Cloning of a cDNA Encoding a Plasma Membrane-Associated, Uronide Binding Phosphoprotein with Physical Properties Similar to Viral Movement Proteins

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Oligogalacturonides are structural and regulatory homopolymers from the extracellular pectic matrix of plants. In vitro micromolar concentrations of oligogalacturonates and polygalacturonates were shown previously to stimulate the phosphorylation of a small plasma membrane-associated protein in potato. Immunologically cross-reactive proteins were detected in plasma membrane-enriched fractions from all angiosperm subclasses in the Cronquist system. Polygalacturonate-enhanced phosphorylation of the protein was observed in four of the six dicotyledon subclasses but not in any of the five monocotyledon subclasses. A cDNA for the protein was cloned from potato. The deduced protein is extremely hydrophilic and has a proline-rich N terminus. The C-terminal half of the protein was predicted to be a coiled coil, suggesting that the protein interacts with other macromolecules. The recombinant protein was found to bind both simple and complex galacturonides. The behavior of the protein suggests several parallels with viral proteins involved in intercellular communication.

INTRODUCTION

Unlike many types of animal cells, plant cells are surrounded by a massive extracellular matrix that defines their shape and prohibits their movement within the organism. Like the extracellular matrices of animal cells, plant cell walls contain important regulatory information necessary for the control of defense and developmental gene expression (Aldington and Fry, 1993; Coté and Hahn, 1994) and probably for cell fate determination (Berger et al., 1994). Among the regulatory molecules of the plant cell wall, the best characterized are the oligogalacturonic acids (OGAs) and polygalacturonic acids (PGAs). These molecules form a quantitatively important part of the pectic matrix (O’Neill et al., 1990) and are probably the most abundant polymeric regulatory molecules on the earth’s surface.

Originally, OGAs were discovered to be defense gene regulators (Darvill and Albersheim, 1984; West et al., 1984; Ryan, 1988); more recent evidence also suggests that they have a role in development, including morphogenesis (Mohnen et al., 1990). In particular, it seems that OGAs can affect several processes influenced by or depending on the hormone auxin (Bellincampi et al., 1996). Most OGA-responsive genes are sensitive only to a narrow range of OGAs whose degree of polymerization (DP) is between 10 and 15 (Ryan, 1988). It is likely that as a result of size and charge, larger OGAs (of DP > 15) do not easily penetrate the plant cell wall under experimental conditions. Much regulatory information residing in the plant extracellular matrix almost certainly remains undiscovered as a result of this restraint (Reymond et al., 1995). Currently, all that is clear is that there is no single OGA regulator but rather a spectrum of unmodified and modified OGA signals.

Although the biological activity of OGAs has been characterized in many systems (reviewed in Coté and Hahn, 1994), little is known about how signaling from these molecules leads to gene expression. In common with many other classes of defense gene elicitors (Nürnberger et al., 1994), OGAs are known to stimulate K⁺ efflux and increase cytosolic [H⁺] and [Ca²⁺] in plant cells (Thain et al., 1990; Mathieu et al., 1991). A G protein has been implicated in OGA signal transduction (Legendre et al., 1992), as have inositol phosphates (Messiaen and Van Cutsem, 1994). With the diverse effects of exogenous OGAs on defense and development, the possibility for multiple signal pathways exists.

Binding sites for oligouronides appear to be of very low abundance on the plant cell surface (Diekmann et al., 1994). Currently, no receptors for OGAs are known, and their
discovery remains a challenge. The fact that OGAs form intermolecular complexes in solution hinders biochemical approaches designed to characterize binding sites because labeled pectic ligands bind to themselves (Kohn, 1985). Many types of OGA/pectin binding proteins are expected to exist in the plant cell. These include various proteins and enzymes anchored to the pectic matrix, chaperone-like proteins involved in wall assembly or rearrangement, receptors for uronide signals, and perhaps other proteins involved in cell-to-cell communication. In all these cases, it would be interesting to isolate and characterize such uronide binding proteins from plant tissues.

We have been studying the interaction of OGAs and PGAs with proteins in isolated plasma membranes. Galacturonides, at micromolar concentrations, enhance the in vitro threonine phosphorylation of a small protein (pp34) that copurifies with potato and tomato plasma membranes (Farmer et al., 1989, 1991). Large OGAs with a DP > 13 are necessary to stimulate the phosphorylation of this protein in vitro (Reymond et al., 1995). The OGA size requirement for stimulated phosphorylation, together with its association with the plasma membrane, suggests that the protein may play an as yet unknown role in signal transduction for larger uronides. We studied the distribution of the polypeptide and its in vitro phosphorylation in all of the angiosperm subclasses defined by Cronquist (1988). The results obtained encouraged us to attempt to clone the protein with a view to investigating its potential direct interaction with uronides. The physical properties of the protein were similar to those of viral cell-to-cell movement proteins, leading us to speculate that the protein somehow may be involved in intercellular communication.

RESULTS

PGA Enhances the In Vitro Phosphorylation of a Protein from Numerous Dicotyledonous Plants

The OGA/PGA-enhanced phosphorylation of a plasma membrane-associated protein allows the investigation of pectic matrix–cell surface interactions. This rapid and simple assay based on labeling proteins with radioactive phosphate permits the study of the interaction of any uronide, modified or unmodified, with the plasma membrane (Farmer et al., 1991). The PGA-stimulated in vitro phosphorylation of plant plasma membrane-associated proteins has been reported in only three species to date: tomato and potato from the subclass Asteridae (Farmer et al., 1989, 1991) and soybean from the widely separate subclass Rosidae (Reymond et al., 1995).

