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DEPARTMENT OF BIOLOGICAL SCIENCES

October 27, 1999

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Mr. Andy Padilla
U.S. Department of Energy
Oakland Operations Office
Contracts, Acquisition and Property Division
1301 Clay Street, Rm. 700N
Oakland, CA 94612-5208

Reference: Grant DE-FG03-91ER20048

Title: Enzymology and Molecular Biology of Cell Wall Biosynthesis

Principal Investigator: Dr. Peter M. Ray

Department: Biological Sciences

Subject: Final Report

Dear Mr. Padilla

Enclosed is the final report, which is submitted in accordance to the terms of the referenced grant. There were no patents or inventions.

Should you have any questions or requests for additional information, please call me at the number indicated below.

Sincerely,



Patricia Ramirez
Financial Manager
(650) 723-6833

Enclosure

cc: Blanca Revuelta, Office of Research Administration

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Final technical report

DOE grant DE-FG03-91ER20048

Title: Enzymology and molecular biology of cell wall biosynthesis

The following aspects of enzymology of cell wall synthesis were pursued under support of the cited grant.

Isolation of plasma membrane-localized glucan synthase II (GS-II) of pea

We achieved post-entrapment fractionation of pea GS-II by 2 methods, isoelectric focusing and glycerol gradient centrifugation (3). The results indicated that 22, 30, 80, and 100 kDa polypeptides that occur in product-entrapped preparations are not responsible for GS-II activity, but did not eliminate the possibility that some or all of them might be accessory components that have an indirect, non-catalytic role in callose formation or deposition. Polypeptides of 55 and 70 kDa, which are major components of product-entrapped material, persisted in their association with GS-II activity through all fractionations. Antisera against each of them immunoadsorbed GS-II activity (3). Electrophoresis of native proteins in polyacrylamide without SDS indicated that (in digitonin-solubilized material) these polypeptides occur as a very large multiprotein complex in which minor amounts of the 30- and the 100-kDa polypeptides are also found (3). We more recently detected a 220-kDa polypeptide that also appears to be associated with GS-II activity and with this complex (Dhugga and Ray, unpublished results). This at first suggested a connection with yeast β -1,3-glucan synthase ~215 kDa subunits, but this was contraindicated by the results of gene cloning to be described shortly.

Cloning of genes for possible plant GS-II components.

With our anti-pea GS-II antisera we screened a pea cDNA expression library for sequences encoding the presumptive 55, 70 and 220 kDa GS-II components. The results, summarized in Table 1 (Dhugga and Ray, unpublished), contained several surprises. Each of these screens yielded 2 different sequences. One sequence in each case closely matches that of a tonoplast ATPase (V-ATPase) subunit, either of corresponding molecular mass (with the anti-55 and -70 screens) or of 55 kDa in the case of the anti-220 screen. We understand that Bruce Stone's group in Australia obtained a similar result when they tried to clone the gene for a presumed component of ryegrass glucan synthase. As far as we know they did not publish this, presumably because they thought the result was erroneous. Further work in our lab suggested that these V-ATPase subunits are not just contaminants of our antigens but may actually be components of GS-II. For example, immunocytochemical tests indicated that these V-ATPase subunits occur on the plasma- (as well as the vacuolar) membrane. This finding was confirmed independently by Robinson's group in Göttingen, Germany (13).

The second sequence picked by our anti-70 kDa antiserum corresponded to calnexin. The difference in apparent molecular masses of 65 vs. 70 between Delmer's and our work falls within the range of variations found in the peculiar electrophoretic behavior of calnexin (9). The second sequence selected by our anti-55 kDa antiserum is unique. Because the component of our preparations that detectably binds UDP-glucose is 55 kDa (3) we think that this polypeptide is probably the substrate-binding, catalytic subunit of pea GS-II.

