An Antibody Fab Selected from a Recombinant Phage Display Library Detects Deesterified Pectic Polysaccharide Rhamnogalacturonan II in Plant Cells

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Rhamnogalacturonan II (RG-II) is a structurally complex, low molecular weight pectic polysaccharide that is released from primary cell walls of higher plants by treatment with endopolygalacturonase and is chromatographically purified after alkaline deesterification. A recombinant monovalent antibody fragment (Fab) that specifically recognizes RG-II has been obtained by selection from a phage display library of mouse immunoglobulin genes. By itself, RG-II is not immuno-genic. Therefore, mice were immunized with a neoglycoprotein prepared by covalent attachment of RG-II to modified BSA. A cDNA library of the mouse IgG1κ antibody repertoire was constructed in the phage display vector pComb3. Selection of antigen-binding phage particles resulted in the isolation of an antibody Fab, CCRC-R1, that binds alkali-treated RG-II with high specificity. CCRC-R1 binds an epitope found primarily at sites proximal to the plasma membrane of suspension-cultured sycamore maple cells. In cells deesterified by alkali, CCRC-R1 labels the entire wall, suggesting that the RG-II epitope recognized by CCRC-R1 is masked by esterification in most of the wall and that such RG-II esterification is absent near the plasma membrane.

INTRODUCTION

The walls of plant cells are composed of a complex array of macromolecules, including polysaccharides, proteins, and phenolic compounds (reviewed in Carpita and Gibeaut, 1993). Recent studies indicate that the wall is not a static structure but that its composition and the location of its components change in response to pathogens, symbions, and other environmental factors (Rae et al., 1992; Ye et al., 1992; Bolwell, 1993). Changes in cell wall structure also occur in the course of normal plant development (Koch and Nevins, 1989; Arribas et al., 1991; Freshour et al., 1996). Monitoring these changes is difficult using traditional biochemical approaches that rely on chemical and enzymatic extraction of cell wall molecules (McNeil et al., 1984; Talbott and Ray, 1992). These extraction procedures intrinsically disrupt the structure of the wall polymers. Examination of cell wall polysaccharides in vivo can be facilitated by the isolation of monoclonal antibody-derived probes that can be used as tools for localization. When the antibody epitopes have been well defined, the antibodies can be used for specific biochemical analysis of whole cells or microscopic sections of cells.

The value of cytological probes for elucidating the cell- or tissue-specific distribution of cell wall components is widely recognized (Roberts et al., 1985). However, histological studies using small-molecule chemical stains, polysaccharide binding enzymes, or polyclonal antibodies are often difficult to interpret because of their broad and/or imprecise binding specificity (Hughes and McCully, 1975; Brundrett et al., 1988; Moore and Staehelin, 1988; Liu and Berry, 1991; Benhamou, 1992). Monoclonal antibodies are epitope specific and have been used to localize wall proteins, glycoproteins, and polysaccharides (Knox et al., 1990; Ye and Varner, 1991; Zhang and Staehelin, 1992; Smallwood et al., 1994; Freshour et al., 1996). Immunochemical studies performed using anti-carbohydrate antibodies have established that oligosaccharide epitopes can be diagnostic of development-associated events. For example, monoclonal antibodies raised against various extracellular matrix arabinogalactan proteins appear to predict the developmental fate of cells in roots (Knox et al., 1991) or floral meristems (Pennell et al., 1991). Our laboratory has used monoclonal antibodies raised against rhamnogalacturonan I (RG-I) to follow the developmental- and cell type-specific regulation of carbohydrate epitopes in Arabidopsis roots (Freshour et al., 1996).

Rhamnogalacturonan II (RG-II) is a pectic polysaccharide structurally unrelated to RG-I that has been isolated from the
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primary walls of all higher plant cells that have been examined, including examples of monocots, dicots, and gymnosperms (Thomas et al., 1987, 1989; Stevenson et al., 1988). Detailed structural characterization has been performed on alkali-treated RG-II; purification of the pectic polysaccharides is facilitated by alkali deesterification. Although of low molecular mass (~5000 kD), RG-II is structurally complex, consisting of at least 11 different monosaccharides, some of which are quite rare. Aeric acid, for example, is the only branched acidic deoxy sugar that has been identified in nature, whereas 2-keto-3-deoxyoctulosonic acid (KDO), a common component of bacterial capsular polysaccharides and lipopolysaccharides, has been found nowhere else in plants (O’Neill et al., 1990). The (1→4)-linked α-D-oligogalactosyluronic acid backbone of RG-II has at least four different side chains with diverse glycosidic linkages. The composition of different preparations of RG-II from various plant sources is remarkably consistent (Stevenson et al., 1988; Thomas et al., 1989; Whitcombe et al., 1995). The structural complexity of RG-II represents a significant investment of the plant’s biosynthetic capacity. Considered together with its wide phylogenetic distribution, the structural complexity of RG-II implies that it plays an important although an as yet undetermined role in cell wall structure and/or function.

Antibodies raised against RG-II have not been reported previously. Polysaccharides are often poor immunogens, and polysaccharide-specific monoclonal antibodies are correspondingly rare. Recombinant antibody phage display, an alternative to hybridoma technology, recently has been developed to isolate monoclonal antibodies (Garrard et al., 1991; Kang et al., 1991; Marks et al., 1991). Phage display permits the use of selective methods to increase the likelihood of finding rare monospecific antigen-binding antibody fragments (Fab). Displaying Fabs on the surface of bacteriophage M13 (Barbas et al., 1991; Hoogenboom et al., 1991) allows selection from >10^9 potential clones, as opposed to screening ~500 clones from a typical hybridoma fusion.