We decided to test whether this phenomenon could be observed in plasma membrane preparations isolated from throughout the Angiospermae. Therefore, we extended our search to the other four dicotyledon (Magnolopsid) subclasses and the five monocotyledon (Liliopsid) subclasses. In each case, we chose at least one plant per subclass and attempted to prepare plasma membranes from at least one tissue type. We probed the plasma membrane preparations with the anti-potato pp34 antibodies produced previously in rabbit granuloma fluid (Jacinto et al., 1993). This was designed to indicate whether the protein occurred in a particular plasma membrane preparation. We also tested each membrane batch for enhanced in vitro phosphorylation in the presence of tomato leaf PGA. Although not fully characterized, this preparation is the uronide that is most active in the phosphorylation assay in potato and tomato plasma membranes (Farmer et al., 1991; Reymond et al., 1995).

Results of a typical experiment are shown in Figure 1. In this case, plasma membrane preparations from potato and buttercup leaves were shown to contain one major protein cross-reacting with the anti-potato pp34 antibodies (Figure 1C) as well as the PGA-enhanced phosphorylation (quantitated with a Molecular Imager; Figure 1B) of one protein of the same apparent molecular mass, ~34 kD in the case of potato and ~31 kD in the case of buttercup (Figure 1A). All dicotyledon subclasses were found to contain proteins cross-reacting with the anti-potato pp34 antibodies, as described in Table 1. However, in the case of endive (Cichorium endivia, subclass Asteridae), no cross-reactivity with the anti-potato pp34 antibodies was observed. In Arabidopsis, a very weak cross-reactivity was observed in leaf plasma membranes, whereas a stronger signal at ~36 kD was detected in inflorescence plasma membrane preparations. The apparent masses of dicot plasma membrane proteins detected by the anti-potato pp34 antibodies fell within a narrow range of ~30 to 36 kD. In all five monocotyledon subclasses, cross-reactivity with anti-potato pp34 antibodies was detected at apparent masses between 30 and 32 kD.

Hoping to detect the presence of proteins cross-reacting with the anti-potato pp34 antibodies in all angiosperm subclasses, we were curious to determine whether PGA-enhanced phosphorylation of these proteins was equally widespread. We observed PGA-enhanced protein phosphorylation in plasma membranes from the four dicotyledon subclasses, Magnoliidae, Hamamelidae, Rosidae, and Asteridae, but not in subclasses Dilleniidae and Caryophyllidae (Table 1). PGA-stimulated phosphorylation was quantitated by using a Molecular Imager. Between 1.5- and 8.4-fold increases in specific protein phosphorylation were observed (Table 1). In every case but one, endive, the apparent mass of the phosphorylated protein correlated with that of the protein recognized by the anti-potato pp34 antibodies. In endive, no cross-reactive proteins were detected. In all monocotyledonous representatives tested, spanning a large evolutionary spectrum of plants, no PGA-enhanced in vitro protein phosphorylation was observed.

Cloning Remorin

To clone the cDNA encoding the potato plasma membrane protein, we purified the polypeptide by following a previously established protocol (Jacinto et al., 1993). The purification be-
gan with 7 kg of potato leaves, from which 63 mg of plasma membrane (protein) was isolated by two-phase partitioning. The final yield of protein was too low to permit accurate quantitation; however, when comparing the density of known amounts of Coomassie blue–stained BSA with that of the protein, we estimated a recovery of ~4 to 8 μg. Initial N-terminal sequencing was not possible as a result of blocking (data not shown), and the remaining polypeptide (~150 pmol) was used for internal sequencing. Eight peptides were successfully sequenced (Figure 2A). Peptide sequences were then used to design polymerase chain reaction (PCR) primers that permitted us to isolate a 330-bp DNA fragment from a potato leaf cDNA library. Using this PCR fragment as a probe, a full-length cDNA was isolated from the same library. The correct cloning of the cDNA was established by aligning sequenced peptides with the cDNA: the eight peptides represent 41% of the protein (Figure 2A). Furthermore, the protein expressed in Escherichia coli was recognized by the anti–potato pp34 antibodies (Figure 3).

The potato cDNA (Figure 2A) predicts a protein of 198 amino acids, with a calculated mass of 21,769 D. The protein is particularly rich in glutamic acid (16.7%) and lysine (17%) residues. Of the first 50 N-terminal residues, 11 (22%) are prolines. The predicted pl of 6.14 agrees with our own measurement of the potato leaf protein (data not shown). Notable features predicted from the cDNA are four extremely hydrophilic domains (Figure 2B). No signal sequence for cotranslational insertion into the endoplasmic reticulum is present. No features indicative of transmembrane domains or membrane anchor signature sequences were predicted, and no sequences indicative of organellar localization were detected. The protein sequence contains no N-glycosylation sites but has several potential serine and threonine phosphorylation sites. The C-terminal half of the protein is predicted to be a coiled coil. The protein contains nine partially conserved sequences of seven amino acids based on the sequence kA[E/D]ekak, where uppercase letters represent conserved residues and lowercase letters represent the most common amino acid found in the sequence. The full-length cDNA was found to be homologous to a cDNA for an Arabidopsis DNA binding protein (Dbp; Alliotte et al., 1989). The two proteins share 67% amino acid identity and 80% similarity (Figure 4). The protein is also homologous to a rice expressed sequence tag. Comparison of the predicted sequence of the potato protein with Arabidopsis Dbp and the rice expressed sequence tag (Figure 4) indicates particularly high homology in the central region of the proteins. This region is very hydrophilic and contains several pairs of charged residues. Like the Arabidopsis Dbp, our protein migrates anomalously ~12 kD above its predicted mass.

