3,4-Dehydroproline Inhibits Cell Wall Assembly and Cell Division in Tobacco Protoplasts

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We investigated the function of cell wall hydroxyproline-rich glycoproteins by observing the effects of a selective inhibitor of prolyl hydroxylation, 3,4-dehydro-l-proline (Dhp), on wall regeneration by Nicotiana tabacum mesophyll cell protoplasts. Protoplasts treated with micromolar concentrations of Dhp do not develop osmotic stability and do not initiate mitosis. The architecture of regenerated cell walls was examined using deep-etch, freeze-fracture electron microscopy of rapidly frozen tobacco cells. Untreated protoplasts assemble a dense fibrillar cell wall consisting of laterally associating subelementary fibrils. In contrast, treatment of protoplasts with Dhp alters the structure of the regenerated wall fibrils in several ways: first, the microfibrils are coated with globular knobs; second, some larger fiber bundles have an open ribbon-like appearance; and third, the smallest subelementary fibrils were not visible. Tobacco cells develop an abnormal morphology as a consequence of this abnormal cell wall structure. Thus, inhibition of prolyl hydroxylation results in the regeneration of a cell wall with abnormal structural and functional properties. These data provide experimental evidence that hydroxyproline-rich glycoproteins are important for the structural integrity of primary cell walls and for the correct assembly of other wall polymers, and that wall structure is an important regulator of cell division and cell morphology.

The structure of Hyp-rich molecules is the result of considerable posttranslational processing of the polypeptide gene product. In carrot extensin, for example, most of the Pro residues are hydroxylated by prolyl hydroxylase, and the resulting Hyp residues serve as a major site for oligosaccharide decoration. Because of this unique biosynthetic pathway, the use of specific inhibitors of prolyl hydroxylation could provide a valuable approach for the study of the function of Hyp-rich wall polymers. Such inhibitors should be particularly effective for HRGPs because hydroxylase inhibitors would also block the attachment of carbohydrate side chains, which have been shown to be partial determinants of HRGP structure (van Holst and Varner, 1984; Stafstrom and Staehelein, 1986).

We previously found that one Pro analog, Dhp, is a potent inhibitor of prolyl hydroxylase in plant cells (Cooper and Varner, 1983). Unlike most other Pro analogs, Dhp acts in vivo at micromolar concentrations to rapidly and irreversibly inactivate prolyl hydroxylase. Dhp-treated carrot root cells continue to synthesize and secrete structurally abnormal extensin HRGPs that might be expected to function abnormally. Dhp treatment also caused the disappearance of the major RPRP from the cell wall of cultured soybean cells, even though the RPRPs are hydroxylated but not glycosylated (Schmidt et al., 1991). Importantly, Dhp is a relatively selective inhibitor of prolyl hydroxylation. Dhp treatment had little effect on enzyme induction or other metabolic processes in wounded carrot root cells (Cooper and Varner, 1983), and studies with an Escherichia coli Pro auxotroph demonstrated that Dhp was the only Pro analog tested that could replace Pro in the synthesis of enzymically active acid phosphatase (Morris and Schlesinger, 1972).

Cell wall regeneration by plant cell protoplasts is a useful system for investigating the biosynthesis, structure, and function of primary plant cell walls because de novo synthesis and assembly of primary cell wall polymers can be synchronously induced in large cell populations. In the case of many plant species including tobacco, protoplasts regenerate a cell wall competent to support continued cell divisions. Cell wall regeneration has thus been investigated in many structural and biochemical studies (Nagata and Takebe, 1970; Hanke and Northcote, 1974; Willison and Cocking, 1975; Burgess and Linstead, 1976; Herth and Meyer, 1977; Willison and

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Abbreviations: AGP, arabinogalactan protein; Dhp, 3,4-dehydro-l-proline; HRGP, hydroxyproline-rich glycoprotein; RPRP, repetitive proline-rich protein.
Grout, 1978; Blashek et al., 1981; Klein et al., 1981; Shea et al., 1989; Mock et al., 1990). Since both extensins and AGPs are synthesized and secreted by protoplasts during wall regeneration (Tanaka and Uchida, 1979; Mock et al., 1990), this system allowed us to investigate the possible role(s) of HRGPs in cell wall structure by examining the effects of Dhp on wall assembly and protoplast development.