Each Fab is composed of the entire antibody light chain (variable and constant light chain domains, \( \text{V}_L \) and \( \text{C}_L \)) and the ~220 amino acids from the N terminus of the antibody heavy chain, also known as the Fd region (variable and first constant heavy chain domains, \( \text{V}_H \) and \( \text{C}_H \)). The Fd and κ polypeptides are linked by one disulfide bond. The resulting heterodimeric Fab contains a single antigen binding site.

The Fab genes are expressed as fusion proteins with a phage coat protein in the pComb3 phage display system (Barbas et al., 1991). The fusion proteins are assembled on the surface of phage particles into which the corresponding plasmid DNA is packaged. Thus, each Fab binding activity is displayed on the surface of a phage particle that carries the genes encoding that Fab. New antibodies can be isolated using phage display by constructing libraries from an animal’s repertoire of antibody specificities. Fabs with the desired binding properties are then isolated from the library by a powerful selection technique known as panning (Smith, 1985).

We have undertaken to expand the variety of monoclonal antibodies available for binding plant cell wall polysaccharide epitopes by developing the ability to select Fabs from phage display libraries by using plant cell wall polysaccharides as ligands for Fab-phage selection. We report here the selection of a Fab that binds specifically to RG-II and its use to localize esterified and deesterified RG-II in cells.

RESULTS

Immunization

RG-II is a relatively low molecular weight polysaccharide and, like other low molecular weight hapten, is poorly immunogenic. A common method to improve the immunogenicity of small molecules is to couple them to a carrier protein (Smith and Ginsburg, 1980). Therefore, we generated covalent neoglycoconjugates (RG-II/Ova and RG-IIcBSA) of sycamore RG-II with ovalbumin or with modified BSA (cBSA). Mice were immunized with RG-II/cBSA, and the serum of one immunized mouse was assayed by ELISA (using class-specific secondary antibodies) for the presence of various classes of antibodies against RG-II/Ova (\( \text{IgG}_1, \text{IgG}_2a, \text{IgG}_2b, \text{IgG}_3, \text{IgM}, \text{IgA}, \) and IgE heavy chains and \( \kappa \) or \( \lambda \) light chains). The RG-II/Ova neoglycoconjugate also was used for panning selection to avoid selection of antibodies against the BSA portion of the immunogen. The highest serum titer was detected for \( \text{IgG}_1 (<1:10,000) \) and \( \kappa (1:10,000) \) chains. These are also the most common antibody classes present in mouse serum (Goding, 1986). Therefore, we constructed an IgG1/κ phage display library as outlined below.

Generation of a Fab-Phage Display Expression Library

The repertoire of antibody genes from the immunized mouse was obtained by polymerase chain reaction (PCR) amplification of cDNA prepared from RNA of splenic lymphocytes. The PCR primers corresponded to conserved \( \text{C}_H1 \) and \( \text{C}_L \) antibody constant domains and to the relatively conserved framework (FR1) region of antibody variable domains (\( \text{V}_L \) and \( \text{V}_H \)). For the heavy chains, four \( \text{V}_H \) FR1 primers were paired with the IgG1 \( \text{C}_H1 \) primer in separate initial PCRs. For the light chains, a single \( \text{V}_L \) FR1 primer was paired with a \( \text{C}_L \) domain primer. Use of these primers has been shown to result in the isolation of a large fraction of the different members of the rearranged variable region repertoire (Huse et al., 1989). The resulting PCR products (and the corresponding clones) are populations of κ chain genes and the Fd portion of heavy chain genes. The primary products of PCR amplification were, as expected, fragments of ~700 bp for all four heavy chain primer pairs. PCR products of similar size were obtained for the light chains.

The light chain PCR products were cloned into plasmid vector pComb3 (Barbas et al., 1991), resulting in a library of \( 10^4 \) transformants. Using DNA from this light chain library as the
vector, the heavy chain PCR products were cloned, yielding 2 \times 10^6 combinatorial clones. Immune-competent mice have \sim 10^8 different antibody specificities. The combinatorial cloning procedure results in random reassortment of the heavy and light chain partners, and the number of combinations is limited primarily by transformation frequencies.

The PCR primers are designed to incorporate unique restriction endonuclease sites so that when the fragments are cloned into expression vector pComb3, in-frame protein fusions of the PelB transit peptide and Fd or \kappa result, each under the control of separate lacZ promoters. In addition, the Fd gene is fused with the C terminus of the phage M13 coat protein III (cplll). When expressed in Escherichia coli, the PelB signal peptide causes the polypeptides to be secreted into the periplasmic space where Fd–cplll and the light chain spontaneously assemble to form an antigen binding Fab. The resulting Fab–coat protein fusion is incorporated onto the surface of M13 Fab–phage fusion particles. The Fab–phage fusion particles, each carrying the corresponding plasmid DNA, can then be selected by panning according to their binding activity.

### Panning Selection

The Fab–phage fusion library was panned against several antigens by a procedure similar to ELISA. Two percent ovalbumin was added to the Fab–phage suspension to block nonspecific protein binding sites in the polystyrene wells. The ovalbumin also provided an excess of soluble competitor so that ovalbumin binding phage were not selected on the immobilized RG-II/Ova conjugate. After vigorous washing, selected Fab–phage particles were eluted by denaturation at acid pH. The number of Fab–phage particles retained on each immobilized antigen was monitored by titrating the number of E. coli cells transfected with the eluted Fab–phage particles. The Fab–phage particles carry plasmid DNA that confers ampicillin resistance. After several panning cycles, an increase in the ratio of the number of phage applied from the repertoire library indicates enrichment of a subset of clones that bind the target antigen.

