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CHARACTERIZATION OF A 1,4-ß-D-GLUCAN SYNTHASE FROM *DICTYOSTELIUM DISCOIDEUM*

Progress Report

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PROGRESS REPORT
Project Years 1 and 2

Characterization of a 1,4-ß-D-glucan synthase from *Dictyostelium discoideum*

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Publications Resulting from Project to Date

Papers:

No papers yet submitted or published.

Abstracts:

Blanton, R.L. 1991. *Dictyostelium discoideum*: A model organism for eukaryotic cellulose synthesis. Cellulose '91, New Orleans, Louisiana. Abstract # 176.

Blanton, R.L. 1991. Cellulose and development in *Dictyostelium discoideum*. International *Dictyostelium* Conference, Vancouver, British Columbia. Section VI, page 7.

Blanton, R.L., P. Dang, and R.D. Allen. 1990. Preliminary investigation of a novel pre-stalk specific gene from *Dictyostelium discoideum*. EMBO/NSF Workshop on the Developmental and Molecular Biology of *Dictyostelium*, Villa Gualino, Torino, Italy. p. 74.

Blanton, R.L., J. Mleczko, and K.J. Morrow. 1990. Isolation and characterization of monoclonal antibodies specific to membranes of differentiated cells of *Dictyostelium discoideum*. EMBO/NSF Workshop on the Developmental and Molecular Biology of *Dictyostelium*, Villa Gualino, Torino, Italy. p. 73.

Papers in preparation or to be submitted within the next six months:

Blanton, R.L. Effects of cAMP and DIF on cellulose synthesis in monolayer cultures of *Dictyostelium discoideum*. To be submitted to *Development*.

Dang, P., R.L. Blanton, and R.D. Allen. Cloning and expression analysis of Dd6, a prestalk specific gene from *Dictyostelium discoideum*. To be submitted to *Differentiation*.

Blanton, R.L., A.D. Morrison, M.J. Grimson, and J.G. Williams. Immunocytochemical localization of the ecmA and ecmB proteins. To be submitted to *J. Cell Science*.

Personnel Involved in Project

Technician

Lisa Sperry was hired at the initiation of the project. Although she had no experience when hired, she is now capable of independently performing many of the experimental procedures involved in the project. Ms. Sperry will continue to work in this laboratory for the third year of the project.

Graduate Students

Sue Pujari held summer research assistantships as part of this project. She initiated the mutant generation and analysis project. Unfortunatley, Ms. Pujari has decided for personal reasons to change from a Ph.D. program to a non-thesis masters and leave the university. William R. Reeves held a summer research assistantship as a Ph.D. student in my laboratory and worked on the whole cell lysate and membrane topology projects. He subsequently decided that he would prefer to work in a biomedical laboratory and left Texas Tech for the medical school graduate program at Texas A&M University. Phat Dang is engaged in the molecular biology work as a thesis master's

student. He is presently supported by the NSF grant. Bethany Passmann will join the lab in the spring semester initially as a thesis master's student.

Undergraduate Students

Several undergraduate research students have worked in my laboratory, some of them for extended periods: Bryan Harris, Dave Blann, and Michelle Shoumaker are all now medical students. Currently in the lab are Jami Adams (who will attend medical school in the fall) and Richard Mabbitt (who will be a graduate student in my lab when he graduates within the next year). Mr. Mabbitt has become an expert operator of the HPLC system and will be engaged in the polymer sizing and product analysis.

