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Maize Mesocotyl Plasmodesmata Proteins Cross-React with Connexin Gap Junction Protein Antibodies

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Polypeptides present in various cell fractions obtained from homogenized maize mesocotyls were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotted, and screened for cross-reactivity with antibodies against three synthetic polypeptides spanning different regions of the rat heart gap junctional protein connexin43 and the whole mouse liver gap junctional protein connexin32. An antibody raised against a cytoplasmic loop region of connexin43 cross-reacted strongly with a cell wall-associated polypeptide (possibly a doublet) of 26 kilodaltons. Indirect immunogold labeling of thin sections of mesocotyl tissue with this antibody labeled the plasmodesmata of cortical cells along the entire length of the plasmodesmata, including the neck region and the cytoplasmic annulus. Sections labeled with control preimmune serum were essentially free of colloidal gold. An antibody against connexin32 cross-reacted with a 27-kilodalton polypeptide that was present in the cell wall and membrane fractions. Indirect immunogold labeling of thin sections with this antibody labeled the plasmodesmata mainly in the neck region. It is suggested that maize mesocotyl plasmodesmata contain at least two different proteins that have homologous domains with connexin proteins.

INTRODUCTION

The process of cell-to-cell communication in higher organisms has been suggested to be vital for the control and coordination of cellular biosynthetic, bioenergetic, proliferative, and developmental activities (Carr, 1976; Goodwin, 1976; Lawrence et al., 1978; Loewenstein, 1979, 1981, 1987; Hertzberg et al., 1981; Goodwin and Lyndon, 1983; Warner et al., 1984; Caveney, 1985; Erwee and Goodwin, 1985; Palevitz and Hepler, 1985; Guthrie et al., 1988).

In animals, intercellular communication is achieved by way of specialized structures called gap junctions (Hertzberg et al., 1981; Loewenstein, 1981; Revel et al., 1985; Caspar et al., 1988), whereas in plants it is achieved by way of complex *trans*-wall membranous structures called plasmodesmata (Gunning, 1976; Robards, 1976; Robards and Lucas, 1990). The plasmodesmata are membrane specializations with a cross-sectional diameter of about 40 nm to 60 nm. They are delimited by the plasmalemma, which is continuous from cell to cell (Robards, 1976; Hepler, 1982; Overall et al., 1982; Robards and Lucas, 1990). A strand of endoplasmic reticulum, the desmotubule (Robards, 1976; Overall et al., 1982; Robards and Lucas, 1990) is contained within the tubular structure. The gap junctions in animals consist of clusters of much smaller channels (connexons), each with an outer cross-sectional diameter of about 7 nm (Caspar et al., 1988). The membranes of the adjoining cells are not continuous, and the cell-cell channel is formed by end-to-end interaction between connexons. Structurally, the two systems thus appear to be very dissimilar. Studies with fluorescent probes of different molecular weights have shown that both gap junctions and plasmodesmata have similar exclusion limits (Flagg-Newton et al., 1979; Schwartzmann et al., 1981; Tucker, 1982; Erwee and Goodwin, 1983, 1985; Goodwin, 1983; Terry and Robards, 1987; Meiners et al., 1988). Furthermore, the conductance of both the animal and the plant junctional structures is regulated by similar biochemical mechanisms (Erwee and Goodwin, 1983; Gainer and Murray, 1985; Yada et al., 1985; Baron-Epel et al., 1988; Meiners et al., 1988; Tucker, 1988). Thus, in spite of the structural differences, gap junctions and plasmodesmata have a number of functional similarities (Gunning and Overall, 1983; Meiners et al., 1988).

Gap junctions have been studied intensively and isolated successfully from numerous tissues and sources. They

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have been characterized rigorously and much is known about their biochemical composition and topology (Hertzberg and Gilula, 1979; Hertzberg et al., 1982; Traub et al., 1982; Hertzberg, 1984; Hertzberg and Skibbens, 1984; Kumar and Gilula, 1986; Paul, 1986; Beyer et al., 1987; Dupont et al., 1988; Goodenough et al., 1988; Yancey et al., 1989). In contrast, very little was known until recently about the biochemical composition of plasmodesmata and they have not yet been isolated and characterized.

It was shown recently that cells of various dicotyledonous plants contain a polypeptide of 29 kD that cross-reacts with an antibody to the gap junction protein connexin32 from rat liver (Meiners and Schindler, 1987, 1989). It was suggested that this polypeptide is localized in the plasmodesmata and functions as a junctional protein (Meiners and Schindler, 1987, 1989). The 29-kD plant polypeptide was detected in membrane preparations isolated from protoplasts stripped of their cell walls by cellulases and in total plant extracts (Meiners and Schindler, 1987, 1989). In these studies, performed only with dicotyledons, it was not shown whether the plant polypeptide was a cell wall constituent, as would be expected for a plasmodesmatal protein.

In the present work, performed with the monocotyledon maize, we used antibodies to two different gap junction proteins of the connexin family. Immunoblotting techniques were used to determine whether there are multiple plant proteins exhibiting immunological homology to animal gap junctional proteins of the connexin family. We used immunocytochemistry to determine whether these proteins are components of the plasmodesmata. As probes, we employed an antibody against connexin32, a principle gap junctional protein of rat and mouse liver, and CL-100, EL-186, and CT-360, site-directed antibodies against connexin43, the principle gap junctional protein of rat heart (Laird and Revel, 1990).