Neither of the sequences selected by antiserum against the 220 kDa GS-II-associated band encoded a polypeptide of this molecular mass. Instead one again matched, as mentioned above, the V-ATPase 55 kDa subunit. The other matched Hsp70, a 70 kDa

animal and microbial "stress" or "heat-shock" protein, about which there is a very extensive literature (2, 7, 8, 10) including a few previous reports of Hsp70 homologs in plants (1, 14), including pea (11). In agreement with these results, on Western blots of plasma membrane proteins the anti-220 antiserum recognized not only the 220 kDa antigen band but also a band at 55 kDa and one at 70 kDa. The 220 kDa band may be a heterotetramer in which 2 each of the ATPase subunit and the HSP 70 homolog are covalently coupled by bonds such as peptide bonds that are not cleaved by the dithiothreitol used in our SDS gels.

We have not yet submitted this extensive work for publication because we wish, before doing so, to have more direct evidence as to which of the several cloned putative subunits is physically part of the GS-II complex and which are essential to its enzymic activity.

Golgi glucan synthase-I (GS-I)

Glycerol gradient centrifugation of proteins solubilized from purified Golgi membranes allows at least 6 bands to be identified, ranging between 45 and 120 kDa, that appear to peak along with the peak of GS-I activity (Dhugga and Ray, in preparation). These are minor bands and the task of isolating them for identification is difficult. To try to narrow down the number of candidate polypeptides we have attempted to fractionate digitonin-solubilized Golgi membranes by fast protein liquid chromatography (FPLC). With this technique, for the first time in our experience with GS-I we are able to recover after fractionation a substantial proportion of the initial enzyme activity as a relatively sharp peak, separated from the major peak of membrane protein. We are currently working to optimize this fractionation and anticipate that, in combination with a contrasting method such as glycerol gradient centrifugation, it will soon be possible to choose at most a limited set of polypeptides to be tested for responsibility for GS-I activity as described in the following proposal.

Golgi reversibly glycosylated protein 1 (RGP1).

In a substrate-labeling approach to GS-I similar to that mentioned above for GS-II we identified a 40 kDa polypeptide doublet that becomes labeled, covalently but reversibly, when glucose-labeled UDP-glucose is supplied to membrane fragments under the conditions of the GS-I assay (6). The members of the doublet proved to be minor length variants of the same protein, which we subsequently (5) designated RGP1 (reversibly glycosylated protein-1). Density gradient fractionation of cellular membranes indicated that RGP1 is Golgi-localized (6). Immunological, fractionation and substrate specificity data indicated that RGP1 is not a catalytic subunit of GS-I, and suggested that RGP1 might function instead as a Golgi membrane glycosyl carrier that can supply to Golgi synthases the various sugar residues (other than fucose) that they need for constructing xyloglucan (6).

We purified RGP1 to homogeneity by affinity chromatography, obtained a partial amino acid sequence for it (4), raised a potent monospecific antiserum against it, and by using this as a screen we cloned and sequenced the *Rgp1* gene (5). *Rgp1* has a unique sequence. Keegstra's group at Michigan State University (K. Keegstra, pers. comm.) recently cloned the same gene from the *Arabidopsis* EST library, using the partial amino acid sequence of pea RGP1 that we had exhibited at the 1994 plant physiology (ASPP) meetings (4).

Immunogold staining using our anti-RGP1 antiserum revealed a striking localization to Golgi dictyosomal cisternae in pea and cotton. The results consistently show RGP1 to be localized only to the *trans* cisternae (5). Since the *trans* region has been

identified as the part of the dictyosome in which xyloglucan is formed (12, 15), this finding adds to the indications that RGP1 is involved in polysaccharide (at least xyloglucan) synthesis.

The dramatic Golgi specificity of our anti-RGP1 antiserum has attracted the attention of plant cell biologists interested in questions that involve Golgi membranes and their identification. The antiserum and the cloned RGP1 gene promise to be tools useful in the search to identify Golgi polysaccharide synthases and possibly other components

Table 1. cDNA CLONES OF PUTATIVE GS II SUBUNITS

Antisera raised against isolated 55, 70, and 220 kDa polypeptides that correlate with GS II activity were used to screen a pea cDNA library in an expression vector. Each antiserum selected 2 distinct clones (several isolates of each) from this library.