Research Progress

Introduction

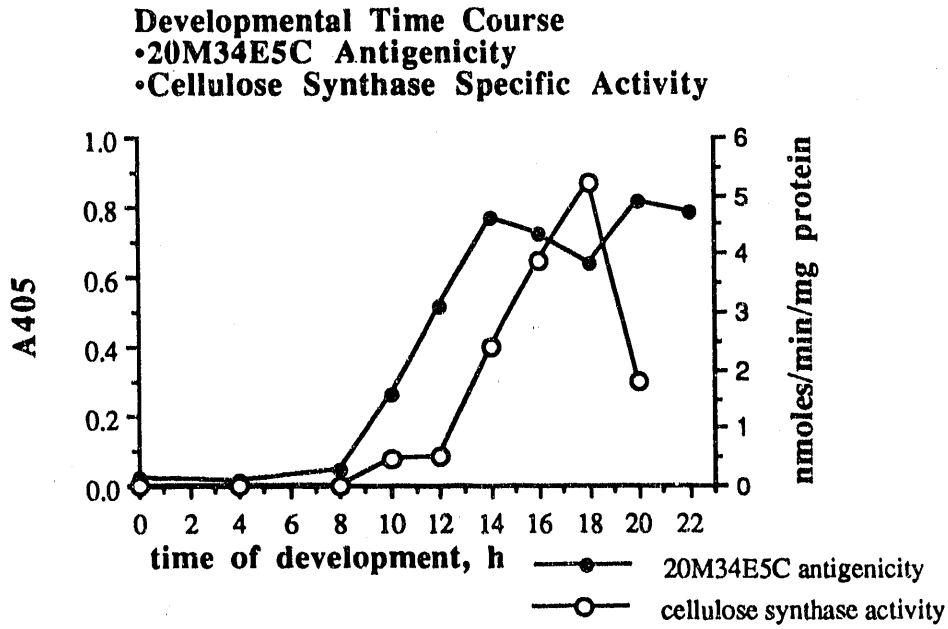
The initial focus of this project was upon: the characterization of potential probes for the cellulose synthase (antibody and nucleic acid), the determination of the cultural induction conditions of cellulose synthesis, the solubilization of the enzyme activity, the development of a non-inhibitory disruption buffer, the generation and isolation of mutant strains deficient in cellulose synthesis, and the development of the capability to determine the degree of polymerization of the in vitro product. I have briefly summarized our most significant findings with only selected data sets being shown in this report in the interest of brevity. Additional illustrations can be provided upon request.

Characterization of potential probes for the cellulose synthase

The start of the DOE project overlapped with the end of a project funded by the Texas Advanced Research program. The TARP project involved the generation of monoclonal antibodies specific for surface antigens of differentiated cells in an effort to isolate a monoclonal antibody that would recognize the cellulose synthase (which in *Dictyostelium* would be a differentiation-specific surface antigen). One of the antibodies, 20M34E5C, we found particularly promising. It was solely specific to differentiating cells and recognized antigens in the pre-stalk region of culminating sorocarps, which would be a region of active cellulose synthesis. In immunocytochemistry of differentiating tracheary elements from *Zinnia*, the antibody recognized membrane regions underlying the wall thickenings, which would be sites of active cellulose synthesis. Furthermore, the antibody recognized a 90 kD protein in Western blots of *Acetobacter*, the molecular weight reported for one of the polypeptides of the cellulose synthase. Given the potential importance of this antibody and the circumstantial evidence that it recognized a protein somehow involved with cellulose synthesis, we continued to work on the antibody as part of the DOE project. Two lines of inquiry were pursued: (1) the characterization of the nature of the antigen recognized in *Dictyostelium* and *Zinnia* and (2) the use of the antibody to isolate a nucleic acid probe.

In the antigen characterization experiments we asked the following questions:

(1) What are the developmental kinetics of antigen expression in *Dictyostelium* and *Zinnia*? Membrane preparations over a developmental time course (*Dictyostelium*) or from cultures induced or not induced to form tracheary elements (*Zinnia*) were applied to microtiter dishes and ELISAs performed. In *Zinnia* the antigen is enriched in differentiating tracheary elements. The results from the *Dictyostelium* time course are shown below.



(2) Is the antibody recognizing the peptide or oligosaccharide part of the antigen? In immunoblots of *Dictyostelium* proteins the antibody recognizes 3-4 bands, which could mean that the antibody is recognizing an antigen common to several proteins, most likely the carbohydrate epitope of glycoproteins. This does not in itself preclude the possibility that the antibody is recognizing the cellulose synthase, but it would reduce the utility of the antibody for expression library screening. In an effort to determine the nature of the epitope recognized by the antibody, we performed periodate degradations, endo-F digestions, and peptide competition experiments. For *Dictyostelium* the results were contradictory: the periodate experiments indicated a sugar epitope, some of the endo-F experiments a polypeptide. With *Zinnia* the experiments clearly indicated a peptide epitope. The rarity of glycoproteins in the eubacteria suggests that the antibody recognizes a peptide epitope in the immunoblots of *Acetobacter*.