We report here that in the mesocotyl of maize seedlings there are at least two proteins, designated PAP26 and PAP27, that are immunologically homologous to the connexin family of proteins. After cell fractionation, PAP26 was found associated only with the cell wall fraction and PAP27 was found both in the cell wall fraction and in the membrane fractions. Both proteins were found to be associated with the plasmodesmata, as determined by immunogold labeling of thin sections visualized by electron microscopy.

RESULTS

Cell Fractionation and Preparation of Washed Cell Walls

Mesocotyl tissue was homogenized in the presence of leupeptin and phenylmethylsulfonyl fluoride and a crude cell wall fraction was separated from the protoplast membranes and proteins by sedimenting at 600*g*, as described in Methods. The protoplast contents were then fractionated by differential centrifugation into heavy and light sedimentable fractions and a soluble fraction. The cell wall-containing fraction was homogenized four times to isolate a cell wall fraction essentially free of cytoplasmic components and to obtain good cell breakage (>99% by examination in a phase contrast microscope). The cell wall fraction was then washed repeatedly until, as shown in Figure 1, the wash exhibited a protein concentration lower than 0.01 OD (as determined by monitoring at 280 nm).

Analysis of Mesocotyl Cell Polypeptide by SDS-PAGE

The polypeptides in each of the fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). As shown in Figure 2, the CBB-stained protein pattern of the washed cell walls (lane 2) is substantially different from that of the other cell fractions (compare lane 2 with lanes 3, 4, and 5).

Immunoblot Analysis of Cell Wall and Protoplast Fractions

Polypeptides in the various fractions were separated by SDS-PAGE and transferred to nitrocellulose paper. Figure 3 shows that when nitrocellulose blots of the various fractions were probed with the connexin43 antibody, CL-100 (raised against the 100–122 peptide of connexin43),

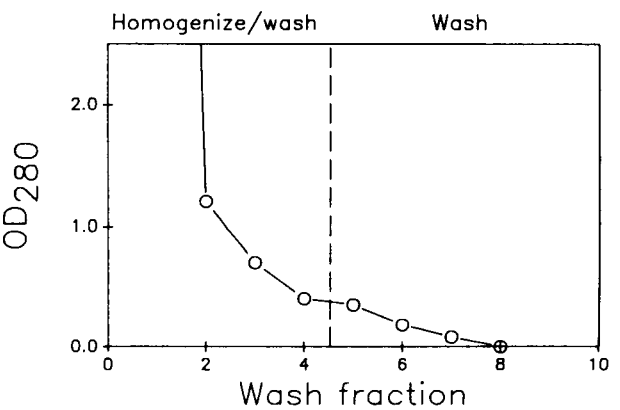

Figure 1. Protein Released during Preparation of Washed Cell Walls.

Relative amount of protein released during four homogenations (left of vertical dashed line) and four washes was determined by measuring the absorbance at 280 nm of the supernatant after each homogenation and wash.

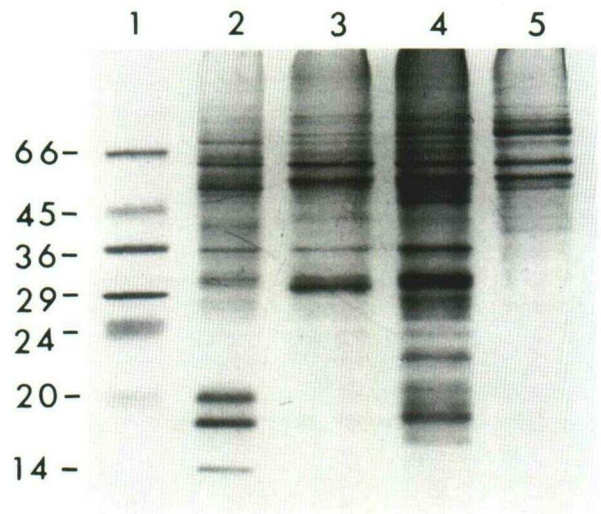

Figure 2. SDS-PAGE Analysis of Polypeptides of Cell Fractions Stained with CBB.

Cell fractions prepared as described in Methods were dissolved in sample buffer at room temperature for 1 hr, and approximately 10 μg of protein per lane were separated on a 1.5-mm-thick, 12.5% SDS-polyacrylamide gel. Lane 1, molecular weight markers; lane 2, washed cell wall fraction; lane 3, heavy membrane fraction; lane 4, light membrane fraction; lane 5, soluble fraction. The molecular weight markers were BSA (66,000); egg albumin (45,000); glycerol-3-phosphate dehydrogenase (36,000); carbonic anhydrase (29,000); trypsinogen (24,000); soybean trypsin inhibitor (20,000); α-lactoalbumin (14,200).

at concentration of 0.6 μg/mL, a strong band (possibly a doublet) exclusively associated with cell wall fraction was observed at 26 kD (lane 2). This staining pattern was not due to mass action because the immunoreactivity did not correspond to any major CBB-stained band (Figure 2, lane 2). Thus, the plant polypeptide appeared to be highly cross-reactive. No additional CL-100 labeling was detected under the conditions employed. It was reported that heating in the presence of SDS and 2-mercaptoethanol caused extreme aggregation of connexin32 from mouse liver (Henderson et al., 1979), but heating of connexin43 from rat heart did not cause aggregation (Manjunath et al., 1985).

Figure 4 shows that solubilizing the plant proteins with sample buffer at room temperature (lane 2) or at 100°C (lane 3) did not cause any significant aggregation of the cross-reacting plant protein. Figure 5 shows that the connexin43 antibodies EL-186, raised against the 186–206 connexin43 peptide (1.7 μg/mL), and CT-360, specific for the 360–382 connexin43 peptide (0.5 μg/mL), did not react detectably with polypeptides in any of the cell fractions or with cell wall proteins (lanes 2 and 4).