MATERIALS AND METHODS

Protoplast Isolation and Culture

Leaf mesophyll protoplasts of *Nicotiana tabacum* (cvs Xanthi and Havana) were isolated and cultured by the method of Maliga (1982). Briefly, leaves of greenhouse-grown plants were surface sterilized and digested in the dark in a modified K3 medium (Maliga, 1982) containing 1.0% cellulysin (Calbiochem), 0.2% macerase (Sigma), and 0.5 M osmoticum (Suc or sorbitol) until mesophyll protoplasts were released. Protoplasts were purified by filtering through either four layers of cotton gauze or 200-μm nylon mesh followed by flotation through 20% (w/v) Suc to remove broken cells and cell wall debris. Purified protoplasts were washed once with W5 (Maliga, 1982), then washed and cultured at a density of 1 to 4 × 10^5 cells/mL in K3 medium supplemented with 0.5 M Glc, 100 mg/L Xyl, 100 mg/L Ara, 0.1 mg/L 2,4-D, 0.2 mg/L benzylaminopurine, and 1.0 mg/L naphthalene acetic acid (Maliga, 1982). Filter-sterilized Dhp (Sigma) was used at a concentration of 50 μM. Fresh Dhp was added every 24 h (for 3-d experiments) or every 48 h (for 14-d experiments) because this inhibitor has a functional half-life of 24 h in vivo (Cooper and Varner, 1983).

Microscopy

Protoplast development was routinely monitored at low magnification with an inverted microscope and at high magnification using phase contrast and Nomarski optics on a Zeiss photomicroscope. For freeze-fracture studies, protoplasts were lightly fixed for 15 min with 2.4% glutaraldehyde in culture medium, then medium was washed away with five 3-fold dilutions with dH2O. This was followed by three complete washes with dH2O and one wash with 10% methanol (as a cryoprotectant) (Boniella, 1983). Cells were layered onto pulverized mica flakes and rapidly frozen to 4 K using the slam-freezing apparatus designed by Heuser (1980, 1983). Quick-frozen samples were fractured and deep-etched in a modified Balzers apparatus and rotary shadowed at 24 K with platinum/carbon, and the replicas were processed as described (Heuser, 1980). Micrographs were taken with a JEOL 200 electron microscope equipped with a goniometer stage.

RESULTS

Dhp Treatment Inhibits Protoplast Development

Freshly isolated tobacco protoplasts are osmotically fragile spheroplasts that lack a cell wall (Fig. 1A). After 2 to 4 d, untreated cells regenerated a primary cell wall and elongated slightly, and more than 80% of untreated protoplasts initiated mitoses. The formation of transvacuolar strands and the cytoplasmic streaming through these strands were both unaffected by Dhp treatment (Fig. 1C, arrowheads). Figure 1D shows an aggregation of plastids around the nucleus observed in Dhp-treated cells after 7 to 10 d. Spheroplasts treated with 50 μM Dhp every 48 h remained osmotically fragile for at least 14 d. The structural instability of cell walls regenerated in the presence of Dhp was indicated by the immediate lysis of Dhp-treated cells caused by decreasing the osmoticum in the culture medium from 0.5 to 0.4 M. At least 85 to 90% of control (untreated) cells withstood this mild hypoosmotic shock after 36 h of wall regeneration. Both cytokinesis and nuclear division were also blocked by Dhp treatment.

Dhp Treatment Modifies the Architecture of Regenerated Cell Walls

We used the rapid-freezing technique, which avoids extensive aldehyde fixation (Heuser, 1980), to observe the architecture of cell walls regenerated by tobacco mesophyll protoplasts. Electron micrographs of replicas prepared by rapid freezing and deep etching revealed the absence of wall polymers on the plasma membrane surface of freshly isolated protoplasts (Fig. 2A). In contrast, Figure 2B shows the mat of fibrils assembled on the surface of untreated protoplasts cultured for 3 d. These microfibrils, with diameters of 8 to 10 nm (large arrowheads), intertwine randomly to form larger 25- to 34-nm fibril bundles. The 8- to 10-nm microfibrils are...
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Figure 2. Cell-surface architecture of untreated and Dhp-treated tobacco protoplasts. A, Deep-etch, freeze-fracture electron micrograph of a freshly isolated tobacco mesophyll protoplast (i, amorphous ice; pm, plasma membrane). B, Deep-etch, freeze-fracture electron micrograph showing cell wall fibrils on the surface of untreated protoplast after 3 d in culture. Large arrowheads indicate 8- to 10-nm microfibrils; medium white arrowhead indicates 3- to 4-nm "subelementary" fibril; small triple arrowheads indicate regularly spaced orthogonal interfibril cross-links. C and D, Deep-etch, freeze-fracture electron micrographs showing the surface of Dhp-treated tobacco protoplasts after 3 d. Note the irregular knobs coating microfibrils in C and D, and the ribbon-like appearance of some large fiber bundles (arrowheads) in D. E to H, Higher magnification views of irregular knobs observed along wall fibrils synthesized by Dhp-treated protoplasts. Note regular spacing of irregular knobs in H. Scale bar represent 400 nm in A to D and 100 nm in E to H.