(3) Does the antibody have an effect upon cellulose synthesis in the in vitro assay involving the crude membrane preparations? There is no consistent effect observed if crude membrane preparations are pre-incubated with the antibody prior to the addition of substrate.

(4) Can the antibody precipitate the enzyme activity from a solubilized preparation? Extensive experiments have been performed to test the ability of the antibody to precipitate the antibody from a solubilized preparation. We have tried different incubation conditions, both Protein A and Protein G as precipitating agents, and the use of a bridging antibody prior to application of the Protein A. We were unable to precipitate the activity. However, as discussed below, the solubilized preparations have not been fully characterized and the enzyme could still be inaccessible to the antibody.

(5) Can the antigen be purified through the use of an immunoaffinity column? We have made immunoaffinity columns and have used them to purify proteins from solubilized cell lysates. These have not yet yielded satisfactory quantities of purified antigen.

The attempt to use the antibody to isolate a nucleic acid probe has involved the following questions:

(1) Can the monoclonal antibody be used to isolate a cDNA clone from an expression library? We screened a slug stage expression library with the monoclonal antibody and did not obtain any positives. However, the polyclonal serum from the mouse used in the fusion that generated the monoclonal antibody showed similar reactivities (including immunoblot patterns) to the monoclonal antibody. The polyclonal mouse serum yielded 8 positives, which all proved to be identical. The recombinant phage were purified and the DNA cloned and sequenced.

The cDNA was used to isolate a genomic clone that is currently being analyzed. A paper reporting the sequence of the full-length cDNA (which has been isolated, cloned, and sequenced) and its expression pattern should be completed within the next two months.

(2) Is the cDNA related to cellulose synthesis? The cDNA was used in a gene disruption experiment and yielded a putative transformant (rather than a disruption it is an over-expressing line) that is altered, but not deficient, in its ability to make cellulose. These experiments will be repeated. The cDNA has no sequence homology with any of the genes of the cellulose synthesis operon of *Acetobacter*. Fusion constructs were prepared and fusion proteins isolated and used to generate a polyclonal antibody. The fusion protein antibody is unable to precipitate the enzyme activity from solubilized preparations.

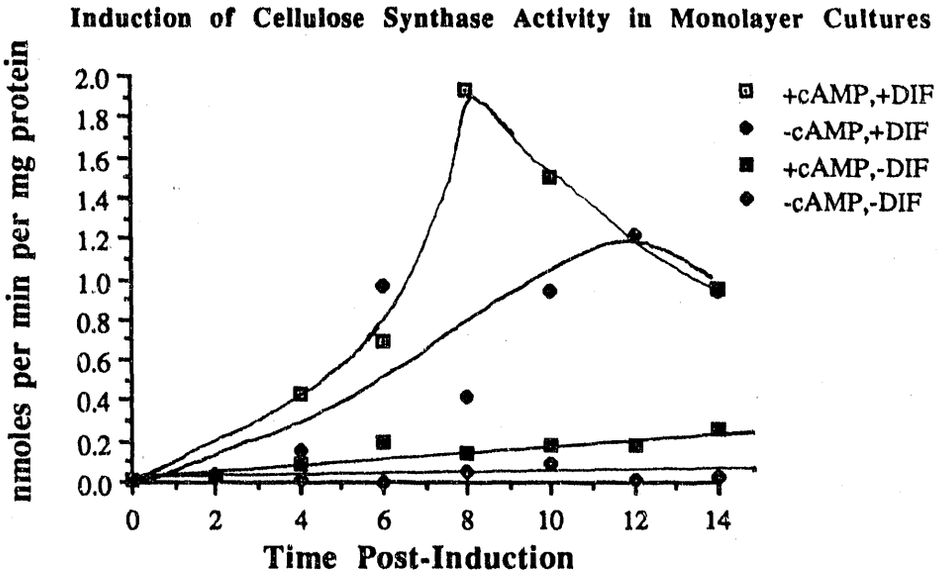
It became apparent that the work with the antibodies and cDNA clone required additional resources. In addition, the extension of the work to problems that were not of direct relevance to the goals of the DOE project led me to apply to the NSF for support of the developmental regulation studies. The continued characterization of the antibody and probes and the attempts to use the cloned genes from *Acetobacter* to isolate the cellulose synthase gene from *Dictyostelium* are now funded by the NSF grant. Of course, the two projects complement one another, but my intent is to have the DOE project focus more on the biochemical aspects of the cellulose synthase. The extension of the antibody work to *Zinnia* is being accomplished by Dr. Candace Haigler in this department. The antibodies have also been sent to Drs. Benziman, Delmer, Fevre, Wasserman, and Matthysse for testing against purified cellulose synthases from *Acetobacter*, *Saprolegnia*, and *Agrobacterium* and against purified plant callose synthases.