We propose the name remorin for this potato protein. A remora is a fish that attaches itself to the surface of other larger organisms; the name alludes to the interaction of the protein with the plasma membrane and with uronides (discussed below) but does not imply a function. The designation used previously, pp34, is no longer useful because it suggests a mass much higher than the calculated mass (21.8 kD) and
Table 1. Presence and PGA-Stimulated Phosphorylation of Remorin in Angiosperms

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Family</th>
<th>Species</th>
<th>Tissue</th>
<th>Anti-pp34 Cross-Reactivity</th>
<th>Relative Mass (kD)</th>
<th>PGA-Stimulated Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnoliidae</td>
<td>Ranunculaceae</td>
<td>Ranunculus ficaria</td>
<td>L</td>
<td>Yes</td>
<td>31</td>
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<tr>
<td>Hamamelidae</td>
<td>Fagaceae</td>
<td>Fagus sylvatica</td>
<td>L</td>
<td>Yes</td>
<td>31</td>
<td>3.8</td>
</tr>
<tr>
<td>Caryophyllidae</td>
<td>Chenopodiaceae</td>
<td>Spinacia oleracea</td>
<td>L</td>
<td>Yes</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Dilleniidae</td>
<td>Brassicaceae</td>
<td>Arabidopsis thaliana</td>
<td>L</td>
<td>Weak</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Rosidae</td>
<td>Fabaceae</td>
<td>Pisum sativum</td>
<td>L</td>
<td>Yes</td>
<td>34</td>
<td>8.4</td>
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<tr>
<td>Asteridae</td>
<td>Solanaceae</td>
<td>Solanum tuberosum</td>
<td>L</td>
<td>Yes</td>
<td>34</td>
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<td></td>
<td>Asteraceae</td>
<td>Cichorium endivia</td>
<td>L</td>
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<td>32</td>
<td>2.5</td>
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<tr>
<td>Alismatidae</td>
<td>Hydrocharitaceae</td>
<td>Egeria densa</td>
<td>L</td>
<td>Yes</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Areceae</td>
<td>Arecaceae</td>
<td>Monstera delicosa</td>
<td>L</td>
<td>Yes</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Commelinidae</td>
<td>Gramineae</td>
<td>Zea mays</td>
<td>L</td>
<td>Yes</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Liliidae</td>
<td>Asphodelaceae</td>
<td>Chlorophytum elatum variegatum</td>
<td>L</td>
<td>Yes</td>
<td>31</td>
<td>0</td>
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<tr>
<td>Zingiberidae</td>
<td>Marantaceae</td>
<td>Ctenanthe oppenheimiana</td>
<td>L</td>
<td>Yes</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

a For growth conditions, see Methods. L, leaf; I, inflorescence.

b The antibody used is characterized in Jacinto et al. (1993).

c Relative mass of a cross-reactive polypeptide that was recognized by the anti–potato pp34 antibody on an immunoblot. In all cases except for Cichorium endivia, the cross-reactive band corresponded to the PGA-stimulated phosphoprotein.

d Fold increase in phosphorylation in the presence of tomato leaf PGA.

Remorin Binds Uronides

To understand the uronide-enhanced in vitro phosphorylation of remorin, we decided to test whether the interaction between uronides and the protein was direct or indirect. To test for a direct interaction between pectic polysaccharides and remorin, we developed a binding assay based on biotinylated uronides. We employed a homogeneous OGA with a DP of 20, purified according to Spiro et al. (1993), and a fractionated pectin (G-50 PIIF; Bishop et al., 1984). These uronides were labeled at the reducing ends with biotin and purified by affinity chromatography on avidin columns to remove unlabeled uronides (see Methods). The number of biotin molecules per uronide was not quantitated, but it is likely to be one because labeled OGAs retain their discrete mobilities close to the parent OGA in gel electrophoresis (data not shown). Remorin was expressed as a fusion protein with glutathione S-transferase (GST) in E. coli (Figure 5A). To test whether GST-remorin could bind uronides, the fusion protein–containing bacterial extracts were electrophoresed, blotted onto nitrocellulose, and probed with a biotinylated pectic fragment. Biotin–horseradish peroxidase does not bind to GST-remorin fusion protein, nor does biotin displace biotinylated OGA bound to GST-remorin (data not shown).

Figure 5 shows that GST-remorin binds to both eicosagalacturonic acid (Figure 5B, lane 1) and fractionated pectin (Figure 5B, lane 2). As a control, extracts containing overexpressed GST were also probed in the same manner with the biotinylated pectic fragment (Figure 5B, lane 3). No uronide binding proteins were found in these extracts. GST-remorin was not labeled when protein blots were treated simply with avidin–horseradish peroxidase in the absence of ligand (Figure 5B, lane 4; see Methods). To demonstrate clearly that labeled uronides bind to GST-remorin in the absence of other proteins, we performed a similar experiment with purified GST-remorin. The fusion protein was probed with biotinylated fractionated pectin. This ligand bound to purified GST-remorin (Figure 6), whereas no signal was detected with purified GST.