Dhp treatment alters the morphology of wall fibrils synthesized by regenerating tobacco protoplasts. As shown in Figure 2, C and D, deep-etch surface replicas of rapidly frozen, Dhp-treated protoplasts cultured for 3 d showed microfibrils coated with large irregularly shaped knobs (up to 50 nm in diameter). These knobs have no discernible substructure (Fig. 2, E–H), and in some cases occur at regularly spaced intervals along the fiber axis. Cell walls regenerated by Dhp-treated protoplasts also lack the 3-nm subelementary fibrils and the interfiber cross-links seen in replicas of control cell walls. In some cases, the largest wall fibers, which appear as solid bundles of microfibrils in untreated protoplasts (Fig. 2B), are altered to display an open ribbon-like structure (Fig. 2D).

Dhp Treatment Alters the Morphogenetic Potential of Cultured Protoplasts

The cell wall regenerated by untreated tobacco mesophyll cell protoplasts is functionally competent to support contin-
used cell division. In more than 50% of these untreated cells, mitoses led to the formation of actively dividing clusters of cells after 14 d (Figs. 1A and 3A). In contrast, about 90 to 95% of mesophyll protoplasts treated for 3 d with 50 μM Dhp (at 0, 24, and 48 h) expanded greatly but never initiated mitosis (Fig. 3B). A few Dhp-treated cells divided slowly and developed into clumps of huge, elongated, sausage-shaped cells (Fig. 3, C and D). Protoplasts that were treated for 6 d with Dhp expanded greatly but did not initiate cell divisions (Fig. 3, E and F). These 6-d treated protoplasts developed an extremely thick irregular cell wall with strands of wall material extruded into the culture medium (Fig. 3E). Occasionally, 6-d treated cells gave rise to subprotoplasts by budding (Fig. 3F).

**DISCUSSION**

**Regenerating Wall Architecture Revealed by Rapid-Freeze/Deep-Etch EM**

Numerous studies have used deep-etch, freeze-fracture EM to investigate the ultrastructure of regenerated cell walls (Willison and Cocking, 1972, 1975; Grout, 1975; Willison and Grout, 1978). However, all of these previous studies have examined cells fixed extensively with glutaraldehyde, which may act to artifactually alter wall architecture. As seen in these previous studies, untreated protoplasts assemble a dense mat of fibrils, presumably cellulose microfibrils, on their surface (Willison and Cocking, 1972, 1975; Herth and Meyer, 1977; Willison and Grout, 1978; Klein et al., 1981). The smallest fibrils revealed using the rapid-freezing technique are about 3 to 4 nm in diameter. Since the thickness of the platinum coating is about 2 nm (Heuser, 1983), these smallest fibrils have the dimensions of the subelementary fibrils (Franke and Ermen, 1969; Hanna and Côte, 1975), which previously have been observed in regenerated cell walls only by negative staining (Herth and Meyer, 1977). The failure of previous workers to visualize subelementary fibrils on the surface of freeze-fractured protoplasts may be due to their use of prolonged glutaraldehyde fixation or to ice-crystal formation associated with conventional freezing methods.