The determination of the cultural induction conditions of cellulose synthesis

One of the most interesting aspects of cellulose synthesis in *D. discoideum* is its inducibility: cells that have never made cellulose will be induced to synthesize cellulose. This induction can be studied in the absence of other morphogenetic processes in monolayer culture systems. In these cultures amoebae are plated as a cell monolayer in a liquid medium. If cells of the mutant strain that is incapable of synthesizing the stalk cell-inducing morphogen DIF (differentiation inducing factor) are plated, then the formation of stalk cells will depend upon the addition of DIF. The monolayer cultures may therefore be used to study the conditions that are required for the induction of cellulose synthesis. They also provide an opportunity to study the in vivo effects of inhibitors of cellulose synthesis.

We performed cellulose synthase assays using crude membrane preparations isolated over an induction time course of monolayers treated with DIF and cAMP, alone and in combination. The results of this experiment are shown on the next page. Not only did we find that maximal cellulose synthesis requires the presence of both DIF and cAMP, but microscopical observations indicated that this culture system allows the distinction of two modes of cellulose synthesis. During fruiting body formation, pre-stalk cells synthesize cellulose to form the stalk tube as they migrate up the stalk. Once they reach the top of the stalk tube, the pre-stalk cells migrate into the stalk tube and begin to synthesize cellulose-containing cell walls. In the monolayer cultures it appears that the stalk tube material is secreted on the surface of the culture dish prior to the synthesis of the stalk cell walls. This stalk tube-like synthesis was present in cultures induced solely with cAMP. In cultures treated only with DIF stalk cells, but no other material was formed. In cultures induced with both DIF and cAMP both the stalk tube-like material and stalk cell walls are formed. These results will be prepared for publicaton within the next two months.

Preliminary experiments with cellulose synthesis inhibitors have not shown any effects of DCB, Tinopal (Calcofluor), or phthoxazolin (a newly described cellulose synthesis inhibitor) on stalk cell wall formation. However, Tinopal and coumarin do inhibit the in vitro activity; DCB and phthoxazolin do not.



The solubilization of the enzyme activity

The successful solubilization of the cellulose synthase is a critical first step for any further purification efforts, such as product entrapment. Results of our detergent trials are summarized in the table.

Results of Detergent Trials

Detergent	Range Tested mg/mL	Best % Solubilization Protein	Activity
CHAPs	10.0-0.5	58%	63%
CHAPSO	10.0-0.5	99%	13%
Cholate	8.0-0.8	52%	32%
Digitonin	20.0-1.0	60%	1%
Lubrol PX	0.1-0.005	42%	0
Lysophosphatidyl-choline	4.0-0.2	61%	11%
Octyglucoside	16.0-1.6	50%	53%
Triton X-100	2.0-0.1	65%	4%
Zwittergent-8	2.0-0.05	71%	3%
Zwittergent-10	10.0-0.01	65%	4%
Zwittergent-12	2.0-0.05	21%	0
Zwittergent-14	0.8-0.08	22%	0
Zwittergent-16	1.0-0.01	50%	5%

We found the best conditions to be 6 mg/mL CHAPs in a detergent:protein ratio of 1:1. In these conditions, 24-58% of the protein is solubilized, with 24-63% of the recovered activity present in the supernatant. However, there are two caveats to these results. One is that we found it necessary to relax our definition of solubilization. Most investigators use an hour-long 100,000 xg centrifugation as their criterion. We found it necessary to use as our criterion the activity remaining in the supernatant following the centrifugation conditions that would pellet the vesicles of the crude membrane preparations (27,000 xg for 10 min). Also, the recovered activity is rarely as high as 50% of the activity of the crude membranes. No significant improvement in activity

could be obtained by the addition of phospholipids to the reaction mixture nor by using detergent removal protocols. Some of the detergents appeared to inhibit the cellulose synthase activity.