Figure 6 shows that when the electrotransferred polypeptides from the various fractions were immunoblotted with a connexin32 antibody at a concentration of 2 μg/mL, the strongest labeling was seen in the light membrane fraction at an apparent molecular mass of 27 kD (lane 5) and in the cell wall fraction at the same molecular mass (lane 3). A weaker response was also seen in the heavy membrane fraction (lane 4), with only a very faint reaction in the soluble fraction (lane 6). Some other weaker bands at higher molecular weights were seen in all mesocotyl cell fractions, notably a moderate strong band around 66 kD (lanes 3 to 6). It is not clear whether these bands are aggregates of the 27-kD polypeptide or different proteins. As a control, the connexin32 antibody labeled a band at 27 kD in a liver gap junction preparation and several presumed aggregates of the 27-kD band (lane 2).

To investigate whether the connexin43 antibody CL-100 and the connexin32 antibodies labeled different cell wall polypeptides, a blot of cell wall proteins was first probed with CL-100 and then reprobed with anti-connexin32. Figure 7 shows that when the blot was first probed with CL-100, only one band at 26 kD was labeled (lane 3). When this blot was reprobed with anti-connexin32, a band exhibiting a slightly higher apparent molecular mass of approximately 27 kD was labeled (lane 2).

Immunoelectron Microscopy

Immunocytochemistry of resin-embedded tissue sections using the CL-100 and CT-360 antisera, affinity-purified

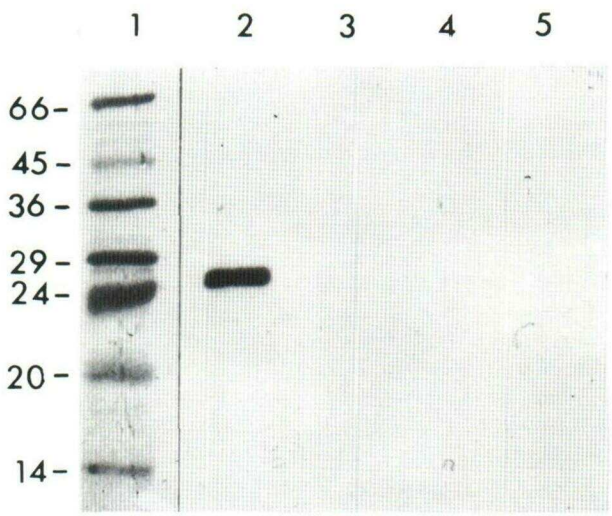

Figure 3. Immunoblot Analysis of Cell Fractions Resolved by SDS-PAGE with Affinity-Purified Connexin43 Antibody CL-100.

Antibody binding was determined by using alkaline phosphatase-conjugated goat anti-rabbit IgG and staining for alkaline phosphatase activity. SDS-PAGE was performed as described in the legend to Figure 2. The protein concentration of each lane was 30 μg. Lane 1, molecular weight markers; lane 2, washed cell wall fraction; lane 3, heavy membrane fraction; lane 4, light membrane fraction; lane 5, soluble fraction.

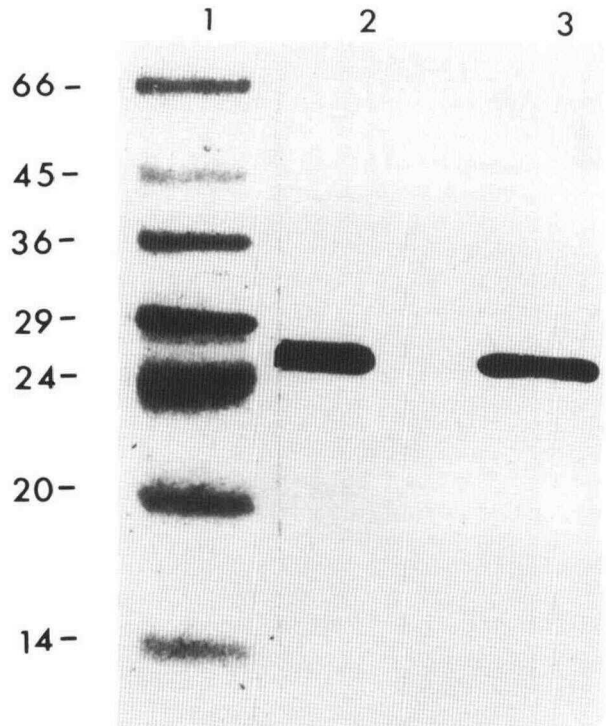

Figure 4. Effect of Sample Boiling in SDS-Sample Buffer on the Apparent Molecular Weight of the Plant Polypeptide Recognized by the CL-100 Antibody.

Proteins in the cell wall fractions were solubilized by incubating the samples in sample buffer for 1 hr at room temperature (lane 2) or by boiling for 3 min (lane 3). Samples were then cleared of insoluble material by centrifugation and loaded on the gel. Electrophoresis and immunoblotting were performed as previously described. Molecular weight standards are in lane 1.