The molecular architecture of regenerated cell walls assembled by untreated protoplasts resembles the wall organization observed in deep-etch micrographs of “real” cell walls in several important respects (McCann et al., 1990; Satiat-Jeunemaitre et al., 1992; J.B. Cooper and J.E. Heuser, unpublished data). Both consist primarily of 8- to 10-nm fibers, presumably cellulose fibers, which laterally associate to form larger fiber bundles. Regular cross-links between the 8- to 10-nm fibers were observed in images of regenerating cell walls (Fig. 2B, triple arrowhead), indicating that the formation of these ladder-like structures between wall fibers is a feature of normal cell wall regeneration. Both the length of these cross-links (10–15 nm) and their spacing along the fibers (20–30 nm apart) are similar to the analogous cross-links observed in monocot cell walls that are thought to be composed of xyloglucan (McCann et al., 1990; Satiat-Jeunemaitre et al., 1992). The primary difference between the deep-etch images of regenerating protoplast walls and “normal” cell walls is an overall lower density of 8- to 10-nm fibers in regenerating walls. A consequence of this lower fiber density is the very large apparent pore size in regenerating walls, roughly measured between 30 and 200 nm compared with 10 to 20 nm in normal cell walls (McCann et al., 1990; J.B. Cooper and J.E. Heuser, unpublished data). Our images of wall fibers on the surface of regenerating protoplasts indicate that each 8- to 10-nm fiber is composed of laterally associating smaller fibrils that are themselves composed of laterally associating subelementary fibrils. Furthermore, each smaller fibril can associate with more than one 8- to 10-nm fiber.

**Hyp Biosynthesis Is Required for Development of Wall Tensile Strength and for Mitosis**

Tobacco mesophyll cell protoplasts have provided an excellent model system for investigating the assembly and function of primary cell walls. As originally described by Nagata and Takebe (1970), within about 36 h untreated tobacco protoplasts develop a fibrillar cell wall that stains brightly with Calcofluor and withstands hypoosmotic shock. Treatment of regenerating protoplasts with a selective inhibitor of prolyl hydroxylase severely inhibited protoplast development. Treated cells remained as osmotically fragile spheroplasts for up to 2 weeks. All of the treated cells lysed immediately with a relatively modest decrease (20%) in the osmolarity of the culture medium. This result indicates that posttranslational hydroxylation of HRGP precursors is re-

**Figure 3.** Morphological consequences of abnormal wall architecture. A, Dividing cell clumps formed by untreated tobacco protoplasts after 14 d (bar = 200 μm). B, Enlarged cell morphology observed in about 40% of the cells 2 weeks after a 3-d pulse treatment with 50 μM Dhp (bar = 200 μm). C and D, Elongated cell clumps formed 14 d after a 3-d pulse treatment with 50 μM Dhp (bar = 200 μm). E and F, Abnormal “monster” cells produced 2 weeks after protoplasts were pulse treated for 6 d with 50 μM Dhp showing disorganized cell wall material extruded into the culture medium (bar = 100 μm).
quired for the assembly of a cell wall that can withstand the physical stresses exerted by a turgid plant cell. Two simple interpretations for these data are that (a) (at least) one class of HRGPs serves as an important load-bearing element in primary cell walls, and that this element is absolutely required for the walls' structural integrity; or (b) secretion of abnormal HRGPs has a secondary effect on the synthesis, secretion, and/or assembly of other structurally important wall polymers. Our data support the hypothesis of Shea et al. (1989) that noncellulosic wall polymers (e.g. HRGPs) may be involved in the acquisition of tensile strength by regenerating plant protoplasts.

An important measure of the functional integrity of regenerated cell walls is the capacity to support continued cell divisions. Inhibition of peptidyl Pro hydroxylation by Dhp blocks the initiation of mitosis in tobacco mesophyll protoplasts. This inhibition of cell division by Dhp may explain the observed inhibitory effect of Dhp on growth of soybean cell cultures (Schmidt et al., 1991). Our results on mitotic inhibition by Dhp differs significantly from results obtained by treating tobacco protoplasts with inhibitors of cellulosic biosynthesis (coumarin and 2,6-dichlorobenzonitrile). Cellulose synthesis inhibitors block cytokinesis without affecting nuclear division, thus leading to the formation of multinucleate cells (Meyer and Herth, 1978; Galbraith and Shields, 1982). Our data support and extend the hypothesis that the cell wall is required for cell division in protoplasts (Meyer and Abel, 1975; Shilde-Rentshler, 1977). The observed inhibition of mitosis caused by Dhp treatment is probably not simply a consequence of generally altered wall structure, since dichlorobenzonitrile-adapted cells with a radically altered wall organization (i.e. no cellulose) grow and divide normally (Shedletzky et al., 1990).

In one respect, the organization of the cytoplasm in Dhp-treated cells is different from that seen in untreated control cells: the plastids in treated cells aggregate around the nucleus (Fig. 1D). The formation of these plastid "rosettes" was also observed in protoplasts treated with cellulose synthesis inhibitors (Meyer and Herth, 1978).

