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Communication

Peptidyl Proline Hydroxylation and the Growth of a Soybean Cell Culture[1]

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ABSTRACT

Peptidyl proline hydroxylase inhibitors block the growth of cultured soybean (*Glycine max*) cells and bring about the disappearance of the major salt-extractable hydroxyproline-rich protein, the 33 kilodalton repetitive proline-rich protein (RPRP2). Three polypeptides of 28, 20, and 14 kilodalton that cross-react with an antibody to RPRP2 accumulate in the culture during steady-state growth. In the presence of the proline hydroxylase inhibitors, all of these repetitive proline-rich proteins disappear. These results indicate that the hydroxyproline-rich proteins play a role in cell growth, and that hydroxylation may regulate the steady-state level of at least one of these proteins by influencing its turnover.

Hydroxyproline-containing proteins are found throughout the plant kingdom, generally associated with the extracellular matrix (9). Some of these proteins have been solubilized and characterized (12, 17), and while morphoregulatory roles have been suggested, there is only limited supportive evidence (discussed in ref. 18). Considering that these proteins might be functionally dependent on the hydroxylation of their prolines, we have tested the effect of two specific inhibitors of prolyl hydroxylase (3, 16) in a soybean cell culture. We report here that the inhibition of peptidyl proline hydroxylation brings about the cessation of cell growth, accompanied by the disappearance from the culture of the major salt-extractable hydroxyproline-rich proteins, the RPRPs[2] (2). These results suggest a role for hydroxyproline-rich proteins in regulating cell growth.

MATERIALS AND METHODS

Aliquots of a soybean (*Glycine max*) cell culture, grown as previously described (2), were centrifuged at appropriate times to separate medium and cells. The medium was precipitated overnight with 4 volumes of ethanol, the precipitate washed once with 80% ethanol and extracted in 150 mM LiCl, 20 mM Tris·HCl (pH 8), 2.5 mM $NaHSO_3$ (0.5 mL per 5 mL medium). The cells were collected on a coarse scintered funnel, washed thoroughly with 10 mM Tris·HCl (pH 8), 2.5 mM $NaHSO_3$, frozen in liquid N_2, and stored at −70°C. Growth of the culture was determined from the fresh weight of the cells obtained before freezing. To separate cell walls and cytoplasm, the frozen cells were broken in a polytron in five volumes of 0.25 M sucrose, 40 mM Tris·HCl (pH 8), 3 mM EDTA, 5 mM dithiothreitol, 0.5 mM *p*-toluenesulfonylfluoride. The cell walls were collected on a coarse-frit glass funnel (Millipore XX1004700), and the cytoplasm was clarified by centrifuging for 1 h at 100,000*g* in a Spinco ultracentrifuge. The cell walls were washed several times with 10 mM Tris HCl (pH 8), 2.5 mM $NaHSO_3$, and sonicated in 3 mL (per 0.6–0.7 g of initial cells) of 50 mM HCl, 0.2 M $CaCl_2$, 2.5 mM $NaHSO_3$. Extraction was continued for 3 h at room temperature, the insoluble residue was removed by filtration on nitrocellulose and the extract was neutralized with 1.2 M Tris base. Aliquots were run on SDS-16% acrylamide gels together with several levels of purified RPRP2 (4). The gels were transferred to Immobilon-P (Millipore Co.) in AMPSO buffer pH 9.8 (19) and probed with a rabbit polyclonal antibody (1→2000 dilution) made against the 33 kD RPRP (4). The procedure allowed the detection of 0.4 ng of RPRP and was linear (by visual estimate) to 4 ng. The specificity of the antibody was established by the absence of cross-reaction with a blot containing 100 ng of soybean extensin (2). The HCl-$CaCl_2$ cell wall extracting solution was chosen when it was found to provide a yield of RPRP2 at least equivalent to that obtained with 0.2 M $CaCl_2$, 10 mM Tris (pH 7.6) in extractions of several tissues including log and stationary phase cells in culture, 31 h soybean axes, and hypocotyl of 5 d seedlings. The nitrocellulose filter did not absorb any RPRP2 and the filtration was important in removing fine particulate matter.

RESULTS

Growth of the Cell Culture

To test for a role of the hydroxyproline-rich proteins in cell growth, we supplemented log phase cells of the soybean cell culture either with 3,4-dehydroproline or with ethyldihydroxybenzoate. The proline analog, originally studied as an inhibitor of collagen biosynthesis (8, 14, 15), has been shown in limited studies with mung bean seedlings (6) and more completely in experiments with slices of carrot (3) to be a specific inhibitor of proline hydroxylation, probably by its incorpo-

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[2] Abbreviations: RPRP, repetitive proline-rich protein; EDHB, ethyl-3,4-dihydroxybenzoate.