Table 2 presents the results of preliminary competition studies that were performed in an effort to begin to understand the nature and specificity of ligand binding to remorin. Ligands chosen as competitors were a series of uronides that included tri-α-1,4-galacturonic acid and poly-β-1,4-mannuronic acid, which are inactive in stimulating the phosphorylation of remorin (Farmer et al., 1991; Reymond et al., 1995), as well as poly-L-α-1,4-galacturonic acid, a molecule closely related to poly-α-1,4-galacturonic acid. A number of other polyanions were tested, including carboxymethylcellulose, poly-L-glutamate, and heparin. Molecules that were ineffective in competing with fractionated pectin were poly-α-1,4-galacturonic acid, tri-α-1,4-galacturonic acid, poly-β-1,4-mannuronic acid, carboxymethylcellulose, and poly-L-glutamate. Fractionated pectin and the...
Recombinant Remorin Is Recognized by the Anti-Potato pp34 Antibodies.

A protein gel blot was developed with the anti-potato pp34 antibodies. Lane 1 contains the positive control, potato plasma membrane; lane 2, extract from E. coli-containing expression vector pGEX2T; lane 3, the E. coli-expressing remorin-GST fusion in pGEX2T. In all cases, 20 μg of protein was probed. The arrowheads show cross-reactive remorin.

DISCUSSION

The uronide-stimulated phosphorylation of plasma membrane proteins may provide useful clues to the nature of the interaction of parts of the plant extracellular matrix with plasma membrane-associated proteins. Is the uronide-stimulated phosphorylation of proteins widespread, or is it an exception observed in a few distinct species? In this study, we observed the stimulated in vitro phosphorylation of remorin-like proteins in plasma membranes isolated from four subclasses of dicotyledons: Asteridae, Rosidae, Magnoliidae, and Hamamelidae (Table 1). In only two subclasses of dicotyledons, Dilleniidae and Caryophyllidae, were we unable to detect PGA-enhanced phosphorylation. Thus, the interaction between uronides and remorin is widely conserved in dicotyledonous plants.

How do these results correlate with what we know of biological responses to uronides in plants? Biological responses to OGAs have been reproduced in several of the six

**Figure 3.** Recombinant Remorin Is Recognized by the Anti-Potato pp34 Antibodies.

A protein gel blot was developed with the anti-potato pp34 antibodies. Lane 1 contains the positive control, potato plasma membrane; lane 2, extract from E. coli-containing expression vector pGEX2T; lane 3, the E. coli-expressing remorin-GST fusion in pGEX2T. In all cases, 20 μg of protein was probed. The arrowheads show cross-reactive remorin. Numbers at left refer to molecular mass standards (in kilodaltons).
Figure 4. Remorin Is Related to the Arabidopsis Dpb and to a Rice Expressed Sequence Tag.

Sequence alignment of the Arabidopsis Dpb (Alliotte et al., 1989) and a rice expressed sequence tag (rice EST; GenBank accession number D40002) with potato remorin are shown. Identical amino acids are boxed with black squares, and conserved amino acids are shown in lowercase letters. The asterisk indicates the end of the partial rice expressed sequence tag. Dashes indicate gaps to maximize alignment.

subclass of dicotyledonous plants: Asteridae, Rosidae, and Dilleniidae (Coté and Hahn, 1994). In the case of Dilleniidae, biological responses to uronides have been characterized in cucumber (Robertson and Svalheim, 1990) and cotton (Davis and Essenberg, 1995). Currently, we are unaware of any published data concerning the biological effects of OGAs on Arabidopsis. Failure to observe PGA-enhanced phosphorylation of the protein in Arabidopsis (Table 1) and in a second species from the Dilleniidae, cauliflower (data not shown), may reflect genuine differences in the physiology of these plants from other representatives of the Dilleniidae and other subclasses. However, this seems to be unlikely and needs further investigation. Trivial reasons, such as suboptimal assay conditions and/or lability of the system, are equally plausible. The uronide ligand we used, tomato leaf PGA, was probably an appropriate choice because the Arabidopsis cell wall, like that of tomato, appears to be a typical type I wall (Zablackis et al., 1995).

The other dicotyledon subclass in which no PGA-stimulated in vitro phosphorylation was observed was the Caryophyllidae; we chose spinach as representative. Spinach leaves contain a protein of apparent mass ~30 kD that is recognized by the anti-pp34 antibodies (Table 1). We are unaware, however, of any reports of biological activity of OGAs in the Caryophyllidae. In summary, it is clear that remorin-like proteins are widespread in dicotyledons and that in four of six dicotyledon subclasses, in vitro PGA-enhanced phosphorylation of this protein was observed.

After examining the presence proteins that cross-react with the anti-pp34 antibodies and their phosphorylation in dicotyledons, we turned to the other major division of the angiosperms, the monocotyledons. Proteins that cross-react with the anti-potato pp34 antibodies are found in each monocotyledon subclass (Table 1); however, no PGA-enhanced protein phosphorylation was observed in any monocotyledon-derived plasma membrane. Our choice of monocotyledons representing all five subclasses in the Cronquist system included plants as diverse as dense-leaved pondweed, Egeria densa (subclass Alismatidae), representing more ancient monocotyledons, and maize (subclass Commelinidae), a modern monocotyledon with a type II cell wall. We also tested a second ligand, maize leaf PGA (from a type II cell wall), in the phosphorylation assay. This preparation was inactive in our assays of monocotyledon-derived plasma membranes but functioned in dicotyledon plasma membranes (data not shown).

