Lipid binding activities of flax rust AvrM and AvrL567 effectors

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E ffectors are pathogen-encoded proteins that are thought to facilitate infection by manipulation of host cells. Evidence showing that the effectors of some eukaryotic plant pathogens are able to interact directly with cytoplasmic host proteins indicates that translocation of these proteins into host cells is an important part of infection. Recently, we showed that the flax rust effectors AvrM and AvrL567 are able to internalize into plant cells in the absence of the pathogen. Further, N-terminal sequences that were sufficient for uptake were identified for both these proteins. In light of the possibility that the internalization of fungal and oomycete effectors may require binding to specific phospholipids, the lipid binding activities of AvrM and AvrL567 mutants with different abilities to enter cells were tested. While AvrL567 was not found to bind to phospholipids, AvrM bound strongly to phosphatidyl inositol, phosphatidyl inositol monophosphates and phosphatidyl serine. However, a fragment of AvrM sufficient to direct uptake of a fusion protein into plant cells did not bind to these phospholipids. Thus, our results do not support the role of specific binding of AvrM and AvrL567 to phospholipids for uptake into the plant cytoplasm.

Eukaryotic pathogens, including fungi and oomycetes, secrete effector proteins that are subsequently delivered into host cells to facilitate infection.13 Many oomycete effector proteins contain an N-terminal RxLR motif required for delivery into host cells5 and are able to enter plant cells in the absence of the pathogen.6 However, the mechanism of RxLR protein uptake into cells has yet to be determined. In our recent study,7 the AvrM and AvrL567 effectors from the biotrophic flax rust fungus were also shown to enter plant cells in a pathogen-independent process. Further, through deletion analysis, minimum regions of each protein required for uptake were identified.7 Unlike the oomycete RxLR effectors, no motif was found to be strongly conserved between the uptake regions of these two effectors or in other rust effectors.7

A number of proteins with the ability to cross membranes are known and include the HIV Tat protein8,9 and the penetratin homeodomain of the transcription factor Antennapedia.10,11 Through deletion studies it was shown that uptake of these proteins is mediated by small regions of each protein, less than 30 amino acids in size, known as cell penetrating peptides (CPPs). These sequences can also mediate uptake of attached cargoes, including low molecular weight drugs,12 proteins,13 nucleic acids14 and peptides.15 CPPs have diverse sequences and structures and studies on their uptake show that internalization can occur by different mechanisms—possibly even simultaneously—including clathrin-dependent endocytosis,16 lipid raft-mediated macropinocytosis,17 clathrin- and caveolin-independent endocytosis18 and direct membrane penetration.19 In the case of the amphipathic penetratin peptide, membrane penetration may occur by integration into phospholipid membranes, with two critical tryptophan residues penetrating into the lipid bilayer and positively charged side chains associating non-specifically with negatively charged phospholipids, disrupting the membrane and possibly facilitating the formation...
of small vesicles or inverted micelles within the membrane before release into the cytoplasm.19-25 On the other hand, for endocytic transport, penetratin may be internalized into plant cells.

Lipid Binding Specificities of AvrM and AvrL567

Based on primary sequence analysis, no lipid binding domains were identified in AvrM or AvrL567. The majority of lipid binding domains lack primary sequence similarities or motifs,27 although they are often characterized by the presence of important positively charged residues. Therefore, the binding activity of both proteins to a range of lipids found in biological membranes was experimentally tested. A lipid blot consisting of 100 pmol of immobilized lipid.

To explore the possibility that lipid binding might be required for effector uptake into plant cells, GST-fusions to uptake mutants identified in our earlier study7 were analyzed for lipid binding activity. In the case of AvrL567, both a C-terminal fragment lacking amino acids 26–37, which are required for cell internalization,7 and an N-terminal fragment consisting of residues 26–50 also showed no binding to phospholipids above background (Fig. 2A). As we previously showed that a protein fusion consisting of amino acids 1–50 (including the signal peptide) was sufficient for uptake,7 this result is not consistent with a role for specific phospholipid binding in entry of AvrL567 into cells. Because AvrM showed its strongest binding towards PtdIns(4,5)P2, when detected using an anti-GST antibody28 and the negative control, His-tagged GFP, demonstrated no binding when probed with anti-GFP as expected.