CL-100, and affinity-purified anti-connexin32 was carried out to determine which subcellular structures of the maize mesocotyl contained cross-reactive proteins. It can be seen in Figure 8 that indirect immunogold labeling with CL-100 antisera (Figures 8A and 8B) or affinity-purified CL-100 (Figure 8C) resulted in colloidal gold labeling of the plasmodesmata of various cells. The colloidal gold label occurred over the entire length of the plasmodesmata including the neck region and the cytoplasmic annulus. Although it appeared to occur mostly over the plasmalemma lining the plasmodesmatal channel, its presence over the desmotubules could not be ruled out entirely. Sections labeled with either CT-360 serum (not shown) or rabbit preimmune serum (Figure 8D) were essentially free of colloidal gold except for a few isolated particles over the cell walls and/or nuclei. The immunolabeling of resin-embedded tissue sections with affinity-purified connexin32 antibodies also resulted in colloidal gold over the plasmodesmata (Figure 8E). With this antibody, however, the label appeared to be localized mainly over the exterior regions of the plasmodesmata, mainly in the neck region. A small amount of labeling also occurred over cell walls and plasmalemma.

Standard Electron Microscopy

Portions of typical cortical cells and associated plasmodesmata from etiolated maize mesocotyl tissue are illustrated in Figure 8F for comparison with the tissue prepared for immunocytochemistry. The cells are interconnected by variable numbers of plasmodesmata, each consisting of a plasmalemma-lined canal containing a desmotubule. The desmotubules near the neck regions of the canals appear to be structurally continuous with strands of endoplasmic reticulum in the cytoplasm.

DISCUSSION

In previous studies using various dicotyledons, it was shown that a plant polypeptide present in membrane preparations isolated from protoplasts and from total plant extracts exhibits immunological homology to connexin32 (Meiners and Schindler, 1987, 1989). Although immunofluorescent localization of the putative soybean connexin in culture demonstrated a peripheral labeling at areas of contact between cells (Meiners and Schindler, 1989), it

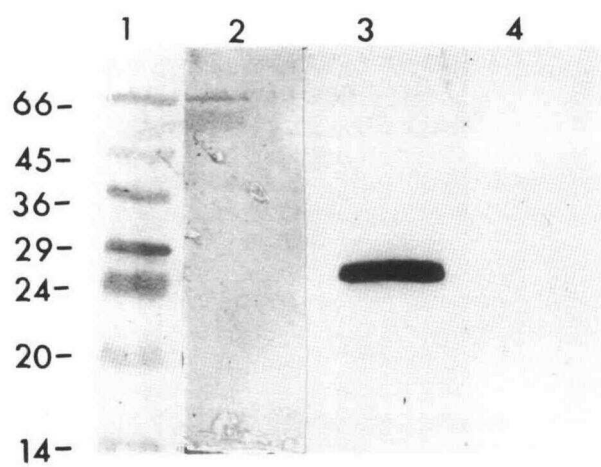

Figure 5. Immunoblot Analysis of Cell Wall Polypeptides with Site-Directed Antibodies against Three Domains of the Heart Connexin43.

Washed cell wall polypeptides were resolved by SDS-PAGE and probed with connexin43 antibodies. Lane 1, molecular weight standards; lane 2, EL-186; lane 3, CL-100; lane 4, CT-360.

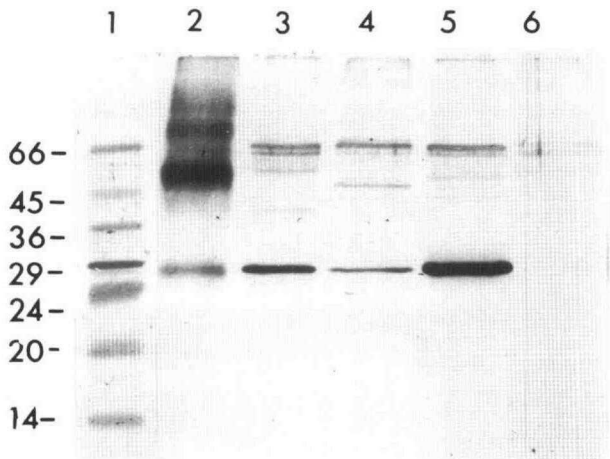

Figure 6. Immunoblot Analysis of Cell Fractions Resolved by SDS-PAGE with Affinity-Purified Antibody against Whole Liver Gap Junction Connexin32.

Lane 1, molecular weight markers; lane 2, 1 μg of liver plaques; lane 3, washed cell walls; lane 4, heavy membrane fraction; lane 5, light membrane fraction; lane 6, soluble fraction.

was not determined to which cell fraction this protein associated. It was suggested that this protein functions as a structural subunit of the plasmodesmata.

In the present study, using etiolated seedlings of the monocotyledon maize, we report on the presence of at least two different plasmodesmatal proteins that have immunologically homologous domains to two members of the connexin family, connexin32 and connexin43. Immunoblots of the polypeptides associated with the different cell fractions demonstrated that one polypeptide of 26 kD, which cross-reacted with an antibody against connexin43, remained associated entirely with the cell wall fraction (Figure 3). When electrotransferred proteins from the different cell fractions were probed with an antibody against connexin32, a second polypeptide of 27 kD was detected. This polypeptide was found to be associated both with the cell wall fractions and with other membrane fractions (Figure 6).

Electron microscope immunocytochemistry using immunogold labeling showed that both antibodies recognize plasmodesmata-associated proteins (Figure 8). We suggest, therefore, that these two plasmodesmatal-associated polypeptides (PAPs) be denoted as PAP26 and PAP27. PAP26 has a molecular mass of 26 kD and is immunologically homologous to connexin43, and PAP27 has an apparent molecular mass of 27 kD and is immunologically homologous to connexin32. Whereas PAP26 appeared to be distributed along the entire length of the plasmodesmata including the neck region and the cytoplasmic annulus, PAP27 appeared to be localized more at the neck region of the plasmodesmata.