In monocotyledons, no confirmed biological responses to OGAs have been reported. Thus, from our perspective, it appears that there may be real differences between monocotyledons and dicotyledons regarding in vitro and in vivo responses to OGAs and PGAs. Monocotyledons, however, have
almost certainly received less attention than dicotyledons with respect to signal transduction studies, and it is premature to conclude that monocotyledons will not respond to exogenous OGAs. Moreover, biological activity of OGAs has been reported in the Gymnospermae (Asiegbu et al., 1994), and it will be worthwhile to test for the presence of remorin in these plants.

In summary, data on the occurrence of uronide-enhanced remorin phosphorylation in dicotyledons correlate broadly with the fact that to date, only this class of angiosperms is known to display biological responses to these molecules. These considerations suggest a potential role of the remorin in the biological activity of OGAs and convinced us to try to clone the remorin cDNA.

Remorin Is a Hydrophilic, OGA Binding Protein That Is Associated with the Plasma Membrane

A cDNA for remorin was cloned from a potato leaf cDNA library. Because the predicted protein has a mass of 22 kD and the plant protein has a relative mass of 35 kD, we were concerned that we had sequenced another protein. This was not the case because all eight recovered peptides correspond to the deduced remorin sequence, and the protein expressed in E. coli was recognized by the anti–potato pp34 antibodies (Figure 3) as well as by an antibody raised against an internal peptide predicted from the remorin cDNA (data not shown). Another strong indication of correct sequencing is the identical electrophoretic behavior of remorin and Arabidopsis Dbp (Alliotte et al., 1989). Both proteins migrate at ~34 kD, although their predicted mass is ~32 kD.

The hydrophilicity of potato remorin (Figure 2B) is remarkable. This is surprising in view of the relatively tight association of the protein with plasma membranes. Nothing in the sequence data provides clues as to the nature of this association. We have examined the interaction of remorin with plasma membranes in a variety of ways, including washing the membranes with sodium carbonate solutions, which is an established method for removing peripheral proteins (Fujiki et al., 1982). Low concentrations of sodium carbonate (10 mM) were insufficient to solubilize the protein, whereas 100 mM sodium carbonate solubilized ~30% of the protein (data not shown). We would have expected a higher efficiency of solubilization of the protein by sodium carbonate if it were a peripheral membrane protein and somewhat less if the protein were integral (Fujiki et al., 1982). Detergents were found to be the best means of solubilizing remorin: concentrations of at least 0.5% (v/v) Triton X-100 are necessary to remove the protein from the plasma membrane.

A striking feature of remorin is the proline-rich N terminus. The configuration of prolines in this region is reminiscent of sites involved in protein–protein interactions; for example, those found in signaling proteins having SH3 binding domains (Pawson, 1995). In this case, the motif X-P-x-P, where X tends to be an aliphatic residue and p is a semiconserved proline, is found at residues 15 to 19 (APPAP) and 37 to 41 (APALP).
Table 2. Binding of Biotinylated Pectin to GST-Remorin in the Presence of Various Polyanions

<table>
<thead>
<tr>
<th>Polyanion</th>
<th>Binding Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No competitor</td>
<td>100</td>
</tr>
<tr>
<td>Fractionated pectin</td>
<td>45</td>
</tr>
<tr>
<td>Tomato leaf PGA</td>
<td>64</td>
</tr>
<tr>
<td>α-1,4-d-Trigalacturonic acid</td>
<td>100</td>
</tr>
<tr>
<td>α-1,4-L-Polygalacturonic acid</td>
<td>100</td>
</tr>
<tr>
<td>β-1,4-D-Polymannuronic acid</td>
<td>100</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>100</td>
</tr>
<tr>
<td>Poly-L-glutamate</td>
<td>100</td>
</tr>
<tr>
<td>Heparin</td>
<td>0</td>
</tr>
<tr>
<td>Single-stranded DNA (26 nucleotides)</td>
<td>100</td>
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<tr>
<td>DNA</td>
<td>95</td>
</tr>
<tr>
<td>RNA</td>
<td>92</td>
</tr>
</tbody>
</table>

a Binding of biotinylated fractionated pectin (30 nM) to GST-remorin (see Figure 5B) was determined in the presence of a 500-fold excess of competitor. The intensity of the remorin band was integrated by using a Molecular Imager.

b One milligram of low molecular weight DNA from herring.

c One milligram of total yeast RNA.

(Strow 2A). Whether these motifs play any role(s) in the association of remorin with other molecules, for example, signaling proteins, remains to be tested. The C-terminal half of the protein is predicted to be a coiled coil. These structures tend to imply protein–protein interaction (Branden and Tooze, 1991) but may also be involved in binding polysaccharides, such as heparin (Margalit et al., 1993). Based on the predicted structure of remorin (the SH3-like domains [Pawson, 1995] and the coiled coil structure), we believe that the protein interacts with other macromolecules.