The above experiments used different antisera to detect protein bound to lipid blots. To allow direct comparison of lipid binding of both effector proteins and detection of lipid binding of truncated fragments of these effectors, proteins were expressed as N-terminal GST fusions and binding was detected using anti-GST antibodies. At 6.5 nM, the AvrM-GST fusion showed similar phospholipid binding to that of the wild-type protein (Fig. 2A). Similarly, AvrL567-GST at 175 nM was found to have the same binding pattern as protein lacking the GST tag. However, at 175 nM, the negative control of unmodified GST showed a similar binding pattern to that of AvrL567, indicating that AvrL567 binding was close to background (Fig. 2A).

Lipid Binding Activities of AvrM and AvrL567 Uptake Mutants

1. Figure 1. Binding of His-tagged AvrM and AvrL567 to lipids. Pip strips from Echelon Biosciences were blocked with 4% non-fat milk/PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4), incubated with purified proteins in blocking buffer overnight at 4°C and washed three times for 10 min each in PBST [PBS + 0.1% (v/v) Tween-20]. The Pip2 grip protein (Echelon Biosciences) was used as a positive control and GFP was used as a negative control. Bound AvrM, AvrL567, Pip2 grip and GFP were detected with anti-AvrM, anti-AvrL567, anti-GST28 and anti-GFP (Roche Diagnostics Ltd., Mannheim, Germany) antibodies, respectively. Blots were rinsed, incubated with anti-rabbit IgG-peroxidase conjugate (A9169, Sigma) and bound proteins were detected using the Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical). All proteins were used at 0.5 µg/µl with the exception of AvrM which was used at 0.25 µg/µl. Compounds spotted on the membrane: 1: lysophosphatidic acid; 2: lysophosphatidylinositol phosphate (PtdInsP) head groups, which may act as receptors initiating effector endocytosis into cells.26 Here, we present data on the lipid binding activities of AvrM and AvrL567 in the context of their internalization into plant cells.

2. Table 1. Lipid Binding Specificities of AvrM and AvrL567

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<tr>
<th>Lipid</th>
<th>AvrM-GST</th>
<th>AvrL567-GST</th>
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<tbody>
<tr>
<td>Phosphatidylcholine</td>
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<td>Phosphatidylinositol</td>
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<td>Phosphatidyl ethanolamine</td>
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<td>+</td>
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<tr>
<td>Phosphatidylinositol phosphate</td>
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deletion of residues 106–153, which are required for uptake, substantially reduced but did not completely abolish lipid binding. Thus this region may be required for lipid binding, either directly or through effects on overall protein folding. Indeed this fusion protein expressed very poorly in E. coli, suggesting possible folding defects. However, no lipid binding activity was detected for a GST fusion containing AvrM residues 106–153, indicating that this region is not sufficient for PtdInsP binding. Similarly, residues 29–156 of AvrM, which are sufficient for uptake in planta, also did not show lipid binding. This indicates that the uptake region is not sufficient for AvrM binding to specific phospholipids, which therefore seems to also require the C-terminal effector domain.

Together, these results do not support the conclusion that specific binding to particular phospholipid headgroups is required for pathogen-independent internalization of AvrM and AvrL567 into host cells. This does not rule out a role for direct membrane association of these uptake peptides. Interestingly, the AvrM uptake region shows similarity to the penetratin peptide in that it contains a pattern of alternating hydrophobic and charged residues, so it may also integrate into the lipid bilayer via formation of an amphipathic structure. Conserved hydrophobic positions in the AvrL567 uptake region may function similarly, but it is also possible that different rust effectors adopt different uptake mechanisms.

References