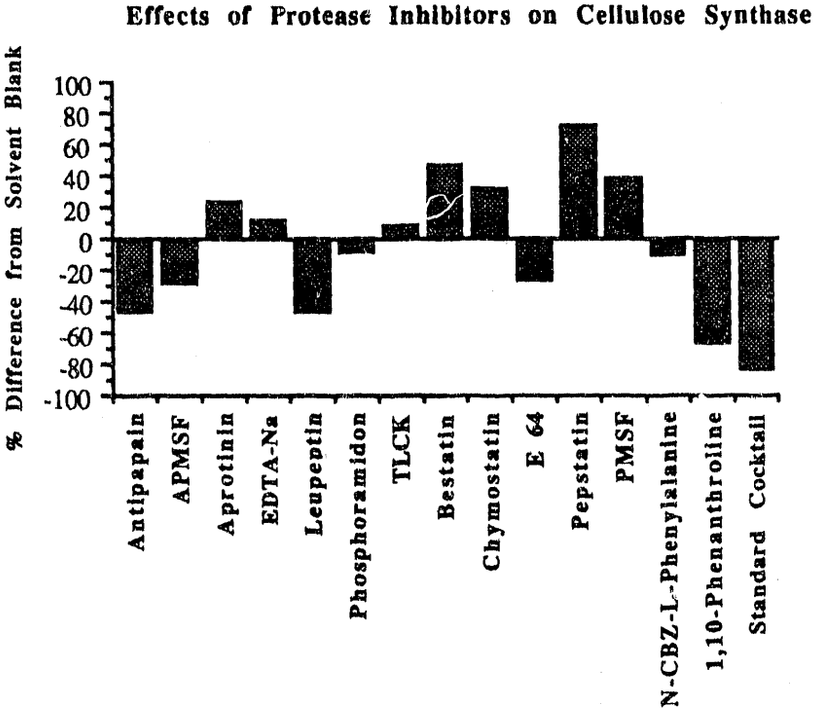
As discussed in the incremental proposal, we hope to continue our solubilization efforts. The activity loss may be restored by c-di-GMP or another soluble activator. Some of our difficulties may be resolved by using more highly purified detergents.

The development of a non-inhibitory disruption buffer

We were interested in developing a disruption buffer that was not inhibitory to the cellulose synthase for two reasons: (1) to be able to do assays on whole cell lysates prepared from a small number of cells and (2) to be able to test for supernatant factors that may enhance the in vitro activity. In our standard procedures the membranes are washed free of the disruption buffer.

We tried a number of other disruption media developed for plant glucan synthases as well as for *Dictyostelium*, but none of these yielded active preparations. We then tested the standard disruption buffer, dropping various components. Interestingly, we found that all components of the buffer were to some degree inhibitory to the cellulose synthase and that the NaHepes buffer alone yielded the most active lysates.

The particularly strong inhibition of the synthase by the protease inhibitor mix (which contains PMSF, N-CBZ-phenylalanine, and 1,10-phenanthroline) led us to test the hypothesis that the cellulose synthase could be activated by proteolysis, as is the case with the chitin synthase. We screened the effects of a number of protease inhibitors on the enzyme activity.



However, we determined that the inhibitory effects of some of the protease inhibitors were due to their being metal ion chelators ($Mg2^+$ is required for maximal enzyme activity). In addition, we determined that inhibition of activity by the protease inhibitors occurred even after varying periods of protease-free incubation, during which proteolytic activation should have occurred. It therefore seems unlikely that the *D. discoideum* cellulose synthase is activated by proteolysis. However,

from these experiments we have developed a broad spectrum cocktail of protease inhibitors for the non-inhibitory disruption buffer.

