most SSBS, however, P30 binds single-strand DNA and RNA with equal affinity (Citovsky et al., 1990).

Cooperative binding of P30 to TMV RNA was proposed to unfold the nucleic acid and convert it into a form that can penetrate through plasmodesmal microchannels (Fig. 3) (Citovsky et al., 1990). Typically, free single-strand nucleic acids exist as bulky and collapsed structures (Citovsky et al., 1989, 1992a); for example, calculations indicate that free-folded TMV genomic RNA molecule has an average diameter of 10 nm (Gibbs, 1976). EM observations demonstrated that binding of P30 to single-strand nucleic acid molecules produces approximately 2-nm-wide unfolded P30-RNA and P30-nucleic acid complexes (Citovsky et al., 1992a). As discussed below, the dimensions of P30-nucleic acid complexes (2 nm) are compatible with the size of plasmodesmata found in plants transgenic for or microinjected with P30 (Wolf et al., 1989; E. Waigmann, W. Lucas, V. Citovsky, P. Zambryski, unpublished data) and, by implication, modified during viral infection. Along with unfolding the transported nucleic acid molecule, P30 binding may protect it from cellular nucleases. Indeed, SSBS binding is known to prevent nucleic acid degradation by externally added endo- and exonucleases (Citovsky et al., 1989).

Although P30 is the only viral protein required for cell-to-cell spread of TMV, it may interact with at least two cellular protein components of the transport pathway. First, P30 potentially interacts with plant cell cytoplasmic receptors that may shuttle the movement complex from the site of its assembly in the cytoplasm to plasmodesmata (Fig. 3). The presence of these receptors, however, has not been proven. Second, P30 likely interacts with plasmodesmal components to increase permeability and promote transport (Fig. 3).

**Putative Cytoplasmic Receptors**

Because P30-nucleic acid movement complexes are presumably formed in the cell cytoplasm where TMV RNA is replicated and translated (reviewed by Palikaitis and Zaitlin, 1986), cell-to-cell movement of the newly formed complexes requires that they first travel to plasmodesmal orifices. How does this cytoplasm-to-plasmodesmata transfer of protein-nucleic acid complexes occur? Much of our knowledge of nucleic acid transport through membrane channels derives from studies of nuclear export and import. Although plasmodesmata and nuclear pores are obviously structurally different, both are the only known complex proteinaceous pores involved in active bidirectional traffic of macromolecules (Robards and Lucas, 1990; Forbes, 1992; Citovsky and Zambryski, 1993). Furthermore, transport of viral nucleic acids through plasmodesmata (Citovsky et al., 1992a), as well as nuclear transport of Agrobacterium T-DNA (Citovsky et al., 1989, 1992b) and pre-mRNAs (Mehlin et al., 1992), presumably involves unfolded nucleic acid-protein complexes. Thus, it is possible that nucleic acid transport through plasmodesmata may functionally resemble nuclear import.

Nuclear import initiates in the cytoplasm with binding of NLSs to specific cytoplasmic receptors, the NBPs, which then direct the transported molecule to the nuclear pore (reviewed...
Movement Protein-Plasmodesmata Interaction

P30-Induced Increase in Plasmodesmal Permeability

Experiments show that P30 (and movement proteins of other plant viruses) interact with plasmodesmata to increase their size exclusion limit (Wolf et al., 1989; Derrick et al., 1992; E. Waigmann, W. Lucas, V. Citovsky, P. Zambryski, unpublished data). Originally, this increase in plasmodesmal permeability was assessed by microinjection of fluorescently labeled dextrans of increasing molecular mass into leaf mesophyll cells of transgenic tobacco plants that constitutively express P30 (Wolf et al., 1989). This study showed that the plasmodesmal size exclusion limit in these transgenic plants is higher (5–6 nm) than that in wild-type tobacco plants (1.5 nm). In a different system, microinjection of tobacco rattle virus into leaf trichomes also caused an increase in plasmodesmal permeability (Derrick et al., 1992).

Recently, P30 interaction with plasmodesmata was further examined by co-injection of purified P30 and fluorescent dextrans into leaf mesophyll of wild-type tobacco plants (E. Waijmann, W. Lucas, V. Citovsky, P. Zambryski, unpublished data). Microinjected P30 increased plasmodesmal permeability to 6 to 9 nm, higher than that observed in P30 transgenic plants (5–6 nm). More importantly, the large fluorescent dextrans appeared as far as 10 to 20 cells away from the site of injection (E. Waijmann, W. Lucas, V. Citovsky, P. Zambryski, unpublished data). This result has two potential explanations. (a) Microinjected P30 itself may move between cells to affect plasmodesmal permeability in noninjected cells. (b) Alternatively, microinjected P30 may trigger a putative intercellular signal transduction pathway that increases the plasmodesmal size exclusion limit in numerous interconnecting cells.