After cell fractionation, we found that a large fraction of PAP27 was associated with the membrane fractions, whereas PAP26 was detected only in the cell wall fraction. The polypeptide present in the membrane fractions could be of nonplasmodesmatal origin or it could be a fragmentation product released from the plasmodesmata during cell wall homogenization. Because plasmodesmata contain membranes that extend from the cytoplasm of the cell, it can be expected that after homogenization a part of the plasmodesmata may be ruptured. It is quite conceivable, therefore, that those parts of the plasmodesmata facing the protoplast might be partially lost from the wall fraction to the membrane fractions. The electron microscopy localization studies support the results from the protein gel blot analysis by showing that PAP27 may be localized more at the neck regions of the plasmodesmata than along the entire length of the plasmodesmatal canal (Figure 8E).

In intact rat heart gap junction membranes, the region of the connexin43 molecule that is recognized by CL-100

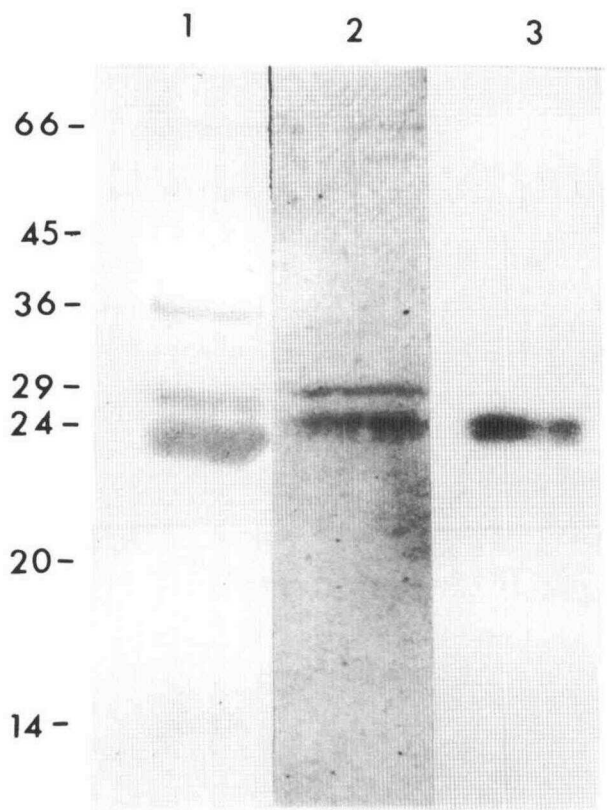

Figure 7. Double Immunoblot Analysis.

Lane 1, molecular weight standards; lanes 2 and 3, polypeptides in washed cell wall fraction resolved by SDS-PAGE, first blotted with CL-100 antibody; lane 2, same polypeptides then reblotted with an antibody against connexin32.