The question is also raised of how the cellulose synthase resists degradation by the abundant endogenous proteases in *Dictyostelium*. In stalk cells, cellulose synthesis is active in the midst of extensive cell autolysis, suggesting that the cellulose synthase is resistant to proteolysis. Interestingly, work with plants has suggested an extreme sensitivity to proteolysis of the cellulose synthase. Our success with obtaining in vitro cellulose synthesis from *Dictyostelium* preparations may relate to a natural resistance to proteolysis.

The generation and isolation of mutant strains deficient in cellulose synthesis.

As indicated by some of the reviewers of the original proposal, the potential contribution of *D. discoideum* to our understanding of cellulose biosynthesis may be greatest at the level of genetics. We gave very high priority to the mutant isolation project and met with the greatest difficulties. Our mutagenesis procedure works well. The difficulties involved the screening process. The basic problem is the fact that cellulose synthesis is an activity of developing cells. This has two implications: (1) a mutation "upstream" of the induction point of cellulose synthesis will manifest itself as a cellulose-deficient mutant (for instance, the DIF-deficient strains are cellulose deficient until they are given DIF); and (2) the mutated cells must be allowed to grow and differentiate before screening. Cells that are diluted and plated will form a plaque that will reach 1-2 cm in diameter before the cells in the center of the plaque will develop. This limits the number of cells that can be plated on a 100 mm culture dish to 20-30; each screen required 150-200 plates. Visual screening was for developmental stages arrested at the stage immediately preceding the initiation of cellulose synthesis. Of the many thousands of colonies screened, about 12 strains arrested at the appropriate stage were isolated. Some of these reverted to normal morphology upon subculturing. In the end, only about 8 strains were suitable. All of these had significant levels of cellulose synthase activity as determined in the in vitro assay. We have also obtained several mutant strains from other investigators. These strains have morphologies consistent with what we would expect for a cellulose deficient mutant. However, all of these strains also had high levels of in vitro cellulose synthase activity.

I consulted with several scientists with extensive experience in mutagenesis efforts involving *Dictyostelium*. All of them acknowledged the difficulty of what we were trying to do. However, two additional screening methods were devised. In the first we planned to screen for spores that were deficient in cellulose. Cellulose forms part of the spore wall, but since the outer spore wall is composed of protein a spore deficient in cellulose would likely still be viable. A cell that is capable of forming a spore will not be mutant in any process upstream of cellulose synthesis. Very large numbers of spores could be screened by using a FACS machine. The detection method was initially to be fluorescein-conjugated cellulase, but this proved to have too high of a negative background. Instead, we tried Calcofluor. Several trial runs were carried out, but the available FACS instrument proved incapable of detecting the Calcofluor fluorescence.

Most recently, we have been trying a method using the monolayer culture system. Here, a cell that forms a stalk cell wall (and is thereby capable of making cellulose) will die since stalk cells are terminally differentiated. Cells incapable of forming stalk cell walls (and presumably cellulose) will remain viable. By carrying these cells through a number of culture cycles, we would expect the cellulose-deficient cells to predominate in the population. The cells can then be screened by standard dilution-plating and visual screening procedures. This method has required the isolation of an axenic strain of the wild-type strain that is capable of developing in the monolayer cultures. Fortunately, another laboratory has provided me with that strain.

The development of the capability to determine the degree of polymerization of the in vitro product

One significant, unanswered question about the nature of the in vitro product is its degree of polymerization. An undergraduate chemistry student in my laboratory has spent the past year developing our capability to do non-aqueous size exclusion HPLC. The standard curve using polystyrene standards has been generated. The DMAC/LiCl solvent system has been tried on test cellulose samples.

Interactions between cellulose and extracellular matrix proteins in *Dictyostelium discoideum*

The slug slime trail, stalk, tube and stalk cell walls of *D. discoideum* are composed of cellulose and proteins. The genes coding for two of the extracellular matrix proteins have been well-characterizedby J.G. Williams and co-workers. In a collaborative project with Dr. Williams I have been engaged in an effort to understand the functional role of these proteins in the stalk tube and stalk cell walls. Our initial efforts have involved EM immunocytochemistry and suggest a possible interaction between cellulose and the proteins. Interestingly, an antibody to the extracellular matrix proteins gives strong reactions in immunoblots and EM immunocytochemistry with plant cell walls. The work with plants is now being done in Dr. Haigler's laboratory.

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