The mechanism by which P30 increases plasmodesmal permeability is unknown. Previous models proposed asymmetrical redistribution of plasmodesmal protein particles (Lucas et al., 1990; Deom et al., 1992) and/or change in their shape (Robards and Lucas, 1990). Recent observations suggest a third possibility. Filaments were shown to connect plasmodesmal protein particles of the outer leaflet of the appressed ER to the particles in the center of the ER (Fig. 2) (Ding et al., 1992b). It is tempting to speculate that these protein particles can slide in and out of the membrane to increase or decrease the size of the permeable microchannel between the particles (Fig. 2); this movement may be modulated by the filamentous structures attached to the particles.

The P30-induced increase in the size of plasmodesmal channels (5–9 nm) (Wolf et al., 1989; E. Waigmann, W. Lucas, V. Citovsky, P. Zambryski, unpublished data) is consistent with transport of 2-nm-wide P30-nucleic acid complexes (Citovsky et al., 1992a). Thus, these data support the current view that P30-induced modification of plasmodesmata is a prerequisite for virus movement. However, the increase in plasmodesmal permeability as revealed by cell-to-cell movement of fluorescent dextrans may be only part of the story. As mentioned, transport through the nuclear pore may be functionally similar to transport through plasmodesmata. Early experiments measuring diffusion of microinjected dextrans determined the size exclusion limit of the nuclear pore as 40 kD (Paine, 1988). Recent studies, however, suggest that even smaller endogenous nuclear proteins, such as 21-kD H1 histones, do not diffuse through the nuclear pore but are imported by an active NLS-dependent mechanism (Breeuwer and Goldfarb, 1990). By this analogy, endogenous macromolecules (as opposed to microinjected dextrans) may not diffuse through the enlarged plasmodesmata. Instead, cell-to-cell transport is an active process mediated by specific plasmodesma localization signals.

P30 Phosphorylation by a Cell Wall-Associated Protein Kinase

Because plasmodesmal transport of P30-TMV RNA complexes probably involves an increase in the size exclusion limit of plasmodesmata, P30 most likely interacts with plasmodesmal components. Although interaction of P30 with specific plasmodesmal proteins has not yet been proven, a cell wall-associated protein kinase from tobacco, a potential plasmodesmal component, directly interacts with P30. This Ser/Thr-specific protein kinase phosphorylates P30 at its carboxyl terminal Ser258, Thr261, and Ser265 residues. Interestingly, these P30 phosphorylation sites do not correspond to...
any known consensus phosphorylation sites for protein kinases (Citovsky et al., 1993).

The cell wall-associated protein kinase activity is organ specific, is present mainly in leaves but only marginally in stems, and is absent in roots and apical buds. In addition, this enzymic activity is developmentally expressed, closely paralleling basipetal (tip-to-base) leaf maturation (Citovsky et al., 1993). This basipetal pattern of development also has been described for formation of secondary (branched) plasmodesmata in tobacco leaves (Ding et al., 1992b). Because the protein kinase activity correlates with secondary plasmodesmata development, this cell wall-associated protein kinase may represent a functional component of secondary plasmodesmata. Possible association of the protein kinase activity with secondary plasmodesmata is further supported by the recent observation that P30, a substrate for this cell wall-associated protein kinase (Citovsky et al., 1993), is specifically and irreversibly accumulated in secondary plasmodesmata in P30 transgenic plants (Ding et al., 1992a). Potentially, P30 phosphorylation is involved in its specific deposition in secondary plasmodesmata.

What biological role, then, does P30 phosphorylation have for the virus and/or for the host plant? To address this question, one should consider the adverse effects of P30 on the physiology of the host plant. For example, the P30-induced increase in plasmodesmal permeability may alter intercellular communication, an important biological process. Thus, inactivation or attenuation of P30 activity may be critical for survival of the host plant and, in turn, of the virus; to this end, phosphorylation may function to deactivate P30 by sequestering it to plant cell walls (Fig. 3). The following observations support this hypothesis. (a) Young apical leaves lack the protein kinase activity responsible for P30 phosphorylation (Citovsky et al., 1993). In P30 transgenic plants, these young leaves were found to accumulate P30 in the soluble fraction (Deom et al., 1990). (b) In contrast, mature leaves with the highest levels of P30 phosphorylation (Citovsky et al., 1993) efficiently accumulate P30 in their cell walls (Deom et al., 1990). (c) In wild-type plants, young apical leaves, potentially unable to sequester P30 by phosphorylation, are more susceptible to virus infection (reviewed by Culver et al., 1991). Thus, the confinement of P30 to secondary plasmodesmata may be mediated by a protein kinase that specifically associates with these intercellular connections.

LOOKING AHEAD

Over the next few years, the use of plant virus movement proteins will hopefully enrich our knowledge of plasmodesmal transport. As research continues, cellular proteins potentially involved in this transport pathway will be identified and characterized. The elucidation of the cell-to-cell transport mechanisms will allow us to address many critical questions in plant biology. For example, analogous to animal systems, changes in plasmodesmal transport may create compartments in which cells communicate with each other but not with cells from other compartments within the same tissue; such “communication” compartments may function during plant development and morphogenesis. On the applied level, knowledge of plasmodesmal transport may lead to novel approaches for production of agronomically important virus-resistant plants. Thus, the multifaceted attack now in progress on the structure of plasmodesmata and plasmodesmal transport pathways will provide new and exciting answers for the question of how plant cells communicate with each other.

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