Clone class	Screened for polypeptide of kDa	Cloned sequence homologous to	Possible function in GS II complex
1	55	V-ATPase 55 kDa subunit	substrate binding
2	55	no match (unique sequence)	catalytic subunit
3	70	V-ATPase 70 kDa subunit	substrate binding
4	70	calnexin	subunit(s) conformation, complex integrity or binding to membrane*
5	220	V-ATPase 55 kDa subunit	as above
6	220	HSP 70	subunit(s) conformation or product extrusion*

*function suggested from role of protein in other systems

REFERENCES

1. **Amir-Shapira D, Leustek T, Dalie B, Weissbach H, Brot N** (1990) Hsp70 proteins, similar to *Escherichia coli* DnaK, in chloroplasts and mitochondria of *Euglena gracilis*. *Proc Natl Acad Sci USA* **87**: 1749-1752
2. **Clarke AR** (1996) Molecular chaperones in protein folding and translocation. *Curr Opin Struct Biol* **6**: 43-50
3. **Dhugga KS, Ray PM** (1994) Purification of (1,3)- β -glucan synthase from pea tissue. Two polypeptides of 55 kDa and 70 kDa copurify with enzyme activity. *Eur J Biochem* **220**: 943-953
4. **Dhugga KS, Ray PM** (1994) Purification of the reversibly glycosylated polypeptides from pea: purified polypeptides exhibit the same properties as the Golgi-bound form. *Plant Physiol* **105**: 126 (Abstract)
5. **Dhugga KS, Tiwari SC, Ray PM** (1997) A reversibly glycosylated polypeptide (RGP1) possibly involved in plant cell wall synthesis: purification, gene cloning, and trans-Golgi localization. *Proc Natl Acad Sci USA* **94**: 7679-7684
6. **Dhugga KS, Ulvskov P, Gallagher SR, Ray PM** (1991) Plant polypeptides reversibly glycosylated by UDP-glucose. Possible components of Golgi β -glucan synthase in pea cells. *J Biol Chem* **266**: 21977-21984
7. **Frydman J, Höhfeld J** (1997) Chaperones get in touch: the hip-hop connection. *Trends Biochem Sci* **22**: 87-92
8. **Hartl FU** (1996) Molecular chaperones in cellular protein folding. *Nature* **381**: 571-580
9. **Huang L, Franklin AE, Hoffman NE** (1993) Primary structure and characterization of an *Arabidopsis thaliana* calnexin-like protein. *J Biol Chem* **268**: 6560-6566
10. **Johnson JL, Craig EA** (1997) Protein folding in vivo: unraveling complex pathways. *Cell* **90**: 201-204
11. **Marshall JS, Derocher AE, Keegstra K, Vierling E** (1990) Identification of heat shock protein hsp 70 homologues in chloroplasts. *Proc Natl Acad Sci USA* **87**: 374-378
12. **Moore PJ, Swords KMM, Lynch NA, Staehelin LA** (1991) Spatial organization of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *J Cell Biol* **112**: 589-602
13. **Robinson DG, Haschke H-P, Hinz G, Hoh B, Maeshima M, Marty F** (1996) Immunological detection of tonoplast polypeptides in the plasma membrane of pea cotyledons. *Planta* **198**: 95-103
14. **Yalovsky S, Paulsen H, Michaeli D, Chitnis PR, Nechushtai R** (1992) Involvement of a chloroplast HSP 70 heat shock protein in the integration of a protein

(light-harvesting complex protein) into the thylakoid membrane. Proc Natl Acad Sci USA
89: 5616-5619

15. **Zhang GF, Staehelin LA** (1992) Functional compartmentation of the Golgi apparatus of plant cells: immunocytochemical analysis of high pressure-frozen and freeze-substituted sycamore maple suspension culture cells. Plant Physiol **99**: 1070-1083