To investigate the potential interaction of uronides with remorin, we decided to test the ability of the recombinant protein to bind to oligogalacturonides. During the course of our studies, we never observed an enzymatic activity of remorin toward PGA or pectin-derived polymers; thus, the protein is unlikely to degrade the galacturonic acid–containing polymers we used as probes. Binding of biotinylated uronide fragments to the GST–remorin fusion was observed (Figure 5B). We chose two ligands that represented either simple OGAs (a fragment with a DP of 20, eicosagalacturonic acid) or fractionated pectin (Bishop et al., 1984), a molecule likely to be representative of structures existing in the extracellular matrix. A purified GST–remorin fusion protein also binds uronides (Figure 6), indicating that other components of the E. coli extracts are not responsible for the binding.

Initial competition studies were performed with crude bacterial extracts containing the GST–remorin fusion protein. The results obtained (Table 2) were complex, and only limited conclusions can be drawn. Tomato leaf PGA and fractionated pectin were only partially effective in reducing the binding of the biotinylated pectic probe. Why were these ligands not better competitors? The reason is not clear, but the most probable explanation comes from the established fact that OGAs readily interact with themselves to form intermolecular complexes (Kohn, 1985). If intermolecular complex formation occurs in the binding assays, the competing ligand might interact with the bound label without displacing it. That is, the remorin itself may bind to intermolecular PGA complexes as well as to the free ligand. The possibility that intermolecular complexes of OGA are themselves regulatory ligands has already been proposed (Farmer et al., 1991).

Heparin seems to be a strong competitor for the pectic ligand. Direct interaction between heparin and remorin would be consistent with a previous report of the heparin in stimulated in vitro phosphorylation of remorin (Farmer et al., 1991). A PGA binding peroxidase was recently shown to bind tightly to heparin, whereas other peroxidases that did not bind to PGA also did not bind to heparin (Penel and Greppin, 1996).

As demonstrated previously with the Dbp protein (Alliotte et al., 1989), labeled DNA was found to bind to remorin (data not shown). The failure of DNA and RNA to displace labeled uronides (Table 2) may suggest that remorin has two separate binding sites for these molecules, but again, the complexity of binding studies as a result of ligand–ligand interaction (Kohn, 1985) necessitates further work. The copurification of remorin with plasma membranes from all subclasses of angiosperms (Table 1), together with our inability to detect the protein in potato and tomato nuclei by immunoblotting (data not shown), leads us to expect that the principal cellular pools of remorin in the tissues we have examined are unlikely to be associated with DNA. In summary, the competition results provide a correlation between the ability of closely related molecules to stimulate in vitro phosphorylation of remorin and their ability to bind the recombinant protein. Most importantly, this study indicates that the interaction between remorin and OGAs is direct. The direct binding of OGA to remorin was not expected because typically it is protein kinases that are ligand-activated, not their substrates. Although the significance of ligand-stimulated phosphorylation of remorin is not known, there is no reason that such mechanisms may not exist or play roles in regulation. Several possible mechanisms whereby OGA binding might lead to increased phosphorylation of remorin can be imagined. OGA might cause a conformational change in remorin, leading to its interaction with a protein kinase and subsequent phosphorylation. We cannot rule out the simultaneous binding of OGA to remorin and to its kinase, which at present remains uncharacterized.

How does remorin bind uronides? To examine whether the predicted remorin protein sequence provided clues, we compared this sequence with the crystal structure of a protein known to bind PGA: pectate lyase C (PelC) from the bacterial plant pathogen Erwinia chrysanthemi (Yoder and Jurnak, 1995).
remorin  

\[
\text{KKVE-34-KEKE-21-KK-16-K5-K-2E-2-}
\]

PeIC  

\[
\text{D-2-R-5-KVKK-36-EIKE-22-KK-18-K5-R-1-D-25-}
\]

remorin  

\[
\text{E-10-K-19-KK-30-8-R-21-K}
\]

PeIC  

\[
\text{D-9-K-16-KK-27-8-R-4-R-21-R}
\]

Figure 7. Alignment of the PelC PGA Binding Residues and Predicted Remorin Amino Acid Sequence Shows Similarity. 

Amino acid residues thought to be involved in uronide binding in PeIC (Yoder and Jurnak, 1995) are compared with residues in the remorin primary sequence that show similarity in spacing. Otherwise, PeIC and remorin show no overall sequence homology.

Residues thought to be important for uronide binding have been identified in this enzyme. We were surprised to find a similar distribution of these residues in remorin and in the PelC PGA binding groove (Figure 7). Although the two proteins show no obvious sequence homology, the charged residue placement and spacing indicate that these residues may represent convergently evolved uronide binding sites. Directed mutagenesis of the remorin sequence, together with structural studies, is necessary to test whether the charged residues indicated in Figure 7 are dispensable for the binding of uronides to remorin. It is noteworthy that PeIC is a β-strand protein (Yoder and Jurnak, 1995), whereas remorin is predicted to be α-helical. This profound difference in structure is suggestive of very different biological roles for the two proteins.