Figure 1 shows that >1000-fold enrichment was observed by the fourth cycle when RG-II/Ova or BSA was used as the antigen. This indicates that antibodies against epitopes on both RG-II and BSA were elicited by the RG-II/BSA immunogen. Enrichment for Fab–phage particles on either of the cell wall polysaccharides RG-I or xyloglucan (XG) was <100-fold; these polysaccharides were not part of the immunogen. Of 40 clones randomly selected from the fourth cycle of panning against RG-II/Ova, 17 had binding activity as determined by ELISA. Partial DNA sequencing of eight of these clones suggested they were closely related or identical to one another (90 to 100% identity of deduced amino acid sequences in each of the sequenced framework regions [FR1, FR2, and FR3] and complementarity determining regions [CDR1 and CDR2] of the variable domain of heavy chains; data not shown), implying that these genes were of clonal origin. The binding activity of the Fab encoded by one randomly selected clone (CCRC-R1) is further characterized.

### Purification of CCRC-R1

Soluble Fab was purified so that the properties of CCRC-R1 could be analyzed. Plasmid DNA of clone CCRC-R1 was extracted from transfected E. coli cells, and the coat protein gene was removed for production of soluble Fab. Approximately 1 mg/L soluble Fab could be purified from the supernatant of shake flask–grown XL1-Blue cells after induction of the lacZ promoter by isopropyl p-D-thiogalactoside (IPTG). Figure 2 shows that cation exchange chromatography eliminated >90% of the contaminating supernatant proteins. Subsequent light chain–specific affinity chromatography resulted in purification of the Fab to homogeneity (Figure 2, lanes 1 to 3). After reducing SDS-PAGE, the purified Fab gave a doublet of \sim 23 kD each, corresponding to the \kappa and Fd polypeptides (Figure 2, lane 3). After nonreducing SDS-PAGE, the purified Fab gave a single band of \sim 46 kD (Figure 2, lane 4), indicating that the Fd and \kappa polypeptides are linked by a disulfide bond to form dimers, as is expected for a monovalent Fab.
Figure 2. SDS-PAGE Analysis of CCRC-R1.
The SDS–polyacrylamide gel was stained with silver. Lane 1 contains crude concentrated bacterial culture supernatant; lane 2, S-Sepharose–purified Fab; lanes 3 and 4, Quick Mab–affinity purified Fab; lane 5, molecular mass standards. The samples in lanes 1 to 3 and 5 were reduced with DTT; the sample in lane 4 was not. The stained gel was scanned with a personal densitometer (Molecular Dynamics, Sunnyvale, CA) and printed using ImageQuant software (Molecular Dynamics).

Binding Characteristics of CCRC-R1

Competition ELISA, with RG-II/Ova as the solid-phase immobilized antigen, was used to characterize the binding specificity and affinity of CCRC-R1; the results are shown in Figures 3 and 4. Indirect ELISA and dot blot analyses are less reliable for this purpose, because different polysaccharides and glycoproteins immobilize with different efficiencies on polystyrene wells and nitrocellulose membranes (J. Puhlmann, M.J. Swain, W. Steffan, M.N.V. Williams, N. Dunning, and M.G. Hahn, unpublished results). Soluble RG-II/Ova inhibits binding of CCRC-R1 to immobilized RG-II/Ova with a 50% inhibitory concentration (IC_{50}) of ~3 μg/mL (Figure 3). Free RG-II also has an IC_{50} of ~3 μg/mL, which corresponds to an apparent dissociation constant (K_a) of ~10^{-6} to 10^{-7} M. The equal affinity of CCRC-R1 for RG-II and RG-II/Ova indicates that CCRC-R1 binds to the RG-II portion of the RG-II/Ova conjugate and not to the protein or to the arm linking the polysaccharide to the protein. Furthermore, CCRC-R1 does not bind to sodium periodate–treated RG-II/Ova (data not shown). This treatment, which oxidizes many glycosyl residues of RG-II, provides additional evidence that CCRC-R1 recognizes RG-II.

The pectic polysaccharides used in this study were isolated after digestion of sycamore cell walls with a homogeneous fungal endopolygalacturonase. Gel exclusion chromatography of the polysaccharides solubilized by this treatment yielded a polysaccharide fraction (fraction A) that was then deesterified with alkali, pH 12, at 4°C for 1 hr to facilitate its further separation by gel exclusion chromatography into RG-I, RG-II, and homogalacturonan (Marfà et al., 1991). Before alkali-catalyzed hydrolysis of its esters, fraction A has an IC_{50} of ~50 μg/mL, which is 15 times greater than the IC_{50} of purified, deesterified RG-II. After alkali treatment, but without further purification, fraction A has an IC_{50} equal to that of purified RG-II. Thus, the epitope to which CCRC-R1 binds is present in fraction A and is made more abundant, rendered of higher affinity, or unmasked by alkali treatment of the polysaccharide.

Binding of CCRC-R1 to RG-II is reduced or eliminated in solutions containing NaCl or other salts at concentrations of 200 mM and above, suggesting that the binding has an ionic component. To ensure that CCRC-R1 is not binding nonspecifically to the negative charges of RG-II, a number of anionic polysaccharides of plant, bacterial, or animal origin were tested in competition assays. Figures 3 and 4 show that RG-I, dextran sulfate, and polygalacturonic acid (PGA) have IC_{50} values >1000-fold higher than that of RG-II. Several other polyanions, including salmon sperm DNA, heparin, Rhizobium mellioti K antigen, carrageenan, and oligogalacturonides (with a degree of polymerization of 6 to 16), were also tested. None of these competes significantly for CCRC-R1 binding to RG-II/Ova at concentrations of up to 10 mg/mL. CCRC-R1 also does not bind to other plant cell wall polysaccharides (XG or citrus pectin) or to cell wall proteins (sycamore and larchwood arabinogalactan proteins, tomato extensin P2, or maize histidine-rich hydroxyproline-rich glycoprotein), as measured by indirect and/or competition ELISA. Thus, CCRC-R1 is specific for an alkali-treated (deesterified) form of the pectic polysaccharide RG-II.