Table I. *Effect of Dehydroproline and Ethyl Dihydroxybenzoate on the Growth of Soybean Cells in Culture*

In each of the experiments, 20 mL of 5 or 6 d cells having a fresh weight between 250 and 500 mg were incubated as indicated. Cells were harvested at the time periods noted, washed, and their fresh weight was determined. The data are given as the change in fresh weight relative to a similar aliquot harvested at the start of the incubation period.

Treatment			Fresh Weight Change	Inhibition
Experiment	Time of incubation	Additions		
	h		*mg*	%
1	48	25 μM dehydroproline	264	40
		75 μM dehydroproline	145	67
		100 μM dehydroproline	66	85
		Control	440	
2	28	300 μM EDHB	0	100
		Control	158	
3	48	100 μM EDHB	330	15
		200 μM EDHB	116	70
		300 μM EDHB	39	90
		Control	390	

ration into protein in place of proline with the subsequent formation of a stable dehydroproline peptide-enzyme complex (3). Dihydroxybenzoic acid, in studies with the animal proline hydroxylase, has been found to interact at the binding sites of α-ketoglutarate and ascorbic acid, two of the reactants in the proline hydroxylation reaction (11). The reagent, in the form of the diethyl ester (EDHB), strongly inhibited collagen production in a fibroblast culture (16). The data of Table I show that the addition of either of these reagents causes the cessation of growth of the soybean cell culture in a concentration-dependent manner. The levels required for essentially complete inhibition are similar to those needed for the inhibition of collagen synthesis in animal cells. We examined soybean cells treated by both EDHB and dehydroproline to see if there were any obvious changes in morphology. Figure 1 shows that after 17 h of EDHB treatment the cell walls are intact and that the cells are indistinguishable from those of a control culture. A similar picture was seen after 48 h with both EDHB and with dehydroproline (data not shown).

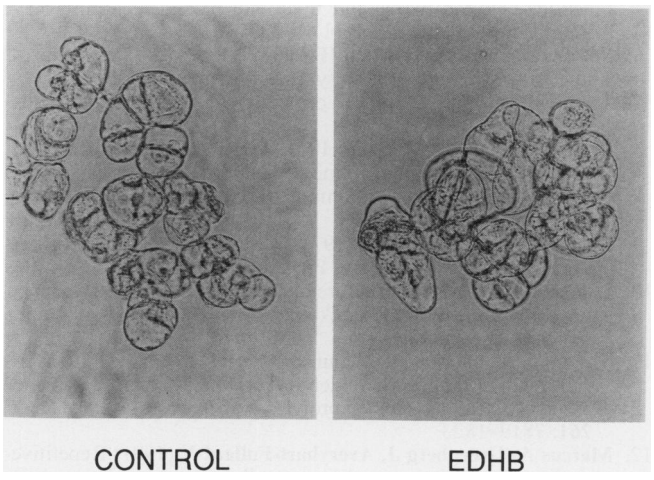

Figure 1. Comparison of 6 d soybean cells by phase contrast microscopy. A suspension of cells (either control or after 17 h in 0.3 mM EDHB) was viewed at a total magnification of ×400 in a Zeiss Axiophot photomicroscope. Photographs were taken using Kodak TX-400, 35 mm film.

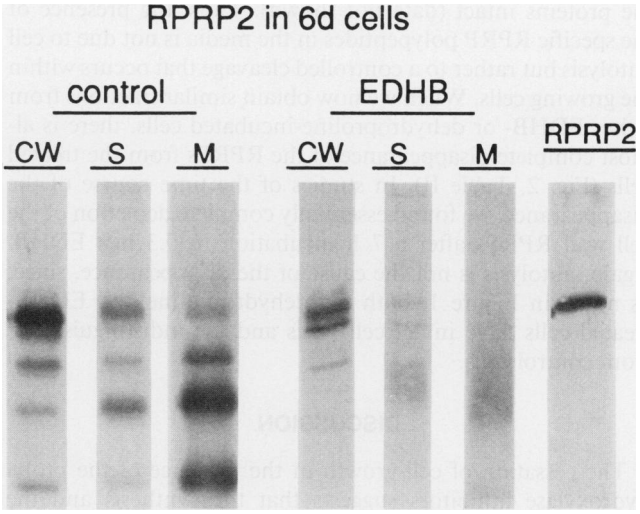

Figure 2. RPRP analysis of soybean cells in culture. Aliquots of the different fractions were run on SDS-acrylamide gels, blotted, and analyzed with anti-RPRP2 antibody as described in "Materials and Methods." The cell wall aliquots (CW) were 0.4 and 3.2 μL, respectively, for control 6 d cells and cells treated for 17 h with 0.2 mM EDHB, with 1 mL of extract equivalent to the walls from 7 mL of cells. The cytoplasmic fractions (S) correspond to 0.8 and 4 μL, respectively, with 1 mL of S representing cytoplasm from 7 mL of cells. The aliquots of the medium were 0.4 and 1.6 μL with 1 mL corresponding to the extract of an 80% ethanol precipitate of 10 mL of medium.