A Potential Role for Remorin

Remorin copurifies tightly with plasma membranes. This membrane association would not be predicted from the cDNA encoding a hydrophilic protein. The protein is phosphorylated in vitro (Farmer et al., 1991) and in vivo (E.E. Farmer, unpublished data) and can bind polyanionic ligands. We are aware of only one other protein reported in the literature that clearly displays these same features: the 30-kD cell-to-cell movement protein of tobacco mosaic virus (TMV-MP), a protein involved in macromolecular trafficking through plasmodesmata (Lucas and Gilbertson, 1994). Despite the absence of predicted membrane-spanning regions, the TMV-MP associates with the plasma membrane like an integral membrane protein (Moore et al., 1992). This behavior is very similar to that of remorin. TMV-MP binds single-stranded DNA (Lucas and Gilbertson, 1994) and thus is similar to remorin, which binds several polyanions. The TMV-MP can be phosphorylated in vitro in the presence of cell-wall-associated proteins (Citovsky et al., 1993) under conditions similar to those we used to phosphorylate remorin. Citosky et al. (1993) propose that the phosphorylation of TMV-MP is a mechanism of deactivating the protein by sequestering it in cell walls. By analogy, we could imagine that the phosphorylation of remorin is involved in localization into the wall. At this stage, however, we must also consider phosphorylation as a possible regulatory mechanism involved in OGA signaling. Despite no apparent sequence homology between the two proteins, the intriguing similarities discussed above lead us (tentatively) to a hypothesis that we are testing.

The apparently hydrophobic behavior of remorin could be explained by its association with plasmodesmata or similar structures present in our plasma membrane preparations. Our working hypothesis is that remorin is a plasmodesma-associated protein potentially involved in cell-to-cell signaling and/or molecular transport. The interaction with uronides, and particularly their effect on remorin phosphorylation, provides candidate signaling molecules with which to probe these potential functions. To test this hypothesis as well as to investigate other possible roles of remorin, transgenic plants with altered levels of this protein are envisaged. Recombinant remorin will also be employed as a tool for the isolation of its kinase(s), with the long-term goal of the in vitro reconstruction of what may be part of the plant cell's (inter)cellular signaling machinery.

METHODS

Plants

The classification used was described by Cronquist (1988) and has been revised (Solnit and Solnit, 1995). Ranunculus ficaria (creeping buttercup) and Fagus sylvatica (beech) came from wild populations in Lausanne. Pisum sativum (garden pea), Solanum tuberosum cv Desiree (potato), Lycopersicon esculentum cv Bonny Best (tomato), Cichorium endivia (endive), and Zea mays (maize) were grown under standard greenhouse conditions (Farmer et al., 1991). Arabidopsis thaliana (mouse-ear cress) was grown in a 16-hr-light/8-hr-dark cycle at 26°C/18°C in a growth chamber. Spinacia oleracea (spinach) came from a local market, and Egeria densa (dense-leaved pondweed) came from an aquarium supplier. Monstera delicosa (cheese plant), Chlorella elatum variegatum (spider plant), and Ctenanthe oppenheimiana (ctenanthe) were grown under tropical climate conditions (>95% humidity) with no supplementary lighting.

In Vitro Phosphorylation

Plasma membrane was purified by zonal centrifugation followed by the dextran-polyethylene glycol two-phase partition method (Yoshida et al., 1983), with both polymers at a concentration of 5.9% (w/v). Potato and tomato plasma membranes were previously characterized (Farmer et al., 1991). Plasma membranes purified from other species were not biochemically characterized. Plasma membrane was thiophosphorylated at 30°C (Farmer et al., 1991) using γ-32P-adenosine-5'-thiophosphate (γ-32P-ATP). To ensure that the use of thiophosphate did not hinder uronide-stimulated protein phosphorylation in plasma membranes from monocotyledons, an alternative substrate, γ-32P-ATP, was used. In each case, comparable results were obtained, and all data presented are for γ-32P-ATP. Tomato leaf polygalacturonate used...
in the phosphorylation assay was the fraction TFA-PIIF, as described by Bishop et al. (1984). Uronides were quantitated according to Blumenkrantz and Asboe-Hansen (1973). Proteins were quantitated with the BCA reagent from Pierce Corporation (Rockford, IL).

Protein Purification and Sequencing

One- to 2-month-old greenhouse-grown potato plants (cv Desirée) were used as a source of plasma membrane (Farmer et al., 1991). Plasma membrane vesicles (corresponding to 63 mg of protein) were prepared from 7 kg of leaves. Remorin was purified from the plasma membranes, as described by Jacinto et al. (1993), and the partially purified protein was electrophoresed in a 12% SDS-polyacrylamide gel. After Coomassie Brilliant Blue R 250 staining and destaining, the ~34-kD band (apparent mass) was excised and exhaustively washed with water. The band was then minced in an Eppendorf tube and dehydrated to near completion using a Speed-vac (Zivy, Basel, Switzerland). Digestion was performed by the addition of an endopeptidase Lys-C solution for 16 hr. For sequencing, automated Edman degradation was performed using a protein sequencing system (model G1005A; Hewlett-Packard, Palo Alto, CA).