Figure 3. Competition ELISA with Pectic Polysaccharides.
The competition assays were performed as described in Methods. Competitor antigens are as follows: RG-II/Ova ( ), free RG-II (■), RG-I (●), fraction A (▲), and alkali-treated fraction A (○). Sycamore cell walls were digested with fungal endopolygalacturonase, and the soluble fraction containing rhamnogalacturonans was isolated (fraction A) by gel exclusion chromatography. Further gel exclusion chromatography following alkali treatment, pH 12 (at 4°C for 1 hr), allowed separation into RG-I and RG-II.
Recombinant Antibody against RG-II

Monosaccharide components of RG-II were individually assayed by ELISA for their ability to compete with CCRC-R1 binding to RG-II/Ova. No competition was observed with D-arabinose, L-rhamnose, L-fucose, D-galactose, or 2-O-methyl D-xylose (at 500 mM concentrations, 82 to 90 mg/mL) or with KDO, galacturonic acid, or glucuronic acid (at 50 mM concentrations, 10 to 13 mg/mL). Salts of the acidic sugars (KDO, galacturonic acid, and glucuronic acid) were used to maintain neutral pH. The buffer for all of these assays contained 100 mM NaCl. Therefore, concentrations of the acidic sugars >100 mM could not be effectively tested, because at these concentrations, binding of CCRC-R1 to RG-II/Ova is inhibited by salt (see above).

Immunochemoal Staining of RG-II in Plant Cells

Having established in vitro the high affinity and specificity of CCRC-R1 binding, we proceeded to use CCRC-R1 to label RG-II in plant cells. Immunofluorescence microscopy detects CCRC-R1 binding to the surface of alkali-treated suspension-cultured sycamore cells, as shown in Figure 5A. Essentially no fluorescein fluorescence was found when the cells were probed with an irrelevant antibody (anti-BSA antibody 11F9-F9; Figure 5B), except at cell junctions, which presumably resulted from the limited accessibility of these junctions to the washing procedure used in these experiments.

Suspension-cultured sycamore cells were either treated with alkali, pH 11, at 4°C for 1 hr or not treated with alkali (non-alkali-treated) and fixed with glutaraldehyde, embedded, and sectioned. The thin sections were then probed with CCRC-R1 and gold-labeled secondary antibody. Representative transmission electron micrographs are shown in Figure 6. Little labeling can be observed when non-alkali-treated cells are examined, as illustrated by Figure 6A. Strong labeling can be observed throughout the cell walls of alkali-treated cells (Figure 6B). The results obtained when the cells were treated with alkali before embedding were similar to those obtained when sections from non-alkali-treated cells were treated with alkali (data not shown).

The clustering of gold particles seen at some locations may not indicate high local concentrations of RG-II; it may be due to aggregation of CCRC-R1, which we have observed to occur in vitro. Fibrous material in the extracellular medium of alkali-treated sections is also labeled with gold (Figure 6B). The fibrous material probably contains extracellular RG-II, which is excreted into the culture medium of suspension-cultured sycamore cells (York et al., 1985). No gold labeling was observed when RG-II was mixed with CCRC-R1 before application of the Fab to sections (data not shown), indicating that CCRC-R1 binding in these sections can be competed by RG-II.

Figure 4. Competition ELISA with Polyanions and Polysaccharides.

The competition assays were performed as described in Methods. Competitor plant cell wall polysaccharides are as follows: RG-II (■), XG (□), and gum arabic (○). Competitor polyanions were polygalacturonic acid (●) and dextran sulfate (△).

Figure 5. Fluorescence Microscopy of Antibody-Stained Suspension-Cultured Sycamore Maple Cells.

Cells were fixed and stained as described in Methods.

(A) Whole cells labeled with CCRC-R1 and goat anti-mouse (κ)-fluorescein isothiocyanate.
(B) Whole cells labeled with anti-BSA monoclonal antibody and goat anti-mouse (κ)-fluorescein isothiocyanate.

Bars = 20 μm.
The gold label is preferentially localized to the plasma membrane of non-alkali-treated cells. The localization of gold particles in 15 micrographs of non-alkali-treated cells was quantitated and analyzed. In non-alkali-treated cells, 694 gold particles were counted; 13% (94) of the particles were in the walls and 42% (293) were near the plasma membrane (plasma membrane proximal). The rest of the particles were randomly distributed in the sections (background labeling). The number of gold particles per area of the sections examined (density of labeling) is compared in Figure 7. The average density of wall labeling is 2.7 particles per μm² in non-alkali-treated cells. However, the average density of label found in the walls of these cells is statistically higher than background labeling (1.0 particles per μm²; \( P = 0.013 \)), indicating that CCRC-R1 labels the walls of non-alkali-treated cells. The plasma membrane–proximal labeling density is markedly higher (30.8 particles per μm²; \( P = 0.0004 \)) than in other parts of the walls of non-alkali-treated cells.

The labeling of the wall by CCRC-R1 is dramatically increased by alkali treatment. Six micrographs of alkali-treated cells were examined. Of 1505 gold particles counted, 75% (1140) of the particles were in the walls and 10% (159) were plasma membrane proximal. The labeling of alkali-treated cells is ~70 times denser in the walls than on other parts of the section (Figure 7). The average density of wall labeling in alkali-treated cells (94.8 particles per μm²) is more than twice that of the average density of plasma membrane–proximal labeling (41.8 particles per μm²), but this difference is not statistically significant (\( P = 0.1 \)). Thus, in alkali-treated cells, the densities of labeling of walls and plasma membrane regions are similar.

The tonoplast membrane was not labeled above background levels in either alkali-treated or non-alkali-treated tissues, indicating that the high density of plasma membrane–proximal labeling is not due to unspecific labeling of membranes. The plasma membrane–proximal labeling densities for non-alkali-treated and alkali-treated cells are not significantly different (\( P = 0.268 \)), indicating that alkaline deesterification does not change the binding of CCRC-R1 to plasma membrane–proximal sites. These results suggest that plasma membrane–proximal RG-II lacks the esters that mask CCRC-R1 binding, whereas these esters are present in RG-II in the rest of the cell wall.