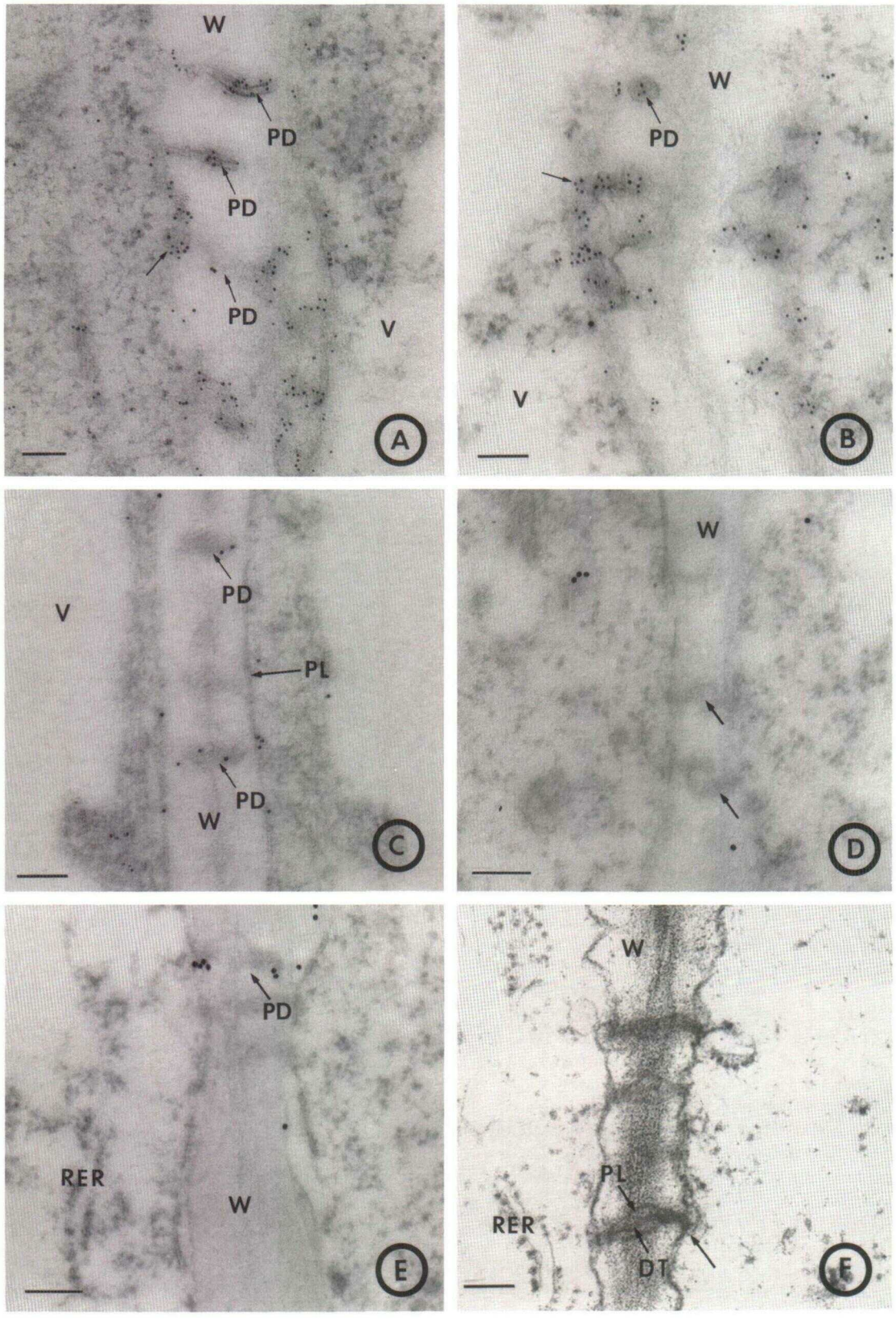

Figure 8. Longitudinal Sections of Plasmodesmatal Aggregates from Maize Mesocotyl Tissue.

has been shown to be exposed to the cytoplasm (Laird and Revel, 1990). However, this region contains sequences that are not shared with other known connexins (Paul, 1986; Beyer et al., 1987, 1990; Zhang and Nicholson, 1989). If one compares the sequences for this region of connexin43 cloned from different sources, rat, human, and chicken, the sequences are nearly identical (Fishman et al., 1990; Musil et al., 1990). The apparent conservation in this region between different animal tissues that express connexin43 (actual sequence data) and between animals and plants (immunological cross-reactivity) suggests that it might be of physiological importance. A further similarity between PAP26 and connexin43 and in contrast to connexin32 is that both PAP26 and connexin43 do not aggregate during sample boiling in the presence of SDS and 2-mercaptoethanol (Henderson et al., 1979; Manjunath et al., 1985; Figure 4).

In contrast to CL-100, the site-directed antibodies EL-186 and CT-360, which recognize an extracellular loop and the C terminus of connexin43 (Laird and Revel, 1990), respectively, did not react with PAP26 (Figure 5) at concentrations that gave good labeling of heart extracts (data not shown). It is obvious that large differences between the PAP26 and connexin43 are expected (at least in some domains) based solely on the difference in the size of these proteins. This could mean that at the relevant sites in PAP26 there is a greater degree of divergence, resulting in no detectable antigenicity. The divergence does not have to be very large for there to be a loss of cross-reactivity because it is known that even though EL-186 binds to a conservative region in the connexin family (Beyer et al., 1987; Laird and Revel, 1990), it is not cross-reactive with connexin32 on protein gel blots (D.W. Laird, unpublished results). However, it is also possible that there is very little homology between PAP26 and connexin43 other than in the region recognized by CL-100. This region is probably involved in regulation, and it is conceivable that the membrane domains are different. In addition, it should be pointed out that our studies with the connexin32 antibody do not allow us to distinguish which regions of PAP27 and connexin32 are homologous. It is possible that we are dealing with homologous regions both in the membrane domains that form the channel and in the cytoplasmic regulatory domains. In contrast, it is likely that there is only a limited degree of homology in certain domains. The determination of the regions of homology and the degree of homology will require sequencing of the respective genes of the plant proteins.

In their studies with cultured soybean cells, Meiners and Schindler (1987, 1989) reported the presence of only one polypeptide that was immunologically homologous to a connexin. In these studies, the extracts were probed with an antibody raised against connexin32 and an antibody raised against a protein isolated from soybean protoplasts. Both antibodies labeled the same band in the soybean extracts. Furthermore, the antibody raised against the plant protein cross-reacted with connexin32. With maize, we also found that the affinity-purified antibody raised against connexin32 only detected one type of polypeptide. In maize, this polypeptide had an apparent molecular mass of about 27 kD (this paper), whereas in soybean the polypeptide that immunostained with the antibody against connexin32 had an apparent size of about 28 kD (Meiners and Schindler, 1987, 1989). The difference may be due to differences between monocots (maize; this work) and dicots (soybean, lettuce, tomato, cucumber, chrysanthemum, rose, petunia, and artichoke; Meiners and Schindler, 1989), or it might be due simply to differences in the isolation technique and/or the electrophoretic technique (Green et al., 1988). We think that the former is probably the case. The maize cell wall peptide, which was detected by the connexin32 antibody, exhibited an identical molec-

Figure 8. (continued).

(A) to **(E)** Plasmodesmata from maize mesocotyl tissue fixed in a solution of 4% *p*-formaldehyde and 0.5% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.2, and embedded in LR White "Hard" resin.

(A) and **(B)** Sections immunolabeled with the CL-100 antisera and goat anti-rabbit IgG conjugated to 12 nm to 15 nm of colloidal gold. Immunolabel is located mostly over the plasmodesmata (PD) with small amounts of background over the cell wall (W) and cytoplasm. Unlabeled arrows indicate label associated with neck regions of plasmodesmata viewed in a glancing section. Vacuoles (V) are free of immunolabel. In **(A)**, Magnification ×38,430; Bar = 0.2 μm. In **(B)**, Magnification ×39,345; Bar = 0.2 μm.

(C) Section labeled with affinity-purified CL-100 antibody and goat anti-rabbit IgG conjugated to 12 nm to 15 nm of colloidal gold. Immunolabel is located mostly over the plasmodesmata (PD). PL, plasmalemma; V, vacuole; W, wall. Magnification ×38,430; Bar = 0.2 μm.