cDNA Cloning

Polymerase chain reaction (PCR) of DNA from a potato leaf (cv Bintje) cDNA library (λ ZAP; Stratagene; a kind gift from D. Johnston, Federal Agricultural Research Station of Changins, Nyon, Switzerland) was used to clone oligonucleotide primers of low degeneracy based on the codon usage preference of potato (from J.M. Cherry, Harvard University, Cambridge, MA). The reaction mixture consisted of 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each deoxynucleotide triphosphate, 20 milliunits of Taq polymerase per µL, and 1 µM each primer. Cycling conditions were as follows: 2 min at 94°C then 1 min at 94°C, 1.5 min at 68°C and 1.5 min at 72°C for 35 cycles, then 2 min at 72°C. A PCR product of 330 bp was obtained by using the primers 5'-GTTGAAATTGTKGAY-3' and 5'-TCCCAAGCWCCAATW-3: where K is T or G, Y is T or C, and W is T or A. The primers were derived from the sequence VEIVDP and AIGAWE of the sequenced peptides KVEIVDPAPPAPGPVEA and VSAIGAWENSK, respectively (Figure 2A). The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced using the T7 Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer’s instructions. The partial PCR product was labeled with digoxigenin (DIG system; Boehringer Mannheim, Mannheim, Germany) and used to probe the potato cDNA library. Labeling, hybridization, washing, and detection by chemiluminescence were performed according to the supplier’s instructions. Both strands of positive cDNA clones were sequenced in their entirety. Sequence alignments and analyses were performed using software from Genetics Computer Group (version 8; Madison, WI; Devereux et al., 1984) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al., 1990). Coiled coil prediction was done by using Coils (version 2.2) program (A. Lupas, Max-Planck-Institut für Biologie, Martinsried, Germany).

Expression of Glutathione S-Transferase Remorin

To generate a glutathione S-transferase (GST) fusion gene, PCR primers containing additional BamHI or EcoRI restriction sites at their 5’ end were used to amplify the complete remorin coding region. The BamHI-EcoRI-amplified fragment was cloned into the expression vector pGEX2T (Pharmacia Biotech) digested with BamHI and EcoRI. The fusion protein was produced in Escherichia coli JM109 cells, according to Bernasconi et al. (1994). An overnight culture of E. coli cells that carried pGEX-remorin was diluted 10-fold in 250 mL of Luria-Bertani broth, and the cells were grown at 37°C with vigorous shaking. After 1 hr, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After incubation for another 6 hr at 30°C, cells were collected by centrifugation, resuspended in 3 mL of extraction buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 2.5 mM DTT, 100 mM NaCl, 20% glycerol), subjected to five cycles of freezing and thawing, and sonicated. The cell lysate was centrifuged to remove bacterial debris, and proteins from the supernatant were analyzed by SDS-PAGE. When necessary, the supernatant was applied to a 5-mL glutathione Sepharose 4B column (Pharmacia Biotech). The retained fusion protein, GST-remorin, was eluted with 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, and concentrated by using Centricon-10 concentrators (Amicon). The purity of GST-remorin preparation was assessed by SDS-PAGE and silver staining of the gel. As controls, crude and affinity-purified preparations of GST were obtained by following the same protocol.

Binding Studies

Uronides used in binding studies included purified oligogalacturonic acids (OGAs) with a degree of polymerization (DP) of 20. The OGAs were purified according to Spiro et al. (1993) and as described in Reymond et al. (1995). Fractionated pectin (G50-PIIF; Bishop et al., 1984) was a gift from C.A. Ryan (Washington State University, Pullman). Uronides were biotinylated by reductive amination (Prakash and Vijay, 1983): A solution of uronide (500 µg) in dimethylformamide (360 µL) containing 0.5 M HCl (10 µL), glacial acetic acid (10 µL), and biotin-LC-hydrazide (200 µg; Pierce) was incubated at 40°C for 3 hr. NaCNBH3 (20 µL; 0.1 M in dimethylformamide) was then added to the reaction mixture, and incubation at 40°C was continued for 16 hr. The reaction mixture was then dialyzed against two changes of 5 L of water in 1000 D molecular weight cut-off dialysis tubing. Labeled uronides were purified on a monomeric avidin column (Pierce) according to the manufacturer’s instructions. The eluate from this column was dialyzed first against 2 L of PBS and then exhaustively against distilled water. Electrophoretic separation of the labeled ligands (Farmer et al., 1991) was used to assess their purity. The uronide content of the purified ligands was estimated according to Blumenkrantz and Asboe-Hansen (1973).

Binding assays were performed based on Silva et al. (1987). Proteins were separated in a 12% SDS-polyacrylamide gel. The gel was then washed twice for 1 hr in renaturation buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 20 mM EDTA, 0.1 mM DTT, 4 M urea) before electroblotting onto nitrocellulose. The blot was probed for 3 hr at room temperature with gentle agitation in binding buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5% nonfat dry milk, 100 µM CaCl2) containing 30 nM labeled ligand. After four 30-min washes in binding buffer, the blot was incubated for 1 hr in binding buffer containing 2 µg/mL avidin–horseradish peroxidase conjugate (Pierce), followed by five 5-min washes in binding buffer and five 5-min washes in binding buffer without nonfat dry milk. Bound ligand was detected with a chemiluminescence assay (ECL; Amersham), and the intensity of the binding was quantified using a Molecular Imager (Bio-Rad). A very weak affinity of avidin–horseradish peroxidase for GST-remorin was
noted, and all binding experiments were conducted with a control using avidin–horseradish peroxidase in the absence of any labeled ligand. Polyanions used for the competition experiments were trigalacturonic acid (Sigma), polymeranuronic acid with a DP of ~23, and polygalacturonic acid with a DP of ~19 (a gift from M. Saxton, University of California at Davis). Carboxymethylcellulose, poly-L-glutamate, and heparin were used as controls using (Sigma), polymannuronic acid with a DP of ~23, and polyguluronic acid (New York: Garland Publishing).

References


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Cloning of a cDNA encoding a plasma membrane-associated, uronide binding phosphoprotein with physical properties similar to viral movement proteins.

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