**DISCUSSION**

We have isolated a monoclonal antibody Fab, designated CCRC-R1, that specifically binds the plant cell wall polysaccharide RG-II. Although several anti-polysaccharide hybridomas...
have been molecularly cloned and expressed (Mainhart et al., 1984; Anand et al., 1991; Adderson et al., 1993), this is a novel use of phage-display libraries or recombinant methods for de novo isolation of an antibody against any plant antigen or against any polysaccharide. This is also the first antibody we have raised against RG-II.

The use of recombinant antibody libraries to obtain antigen-binding clones has several inconveniences but many advantages. Construction of libraries for Fab–phage display, when compared with hybridoma generation, is initially a longer, more tedious process. However, maintenance of antibody-producing clones is easier, because phage particles may be stored at 4°C and transformed E. coli cells or DNA may be stored frozen with little fear of losing the clones upon thawing. Furthermore, recombinant libraries allow the selection of useful antibodies from a much larger number of potential antigen-binding clones than hybridoma techniques permit (>10⁶ versus <1000) and consequently are preferred for antigens that do not elicit a strong immune response.

Whereas screening of a hybridoma fusion is a one-time event, recombinant libraries are immortal and may be reused repeatedly in selection schemes that can be refined based on the results of previous selections. For example, selection of particular unwanted specificities can be excluded by adding an excess of an undesired epitope to the panning suspension. Fab–phage particles recognizing this epitope are "out-competed" by the soluble epitope and thus are not retained on the solid phase. Indeed, inclusion of ovalbumin in the Fab–phage suspension during the panning procedure that yielded CCRC-R1 avoided selection of Fabs that recognize ovalbumin. By contrast, several hybridoma-derived monoclonal antibodies that were isolated in this laboratory, using the same neoglycoprotein conjugates (RG-II/cBSA and RG-II/Ova) for immunization and screening, show much higher affinity for the neoglycoproteins than for free RG-II (W. Steffan, M. Swain, A.G. Darvill, P. Albersheim, and M.G. Hahn, manuscript in preparation).

Another advantage of recombinant antibody libraries is that selection of the Fab–phage also immediately yields the genes encoding the antibody, which may then be readily sequenced and manipulated. For example, antibody genes have been expressed in plants to bring about modified metabolic responses or pathogen resistance (Owen et al., 1992; Taviadoraki et al., 1993). The DNA sequences of isolated clones can also be used to quickly distinguish different Fabs that bind the same antigen. In addition, gram quantities of recombinant Fab protein can be rapidly produced in relatively inexpensive culture media, because recombinant Fab can be biosynthesized in E. coli by using pComb3 or other expression vectors that give higher yields. Large amounts of pure Fabs can be used for epitope purification by affinity chromatography (Austin and Corstvet, 1993) and structural studies of antigen–antibody complexes (Rose et al., 1993).

Whole immunoglobulins are large molecules (molecular mass of ~900 kD for IgM and 180 kD for IgG). Their size may hinder their use in cytological studies, because the antibodies may not be able to penetrate tissues to reach their epitopes effectively (Larsson, 1988). Fabs are significantly smaller (45 kD) than whole antibodies and should therefore be of greater use for immunocytochemistry and especially in fine-structure studies (Baschong and Wrigley, 1990).

Binding of CCRC-R1 to RG-II is salt sensitive, a common feature of antibodies that bind charged antigens (Ruff-Jamison et al., 1991). The alkali-deesterified form of RG-II is recognized by CCRC-R1 15 times more effectively than is the non-alkali-treated form (see Figure 3). The greater affinity of CCRC-R1 for deesterified RG-II is expected because the neoglycoproteins we synthesized to immunize mice and to select RG-II–binding phage were made with deesterified RG-II. Acetyl and borate esters are known constituents of alkali-treated RG-II (Whitcombe et al., 1995; Kobayashi et al., 1996; M.A. O'Neill, D. Warrenfeltz, K. Kates, P. Pellerin, T. Domo, A.G. Darvill, and P. Albersheim, manuscript in preparation). Thus, these were likely still to be present in the alkali-treated preparations we used and therefore were unlikely to be the esters that mask the CCRC-R1 epitope. Characterization of the interaction between CCRC-R1 and RG-II suggests the presence of a variety of ester linkages in RG-II that differ in susceptibility to alkali.

![Figure 7. Density of Immunogold Labeling of Suspension-Cultured Sycamore Maple Cells.](image)

The density of gold label was calculated for each area of each micrograph by dividing the number of gold particles by the area of the sections examined. The values plotted are the average densities for 15 micrographs of non-alkali-treated (untreated) cells and six micrographs of alkali-treated cells. For untreated sections, the average density values for Wall, Plasma membrane, and Other regions were all statistically different from one another as determined by paired two-tailed Student's t tests. For alkali-treated sections, the values for Wall and Plasma membrane were not statistically different from each other, but both were different from Other as determined by paired two-tailed Student's t tests. Heteroscedastic (two-sample unequal variance) analysis indicates that the densities of plasma membrane labeling for untreated and alkali-treated micrographs were not significantly different. These results imply that CCRC-R1 immunogold labeling dramatically increases in cell walls after alkali treatment, whereas labeling of the plasma membrane–proximal regions is not altered by alkali treatment.
In ELISA assays, neither the RG-II–component monosaccharides tested nor oligogalacturonides competed for binding of CCRC-R1 to RG-II/Ova. The rare sugar constituents of RG-II (aceric acid, 3-deoxy-d-lyxo-2-heptulosonic acid, 2-O-methyl fucose, or apoiose) and other oligosaccharides of RG-II are not available for competition assays. The lack of competition by monosaccharides may reflect the complexity of the epitope recognized by CCRC-R1. The epitope identified by many antibodies is structurally complex, and for anti-carbohydrate antibodies, it may contain up to seven glycosyl residues (Kabat et al., 1988).