HRGPs May Function to Organize Other Cell Wall Polymers

Inhibition of Pro hydroxylation by Dhp in regenerating tobacco mesophyll protoplasts caused a distinct alteration in the architecture of regenerated cell walls. Dhp treatment led to the appearance of large globular knobs along the length of the wall fibers and to the disappearance of the smaller (<8 nm) wall fibers and interfiber cross-links. These globular knobs had an irregular morphology and showed no consistent substructure (Fig. 2, E–H). The regular spacing of these knobs along the fiber axes resembles the spacing of the orthogonal interfibril cross-links (McCann et al., 1990). It is tempting to speculate that the loss of these cross-links and the lack of structural integrity in Dhp-treated cell walls are related. The occurrence of open, ribbon-like fiber bundles was sometimes observed in Dhp-treated protoplast walls (Fig. 2D). Similar ribbons of cellulose microfibrils were previously observed in cell walls assembled by tobacco protoplasts cultured in a high-salt medium that also inhibited continued cell division (Herth and Meyer, 1977). Perhaps culturing protoplasts in saline medium inhibits the synthesis and/or secretion of HRGPs, which are necessary for the formation of proper cellulose fiber bundles and for continued cell division. The most direct interpretation of our results is that HRGPs are important for the normal assembly of other cell wall macromolecules, and that HRGPs may play a role in modulating the lateral association of the smallest wall fibrils. This result provides experimental support for the hypothesis of Shea et al. (1989) that gel matrix polymers in the regenerating cell wall could help organize cellulose fibers.

HRGPs and Wall Architecture Determine the Morphology of Plant Cells

The morphology of tobacco cell cultures was radically and irreversibly modified by Dhp treatment. Rather than developing into dividing clumps of ellipsoid-shaped cells, most Dhp-treated protoplasts developed into enormous, irregularly shaped "monster" cells. At low frequency, clumps of large, elongated, and slowly dividing cells were observed (Fig. 3, C and D). Our observations contrast with the data of Schmidt et al. (1991), which showed no effect of Dhp treatment on the morphology of cultured soybean cells. A consideration of the recovery kinetics from Dhp treatment provides the simplest explanation for this difference. Since plant cells recover the capacity for prolyl hydroxylation with a half-time of 24 h (Cooper and Varner, 1983), our experiments used a pulse-treatment protocol (once every 24 h for short-term experiments, or once every 48 h for long-term experiments). Schmidt et al. (1991) utilized a single Dhp treatment at 0 h and observed no long-term consequences on cultured cell morphology.

It is interesting to note that the morphology of the "monster" cells that develop from Dhp-treated protoplasts closely resembles the cell morphology observed when soybean suspension culture cells were treated with colchicine (Umetsu et al., 1975). We speculate that the organization of extracellular polymer networks is structurally coupled with the organization of the cortical cytoskeleton, perhaps through integrin-like or vitronectin-like cell-surface molecules (Zhu et al., 1993). In this context, our results are consistent with a role for extensin HRGPs in stabilizing cortical microtubules (Akashi et al., 1990). Budding cell protoplasts, observed occasionally in 6-d Dhp-treated cultures, have previously been observed as a consequence of abnormal cell wall development (Meyer and Herth, 1975). Our data provide strong support for the idea that the cell wall architecture assembled during the first few days of protoplast culture is a critical determinant of the developmental potential of plant protoplasts.

Functional Significance of HRGPs

More than three decades after the discovery of Hyp as a constituent amino acid of cell wall polypeptides, the potential functions of each of the several distinct classes of Hyp-rich proteins, glycoproteins, and proteoglycans remain speculative (Fincher et al., 1983; Cassab and Varner, 1988; Varner and Lin, 1989; Roberts, 1990; Marcus et al., 1991; Showalter, 1993). We used a selective inhibitor of prolyl hydroxylase, Dhp, to investigate the function of HRGPs synthesized during cell wall regeneration by tobacco mesophyll protoplasts. Interpretations of such pharmacological experiments depend.
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LITERATURE CITED

Hanna RB, Cote WA (1975) The sub-elementary fibril of plant cell wall cellulose. Cytobiology 10: 102–116
Scheide-Rentschler L (1977) Role of the cell wall in the ability of tobacco protoplasts to form callus. Planta 135: 177–181
Willison JHM, Cocking EC (1972) The production of microfibrils at the surface of isolated tomato-fruit protoplasts. Protoplasma 75: 397–403