(D) Section labeled with preimmune serum and goat anti-rabbit IgG conjugated to 12 nm to 15 nm of colloidal gold.

(E) Section labeled with affinity-purified connexin32 antibody and goat anti-rabbit IgG conjugated to 12 nm to 15 nm of colloidal gold. A small amount of label is associated with plasmodesma (PD). RER, rough endoplasmic reticulum; W, wall. Magnification ×38,430; Bar = 0.2 μm.

(F) Plasmodesmata in cortical cell wall (W) of maize mesocotyl tissue fixed in glutaraldehyde and osmium tetroxide and embedded in Spurr's resin. Each plasmodesma consists of a plasmalemma-lined canal (PL) containing a desmotubule (DT). Near the neck region of the connection, the desmotubule appears to be structurally continuous with a strand of endoplasmic reticulum in the cytoplasm (unlabeled arrow). RER, rough endoplasmic reticulum. Magnification ×37,515; Bar = 0.2 μm.

ular weight on a gel run with gap junction protein from rat liver (Figure 6). In contrast, the connexin-homologous protein from soybean root cells showed a band that was clearly larger than that of the rat liver gap junctions run on the same gel (Meiners and Schindler, 1987, 1989).

The molecular mass of the connexin43 on SDS-PAGE gel was about 43 kD, whereas the plant protein that cross-reacted with the connexin43 antibody CL-100 was 26 kD. The band detected on the immunoblot appeared as a doublet. The doublet may indicate the presence of two isoforms of the polypeptide. Post-translational modification such as phosphorylation has been shown to be responsible for the different forms of connexin43 in cardiac myocytes (Laird and Revel 1990; Laird et al., 1991) and in embryonic chick cells (Musil et al., 1990). Although antiproteases were used during tissue preparation, we cannot exclude the idea that the plant 26-kD protein is a degradation product of a larger protein. This may be proven only by comparing the sequence of both proteins. Cloning and sequencing also will allow us to determine the degree of homology between the two plant proteins (PAP26 and PAP27) and between the plant proteins and the proteins of the connexin family.

METHODS

Plant Material

Maize seeds (*Zea mays* cv Jubilee, Roger Bros. Co., Idaho Falls, ID) were soaked for 4 hr in running tap water, planted in moist vermiculite, and grown in the dark for 5 days at 25°C. (Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.)

Cell Fractionation and Preparation of Washed Cell Walls

Mesocotyl segments, 1 cm long, excised just below the mesocotyl node, were homogenized for about 5 min using a pestle and mortar in 2 mL/g of tissue homogenization media, pH 8.5 (HM/8.5) consisting of 20 mM Tris-HCl, pH 8.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 20 μg/mL leupeptin. The phenylmethylsulfonyl fluoride (stock solution 500 mM in DMSO) was always added directly to the tissue in the homogenization medium upon commencement of grinding. All steps were performed at 2°C to 4°C. The cell walls were pelleted by centrifuging (Sorvall RC-5, Du Pont Instrument Co.) the homogenate at 600*g* for 5 min in a swinging bucket rotor (Sorval HB-4). The pellet was rehomogenized with the same homogenization media (2 mL/g of tissue) with the exception that the pH was 7.5 (HM/7.5). The cell walls were sedimented by centrifugation as above. Homogenization and centrifugation were repeated four times until more than 99% of the cells were broken, as was determined by phase contrast microscopy. The cell walls were then washed four times in 2 mL/g of tissue HM/7.5. The final pellet was referred to as the washed cell wall fraction. The supernatant from the first homogenization was centrifuged at 4000*g* for 10 min (Sorvall RC 5B, rotor HB-4). The pellet from this centrifugation was referred to as the heavy membrane fraction. The 4000*g* × 10 min supernatant was further centrifuged for 1 hr at 90,000*g* (Beckman LB-55, rotor type TI-45 with inserts for 42 small tubes). The resultant pellet was referred to as the light membrane fraction pellet and the supernatant as the soluble fraction.

Protein Content Analysis

Samples were solubilized in electrophoretic sample buffer (SB), 2.2% SDS, 11% glycerol, 0.05 M Tris-HCl, pH 6.8, and 5.5% 2-mercaptoethanol, final concentration. The three pelleted fractions were suspended per gram of tissue in 500 μL of 1 × SB while the soluble fraction was diluted in 1:1 (v/v) in 2 × SB. Samples were boiled for 3 min and cleared by centrifugation. The protein content of samples was assayed on filter paper according to Marder et al. (1986), but with the omission of the acetone step.

Electrophoresis and Immunoblotting

All samples were solubilized in SB for 1 hr at room temperature or boiled for 3 min as indicated in the text and then cleared of insoluble material by centrifugation at 5000*g* in a microcentrifuge. The washed cell wall and the heavy and light membrane fractions were suspended per gram of tissue in 500 μL of 1 × SB while the soluble fraction was suspended in 1:1 (v/v) in 2 × SB. Polypeptides were separated in a Mighty Small II 7 × 8-cm vertical slab unit (Hoefer Scientific Instruments) by SDS-PAGE, according to Laemmli (1970), with a 1.5 mm thick 12.5% polyacrylamide gel. Approximately 30 μg of protein per lane were loaded on the gel. Molecular weight markers (Sigma, SDS-7) were bovine albumin (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), soybean trypsin inhibitor (20 kD), and α-lactoalbumin (14.2 kD).