It is formally possible that CCRC-R1 is labeling, in immunocytochemical localizations, something other than RG-II. However, we have been unable to detect binding of CCRC-R1 to any other molecule in vitro, and all binding of CCRC-R1 to thin sections is prevented by an excess of RG-II. Therefore, we assume that the CCRC-R1 binding indicates the presence of RG-II in these tissue sections.

CCRC-R1 strongly labels RG-II in the alkali-deesterified walls of suspension-cultured sycamore cells, whereas much weaker labeling occurs in the walls of cells not treated with alkali (see Figures 6 and 7). The increase in labeling after alkali treatment might be due to chemical modification (e.g., deesterification) of the polysaccharides or to increased accessibility of epitopes in the wall to antibody binding. However, binding of monoclonal antibodies to other cell wall polysaccharides is not altered by alkali treatment of the tissue sections (data not shown). Because the affinity of CCRC-R1 for soluble RG-II also is increased by alkali treatment, it is likely that the stronger labeling of the alkali-treated walls is due to deesterification of the wall polysaccharides in situ in the sections. Thus, in all probability, most of the RG-II in these cell walls contains epitope-masking esters.

The density of CCRC-R1 labeling at or near the plasma membrane is similar with or without alkali deesterification, suggesting that, in vivo, the ester of RG-II that masks the CCRC-R1 epitope in most of the wall is less abundant or absent near the plasma membrane. The apparent difference between RG-II esterification in the wall and at the membrane provides the first evidence we have obtained that there are two distinct populations of RG-II in the cell. This may indicate that RG-II does not possess the epitope-masking ester when secreted at the plasma membrane and, moreover, that this ester is added to RG-II in muro.

Others have observed different levels of pectin esterification in regions of the cell wall. The unesterified pectic epitope recognized by monoclonal antibody JIM5 appears to be more abundant in walls adjacent to the intercellular spaces and the plasma membranes of carrot root cells (Knox et al., 1990). JIM5 binds deesterified homogalacturonan but not RG-II, whereas CCRC-R1 binds RG-II but not deesterified homogalacturonan; consequently, the epitopes that these antibodies recognize are different. However, JIM5 and CCRC-R1 both preferentially label deesterified forms of pectic polysaccharides at the plasma membrane. Subcellular regulation of the esterification of pectic polysaccharides may have fundamental importance to the function of the cell wall.

METHODS

Antigens and Immunization

Plant and bacterial polysaccharides and proteins prepared as described were obtained from colleagues at the Complex Carbohydrate Research Center as indicated below. Endopolygalacturonase-released polysaccharide fraction A, rhhamoglacturonan I (RG-I), rhhamoglacturonan II (RG-II), and oligogalacturonides (degree of polymerization 6 to 16) were obtained from A. Whitcombe and M. O'Neill (Martà et al., 1991). Xyloglucan (XG) was obtained from W. York (York et al., 1985); Rhizobium meliloti capsular polysaccharide (K antigen) was obtained from B. Reuhs (Reuhs et al., 1993). Cell wall proteins (sycamore arabinoxylan protein, Pope, 1977; tomato extensin P2, Smith et al., 1984; and maize histidine-rich hydroxypyrolone-rich glycoprotein, Kieliszewski et al., 1992) were obtained from M. Kieliszewski. Other polysaccharides, proteins, and salmon sperm DNA were obtained from Sunkist Growers Inc. (Corona, CA), Sigma, or FMC Corp. BioProducts (Rockland, ME).

The protein–polysaccharide conjugates RG-II/cBSA and RG-II/Ova were prepared by coupling sycamore RG-II either to the "super carrier" cBSA (Pierce, Rockford, IL) or to ovalbumin by using the isothiocyanate procedure described by Smith and Ginsburg (1980; W. Steffan, A.G. Darvill, P. Albersheim, and M.G. Hahn, manuscript in preparation). The RG-II used for these conjugates was indistinguishable in composition from that previously published by our laboratory (O'Neill et al., 1990; Whitcombe et al., 1995). Conjugates contained ~50% (RG-II/Ova) or ~70% (RG-II/cBSA) polysaccharide as determined by anthrone assay (Dische, 1962).

Female BALB/c mice were immunized intraperitoneally with RG-II/cBSA at the Monoclonal Antibody Facility of the Botany Department at the University of Georgia (Athens, GA). A 200-μL mixture containing 200 μg of RG-II/cBSA in 100 μL of H2O and 100 μL of complete Freund's adjuvant was injected on day 0. The initial injection was followed on days 28 and 56 by injections of a mixture of 100 μg of RG-II/cBSA in 50 μL of H2O and 50 μL of incomplete Freund's adjuvant. A final injection of 50 μg of RG-II/cBSA in 50 μL of H2O was given on day 67. Serum taken by tail bleed was assayed by indirect ELISA as described by Harlow and Lane (1988), using RG-II/ovalbumin (RG-II/Ova)—coated plates and anti–mouse antibody secondary antibody/alkaline phosphatase conjugates. The mouse with the highest titer was killed for hybridoma cloning, and the mouse with second highest titer was killed for molecular cloning of antibody fragments (Fabs); the blood was kept at 4°C, and the spleen was frozen in liquid N2. The serum titer of each antibody class was assayed by ELISA as described above, using class-specific secondary antibody/alkaline phosphatase conjugates (Fisher Scientific, Pittsburgh, PA).