Changes in RPRP2

The predominant hydroxyproline-rich protein of soybean cells is the 33 kD RPRP2. (This protein was previously referred to as RPRP3, with RPRP2 relegated to a minor 28 kD component having the same amino acid distribution [4]. Our data [Fig. 2] suggest that the 28 kD protein is a cleavage product of the 33 kD protein. We therefore now refer to the primary 33 kD gene product as RPRP2.) To test the effect of the proline hydroxylation inhibitors on the endogenous levels of this protein, we incubated cells in the presence of either dehydroproline or EDHB, fractionated the cultures into media, cell walls, and cytoplasm and analyzed the fractions for their RPRP2 content. Figure 2 shows that control cells contain four prominent polypeptides that react with an anti-RPRP2 antibody, migrating nominally at 33, 28, 20, and 14 kD. Extracts of the cell wall contain predominantly the 33 kD protein while the medium is rich in the 20 and 14 kD forms. Incubating either the 33 kD protein or the smaller polypeptides with homogenates of the cells or with the media, leaves

the proteins intact (data not shown). Thus, the presence of the specific RPRP polypeptides in the media is not due to cell autolysis but rather to a controlled cleavage that occurs within the growing cells. When we now obtain similar fractions from either EDHB- or dehydroproline-incubated cells, there is almost complete disappearance of the RPRPs from the treated cells (Fig. 2, Table II). In studies of the time course of the disappearance, we found essentially complete depletion of the cell wall RPRPs after a 7 h incubation in 0.3 mM EDHB. Again, autolysis is not the cause of the disappearance, since, as noted in Figure 1, both the dehydroproline and EDHB-treated cells have intact cell walls and are indistinguishable from control cells.

DISCUSSION

The cessation of cell growth in the presence of the prolyl hydroxylase inhibitors suggests that the synthesis and the secretion of the hydroxylated proline-rich proteins into the cell-wall matrix is crucial to growth. Possibly the hydroxyl groups are necessary as sites of attachment of glycosyl residues to provide a hydrophilic milieu (10), or a shield against proteolytic incursion (7). RPRP2, which comprises at least 80% of the salt-extractable hydroxyproline-containing proteins of the soybean cells, has, however, little or no covalently attached polysaccharide. An alternative idea suggested by the presence of the lower mol wt RPRPs in the cell culture is that the primary gene product (RPRP2) continually undergoes cleavage in growing cells, and that the steady-state level of the protein is determined by the hydroxylation of its prolines. Thus, the disappearance of the RPRPs when proline hydroxylation is prevented would be a consequence either of an accelerated turnover of the nonhydroxylated protein or a decreased rate of accumulation. A distinction between these possibilities should be possible by appropriate pulse-chase analyses. More significant is that in either case, it would seem that the proline-rich proteins *per se* are necessary structural components of the cell wall, independent of a covalent interaction of their hydroxyproline hydroxyl groups. Finally, it remains possible that the disappearance of the RPRPs is not a direct consequence of the prevention of the hydroxylation of these proteins but, rather, a result of a cellular state brought on by preventing the proline to hydroxyproline conversion, *e.g.* the accumulation of reducing potential as a consequence of the inability to oxidize ascorbic acid via proline hydroxylase. While this is a less likely possibility, it is consistent with recent studies suggesting that the redox state of a cell may be a major factor in regulating cellular processes (1, 5).

The cleavage of RPRP2 in growing cells at specific sites is reminiscent of the regulated cleavage of the extracellular matrix proteins of animal cells (13). It seems possible that the hydroxyproline-rich proteins, in addition to being structural elements of the cell wall, might affect cellular processes by their interaction with other wall components. As such, the regulation of their synthesis and cleavage could have broad biological consequences.

Table II. *Effect of 3,4-Dehydroproline and EDHB on the RPRP Content of Soybean Cells in Culture*

In experiment 1, 20 mL of 5 d cells (400 mg fresh weight) were supplemented either with 100 μM dehydroproline or an equivalent volume of water and growth was allowed to continue for 48 h. In experiment 2, 80 mL of a 5 d culture was filtered on a scintered funnel, washed with 80% media, and split into three parts, each of which was suspended in 20 mL of 67% media. After 1 h, one part was refiltered and collected while water and dehydroproline were added to the other two parts as in experiment 1. After 27 h, the cells were harvested and frozen. In experiment 3, 20 mL of 5 d cells were supplemented with 0.2 mM EDHB and growth continued for 17 h. The data are taken from a combined visual estimate of the four prominent bands (see Fig. 2) of extracts prepared as described in "Materials and Methods" and are expressed as total μg for the specific sample.

Experiment	Conditions	RPRP Content		
		Cell wall	Cytoplasm	Medium
			μg	
1	Control	88	8	64
	48 h dehydroPro	6	0.8	2
2	1 h	33		
	27 h control	25		0.6
	27 h dehydroPro	1		0.03
3	17 h control	62	12	125
	17 h EDHB	2.5	0.8	0.3

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