The gels were either stained with CBB or electrotransferred to nitrocellulose membranes (pore size 0.45 μm). Electrotransfer was by a modification of the method of Szewczyk and Kozloff (1985) for the electrotransfer of strongly basic proteins. Electrotransfer was for 1 hr at 170 V in 12.5 mM ethanolamine/glycine buffer, pH 9.5, containing 20% methanol and 0.01% SDS using a Mighty Small blotter (Hoefer Scientific Instruments, San Francisco, CA). The lane containing the standards was cut out and stained with Amido Black.

Immunostaining

After electrotransfer, the blots were blocked for 1 hr with 5% milk powder in Tris-buffered saline (TBS buffer: 200 mM NaCl, 50 mM Tris-HCl, pH 7.4), containing 0.02% azide (blocking solution) and incubated overnight at room temperature with the specified affinity-purified antibody at a concentration of 0.5 to 2 μg/mL in blocking solution. After one very short wash with TTBS (TBS containing 0.1% Tween 20) followed by one short wash with TBS and two washes, 10 min each, with TBS, the blots were incubated

for 1 hr with goat anti-rabbit coupled to alkaline phosphatase (Jackson Immuno Research Laboratories, Inc., West Grove, PA) at 1:4000 dilution in TBS. After three 10-min washes with TBS, the alkaline phosphatase was visualized by treatment with 3.3 mg/10 mL *p*-nitro blue tetrazolium chloride, and 1.7 mg/10 mL 5-bromo-4-chloro-3 indolyl phosphate (both reagents from United States Biochemical Co.) in development buffer (100 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, pH 9.5).

Antibodies

Site-directed antibodies employed in this study were generated against synthetic peptides according to the cDNA clone made by Beyer et al. (1987). They were raised and characterized as described previously by Laird and Revel (1990). Briefly, the antibody CL-100 was raised against a cytoplasmic loop peptide (residues 100 to 122), EL-186 was generated against a peptide (residues 186 to 206) believed to be exposed to the extracellular surface, and CT-360 was raised against the cytoplasmically oriented carboxy terminus (residues 360 to 382). These antibodies were affinity purified against the synthetic peptides as described by Laird and Revel (1990). The antibody to mouse liver connexin32 was prepared and affinity purified as described previously by Traub et al. (1982, 1989).

Tissue Preparation for Standard Electron Microscopy

Tissue from the mesocotyl of etiolated maize seedlings was excised with a razor blade, diced into small pieces (about 2 mm^2), fixed for 56 hr in a solution of 4% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2, and placed under a low vacuum. After 3 hr, the fixative was changed and fixation continued for another 3 hr at atmospheric pressure. After fixation, the tissue was washed for 1 hr in 50 mM sodium cacodylate buffer at 25°C and post-fixed in 2% osmium tetroxide in 50 mM sodium cacodylate buffer overnight at 4°C. The tissue was washed for 1 hr in buffer, dehydrated in a cold, graded acetone series and propylene oxide, and embedded in Spurr's epoxy resin (Spurr, 1969). Thin, serial sections were cut with a diamond knife on an American Optical Ultracut ultramicrotome, collected on copper grids, and contrasted with 2% uranyl acetate and lead citrate. The sections were viewed and photographed with a Hitachi HU-11E-1 or H-500 electron microscope (Hitachi, Tokyo, Japan) operating at 75 kV.

Tissue Preparation for Immunocytochemistry

Tissue from the mesocotyl of etiolated maize seedlings was excised as described above and placed in a fixative of 4% *p*-formaldehyde and 0.5% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.2, for 18 hr at 4°C. The fixative was changed three times during this period. The material was washed for 1 hr in 100 mM sodium phosphate buffer, dehydrated in a cold, graded ethanol series, and embedded in L.R. White "Hard" resin at 62°C for 36 hr. Serial sections (see following paragraph) were collected on nickel grids for post-embedment immunocytochemistry. During the course of this study, maize mesocotyl tissue was sampled at six different times using approximately 15 to 20 seedlings for each sample. From each sample, approximately eight tissue blocks were selected for ultrathin sectioning and 20 grids, each with 4 to 5 sections (about 80 nm thick), were cut from the blocks. Approximately 1 mm to 2 mm of tissue was then removed from the blocks and an additional 20 grids with attached tissue sections were prepared. Grids from both levels were then used for the immunolabeling studies described below. Approximately 1000 grids, each with 4 to 5 ultrathin sections, were examined during this investigation.

Post-Embedment Immunocytochemistry

Nickel grids with attached sections were treated with PBST (140 mM sodium phosphate, 500 mM NaCl, 0.3% v/v Tween 20, pH 7.2) with 1% (w/v) BSA for 30 min to prevent nonspecific binding of the antisera. The sections were incubated for 1 hr with either CL-100 or CT-360 antiserum diluted 1:200 with PBST-BSA, or affinity-purified CL-100 at a concentration of 5 μg/mL or affinity-purified anti-connexin32 at a concentration of 0.1 μg/mL diluted in PBST-BSA. After three 10-min washes with PBST, the sections were incubated for 1 hr with goat anti-rabbit IgG conjugated to 12 nm to 15 nm of colloidal gold diluted 1:25 with PBST-BSA. The sections were washed with PBST for 10 min and double-distilled H_2O (3 × 10 min) and post-stained with 2% (w/v) aqueous uranyl acetate. Controls were run in parallel with the antisera incubations using preimmune rabbit IgG in place of the CL-100 and CT-360 antisera. The preimmune serum used was from the rabbit that eventually was used to raise the CT-360 antibody.

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