Library Construction

Total spleen RNA was isolated (Chomczynski and Sacchi, 1987), first strand cDNA was synthesized with M-MuLV reverse transcriptase (Sambrook et al., 1989), and the resulting cDNA was used as the template in two sequential sets of polymerase chain reactions (PCRs). Separate PCR amplifications were performed for IgG, heavy chains, and light chains; in each case, the 3' primers corresponded to the immunoglobulin Cγ1 or Cλ domains, and the 5' primers corresponded to the relatively conserved FR1 domain of the variable regions. The first set of primers and PCR conditions used are based on those described by Huse et al. (1989). A unique Vλ primer (5'-GGCTCA-
GATGTTGAGCTCGTGATGACCCAGTCTCCA3') was used with the Cκ primer (5'-TCTTCTTCTATTACTACCTCCTCCCTGTGA-3'). A family of four Vκ primers (primers 2 to 5 from Huse et al., 1989) were used in four separate heavy chain amplifications with the Cκ1 primer (primer 12 from Huse et al., 1989). PCR products were gel purified using a GenElute kit (BIO BASIC). Extension PCR was performed for 20 cycles with an annealing temperature of 60°C.

Light chain PCR products were digested with SacI and XbaI, ligated into the corresponding sites in pComb3 (Barbas et al., 1991), and electroporated using a Gene Pulser (Bio-Rad, Hercules, CA) into E. coli (Sambrook et al., 1989). The DNA was sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Panning Selection

Panning selection (Smith, 1985) was performed by a method similar to ELISA. Bacterial cells were removed from overnight cultures of fusion phage libraries by centrifugation for 15 min at 3000g. Phage particles were harvested from the supernatant by polyethylene glycol–NaCl precipitation (Parmley and Smith, 1988) and then resuspended in sterile PBS (0.2 g of NaCl, 0.02 g of KCl, 0.154 M Na2HPO4 and K2HPO4, 0.05 g of Na3PO4, and 0.25 g of KCl per L) containing 2% (w/v) ovalbumin. Five cycles of panning selection were conducted against RG-II/Ova, BSA, RG-1, or XG. The antigens were adsorbed onto wells of Immunon 2 polystyrene ELISA plates (Dynatech Laboratories, Inc., Chantilly, VA) as previously described (Puhlmann et al., 1994), and the nonspecific binding sites in the wells were blocked with 2% (w/v) ovalbumin in H2O.

Phage suspensions were applied to antigen-coated ELISA plate wells and allowed to adsorb for 2 hr at room temperature. Unbound phage were then removed by 10 washes with PBS containing 0.05% (w/v) polyoxyethylene sorbitan monolaurate (Tween-20). Bound phage were eluted by 10 min of incubation with 2 M glycine-HCl, pH 2.2. The eluted phage suspension was immediately neutralized with 1 M Tris base and used to transfet a fresh culture of XL1-Blue cells. An aliquot of the transfected cells was used to determine the titer of antigen-bound Fab–phage particles (carrying plasmid DNA) by plating for ampicillin-resistant colonies. To obtain phage for the subsequent panning cycle, the rest of the transfected cells were superinfected with helper phage VSCM13 grown overnight at 30°C, and progeny phage particles were harvested as described above. After four cycles, E. coli colonies containing individual plasmid clones were toothpicked into SB medium (containing ampicillin and tetracycline) and grown for progeny phage as described above. Binding activity of progeny phage against RG-II/Ova was assayed by indirect ELISA by using sheep anti-M13-biotin (5 Prime–3 Prime, Boulder, CO) and streptavidin–alkaline phosphatase as secondary and tertiary reagents, respectively.

Fab Purification

The M13 coat protein III portion of the heavy chain fusion gene was removed from the selected plasmid by digestion with restriction endonucleases Spel and Nhel, which yield compatible sticky ends (dropout deletion; Barbas et al., 1991). Plasmid DNA was recircularized by ligation and transformed into E. coli XL1-Blue. Soluble Fab protein was produced by growing large-scale cultures of bacteria carrying the resultant plasmid. Cultures for Fab production were grown to high cell density (A600 0.7) in SB medium containing 50 μg/mL ampicillin, induced by addition of IPTG to 0.5 mM, and incubated overnight at 30°C with shaking. Cells were removed by 30 min of centrifugation and the culture medium was filtered through a 0.45-μm filter. The culture medium was concentrated by ultrafiltration (10,000 molecular weight cut-off Durapore membrane; Millipore Corp., Bedford, MA). Proteins were precipitated in 70% (v/v) saturated ammonium sulfate, and the pellet was dissolved in 10 mM Tris, pH 7.5, and dialyzed overnight at 4°C against three changes of the same buffer. This preparation is referred to as crude Fab. Fab was purified by ion-exchange chromatography on a 20 × 170 mm S-Sepharose column (Pharmacia, Uppsala, Sweden; batch elution with 0.5 M NaCl, 10 mM Tris, pH 7.5) followed by affinity chromatography on a Quick Mab mouse κ light chain–specific matrix, according to the manufacturer's instructions (Sterogene Bioseparations Inc., Arcadia, CA).
Characterization of Binding Affinity and Specificity by Competition ELISA

ELISA plates were prepared by coating wells of Immulon 2 polystyrene plates with 1 µg/mL of RG-II-Ova in borate saline buffer (6.19 g of boric acid, 9.5 g of Na₂B₆O₁₉·10H₂O, and 4.29 g of NaCl per L, adjusted to pH 8.5) at room temperature overnight. The wells were then blocked for 1 hr with 2% (v/v) ovalbumin or 5% (v/v) nonfat dry milk (NFDM) in H₂O. Crude Fab preparations were diluted to ~20 ng/mL (in TBLS [100 mM NaCl, 10 mM Tris-Cl, pH 7.5]) containing 0.5% (v/v) NFDM or TBLS containing 0.2% (v/v) ovalbumin and mixed with various concentrations of competitor antigens and incubated at room temperature for 2 hr. These mixtures were applied to the wells, incubated for 30 min at room temperature, and washed four times with TBLS containing 0.05% (v/v) Tween-20. The secondary antibody (goat anti-mouse [k]-horseradish peroxidase [HRP]; Southern Biotechnology Associates, Birmingham, AL) diluted 1:1000 in TBLS containing 0.5% (v/v) NFDM was then applied, incubated for 1 hr at room temperature, and washed six times as before. Fifty microliters of colorigenic solution (100 mM Na₂O₄ in 50 mM sodium acetate, pH 5.2. This solution was incubated for 10 min at room temperature, then diluted into TBLS containing 0.5% (v/v) NFDM and used in competition ELISA.

Fluorescence Microscopy

Eight-day-old sycamore maple suspension-cultured cells were fixed overnight with 5% (v/v) glutaraldehyde in TBLS, deesterified by treatment with 50 mM NaOH at 4°C for 1 hr, and washed with 100 mM sodium acetate, pH 5.5. Cells were blocked for 1 hr with 5% (v/v) NFDM and incubated for 1 hr at room temperature with primary antibody; either partially purified CCRC-R1 (~20 µg/mL) or irrelevant IgG/k antibody was used as negative control (culture supernatant of anti-BSA monoclonal antibody 11F9-F9, isolated in this laboratory). The cells were washed with TBLS over a 75-µm nylon mesh filter and then incubated for 1 hr with secondary antibody: 1:100 goat anti-mouse [k] conjugated to fluorescein isothiocyanate; Southern Biotechnology Associates) before final washing with TBLS. Cells were examined on an epifluorescence optics (Carl Zeiss, Inc., Thornwood, NY). The cells were photographed with T-max 100 film (Kodak, Rochester, NY).

Colloidal Gold Conjugation

Colloidal gold (~15 nm) was prepared by the trisodium citrate reduction method of Frens (1973). The secondary antibody (sheep anti–mouse IgG F(ab´)₂; Pierce) was conjugated to colloidal gold by using a modification of the procedure described by Roth (1985). Briefly, 1 mg of the antibody was resuspended in 100 µL of 200 mM sodium borate, pH 9.0, by centrifugation in an Amicon centrifric-30 microcentrifuge (W.R. Grace & Co., Beverly, MA). Ten milliliters of the 15-nm colloidal gold suspension, adjusted to pH 9.0 with 0.2 M K₂CO₃, was rapidly added to a 15-ml centrifuge tube containing the antibody solution. After a 5-min waiting period, 500 µL of 1% (v/v) polyethylene glycol (P1621; Fisher Scientific) was added to the tube and mixed by repeatedly inverting the tube. The preparation was then centrifuged at 48,000 g for 30 min at 5°C. The mobile pellet was recovered with a minimum of supernatant, resuspended in 500 µL of KBPS (10 mM potassium phosphate buffer, pH 7.0, containing 50 mM NaCl and 0.02% [w/v] polyethylene glycol), and stored until use.

Electron Microscopy

Eight-day-old suspension-cultured sycamore maple cells were either non-alkali-treated or treated with 15 mM NaOH at 4°C. After 1 hr, both alkali-treated and non-alkali-treated cells were rinsed twice (10 min each time) with cold 50 mM potassium phosphate (KBP), pH 6.9. The cells were then fixed in 2.5% (v/v) glutaraldehyde in KBP for 2.5 hr, washed with three changes (15 min each) of KPB and post-fixed for 1 hr with buffered 1% (v/v) osmium tetroxide. The cells were again washed three times (20 min each) with KPB before being dehydrated using a graded aqueous ethanol series (20-35-50-62.5-75-85-95-100-100% [v/v] EtOH; 30 min each). Dehydrated cells were gradually infiltrated with LR White embedding resin (Ted Pella Inc., Redding, CA), according to the following schedule: 20-40-60-80% (v/v) LR White/EtOH each for 24 hr and 100% LR White for 36 hr with a change of resin every 12 hr. Polymerization was catalyzed by exposure to 365-nm UV light for 48 hr. All steps were performed at 4°C.

Thin sections (<100 nm) were cut on an ultramicrotome (MT 6000-XL; RMC Inc., Tucson, AZ) and collected on formvar-coated gold-plated slot grids (Ted Pella Inc.). Immunolabeling of the sections is described below. Sections were poststained for 7 min with 4% (w/v) aqueous uranyl acetate and for 4 min with lead citrate (Reynolds, 1963) and examined at 80 kV with an electron microscope (model EM 902A; Carl Zeiss, Inc.).

Antibody Labeling Procedures for Electron Microscopy

Thin sections mounted on gold grids were hydrated by floating grids, section side down, on 10-µL droplets of KBPS (10 mM potassium phosphate buffer, pH 7.2, containing 50 mM NaCl) for 10 min. Some sections were treated with 15 mM NaOH for 5 min. Nonspecific sites of all sections were blocked for 45 min using 3% (w/v) NFDM in KBPS. The sections were then incubated for 60 min with partially purified CCRC-R1 Fab (~200 µg/mL) followed by a 30-sec rinse with KBPS. Incubation of the sections with sheep anti–mouse IgG F(ab´)₂ conjugated to gold diluted 1:25 (v/v) in KBPS, pH 6.0, was performed for 45 min and followed by 30-sec rinses with KBPS and distilled water.

Analysis of Gold Labeling

The number of gold particles in the cell wall and on other parts of tissue sections were counted for six micrographs of alkali-treated cells and 15 micrographs of non-alkali-treated cells from seven labeling experiments. Those particles that appeared within ~25 nm of either side of the plasma membrane were counted as plasma membrane proximal. Areas were calculated by cutting out the relevant areas of micrographs and weighing them. Densities were calculated for each micrograph; the statistical significance of the differences in average densities was calculated by paired or heteroscedastic two-sample two-tailed Student’s t tests for means (Anderson et al., 1994).
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