DEVELOPMENTAL CHANGES IN MEMBRANE-BOUND ENZYMES OF DICTYOSTELIUM DISCOIDEUM DETECTED BY CONCANAVALIN A-SEPHAROSE AFFINITY CHROMATOGRAPHY

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SUMMARY. Plasma membrane-enriched fractions isolated from Dictyostelium discoideum at early stages of development were detergent extracted and subjected to affinity chromatography on a concanavalin A-Sepharose column. Alkaline phosphatase, 5'-nucleotidase, and cAMP phosphodiesterase activities were totally bound to the column when logarithmically growing cells were examined. As the cells entered development, however, a progressive decrease in the ability of these activities to bind to the affinity column was evident.

When the food supply of unicellular amoebae of Dictyostelium discoideum is exhausted, the cells aggregate to form a multicellular organism which progresses through a well-defined developmental sequence (1). As the amoebae enter the aggregation stage of development, both biochemical (2-6) and morphological (7,8) changes in the cell surface occur. Changes in the protein (9) and glycoprotein (10) composition of the cell surface have also been observed during this period of development. The use of the lectin concanavalin A as a probe for cell surface changes as amoebae develop "aggregation competence" (11) has revealed an increase in the number of lectin binding sites (10,12) and a decrease in the agglutinability of the cells by concanavalin A (13) during this period although the molecular basis for these changes remains unknown.

In this report we examine the interaction of various plasma membrane-associated enzymes with concanavalin A to determine whether these interactions are altered during the early stages of development. As an assay procedure we have measured the ability of the detergent-solubilized enzymes to bind to a concanavalin A-Sepharose affinity column.

MATERIALS AND METHODS. Sodium metaperiodate and DOC[1] were purchased from Fisher. Concanavalin A-Sepharose 4B containing 8mg of concanavalin A per ml of sediment was obtained from Pharmacia. Trypticase peptone was purchased from BBL. Bovine serum albumin, αMG[1] and all p-nitrophenyl glycosides were obtained from Sigma.

[1]Abbreviations used; DOC = sodium deoxycholate; αMG = α-methyl-D-glucoside

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An axenic derivative (Ax-3) of Dictyostelium discoideum was grown and harvested as previously described (8), except that trypticase peptone was substituted for thiotone in the growth medium. Vegetative cells were harvested at densities of 2-4 x 10^6 cells per ml. Aggregation competent cells were obtained by suspending vegetative cells in 17mM potassium phosphate buffer (pH 6.1) containing 2mM $MgSO_4$ at a cell density of 1-2 x 10^7 cells per ml (2). The time of transfer into starvation buffer is considered as zero time of development.

Fractions enriched in plasma membranes were prepared as previously described (14) using 1 x 10^{10} cells per preparation. Excess sucrose was removed from the fraction by 3 washes with 0.1M Tris-Cl buffer (pH 7.5). The washed fraction was then suspended in 10mM Tris-Cl buffer (pH 7.5) containing 2% (w/v) DOC at a protein concentration of 3-4mg per ml. The suspension was vortexed for 15 seconds and allowed to stand on ice for 30 minutes. Insoluble material was then removed by centrifugation at 30,000 x g for 30 minutes and the supernatant (DOC-extract) was used immediately for affinity chromatography.

All affinity chromatography operations were conducted at 4°C. A 1 x 23cm column of concanavalin A-Sepharose was poured and washed successively with 100ml each of 0.1M αMG, 1M NaCl containing 0.1% n-butanol, and 10mM Tris-Cl buffer (pH 7.5) containing 0.2% (w/v) DOC (DOC-Tris buffer). Freshly poured and washed columns were employed for each experiment. The DOC-extract was then applied to the column and unbound components were eluted with 125ml of DOC-Tris buffer. Concanavalin A-bound proteins were then specifically eluted with 125ml of DOC-Tris buffer containing 0.1M αMG. The column flow rate was 0.5ml per minute. Tubes containing the unbound and bound fractions, respectively, were combined and concentrated to a small volume by ultrafiltration in an Amicon cell using a PM-30 membrane. The concentrated fractions were then dialyzed for 24-36 hours against 100 volumes of DOC-Tris buffer.

Cyclic nucleotide phosphodiesterase and 5'-nucleotidase activity were measured as previously described (14). Alkaline phosphatase activity was assayed as described by Loomis (15), except that samples were incubated at 30°C in 0.1M Tris-Cl buffer (pH 9.0). Glycosidase activities were assayed essentially as described by Every and Ashworth (16) using 5mM p-nitrophenyl glycosides as substrates. Protein concentrations were determined by the Lowry procedure (17) using crystalline bovine serum albumin as a standard.

Periodate oxidations were performed by mixing a 1ml sample containing approximately 500µg of protein in DOC-Tris buffer with 0.2ml of 0.5M sodium acetate (pH 6.2) and 15µl of 1M sodium metaperiodate. The resulting suspension was incubated at 4°C in the dark for 16 hours with shaking. The reaction was stopped by adding 0.12ml of 1M ethylene glycol and 1ml of Tris-Cl buffer (pH 9.0) and dialyzing overnight at 4°C against DOC-Tris buffer. Control samples were treated in an identical manner except that no periodate was added.

Conditioned medium was prepared by suspending vegetative cells in starvation buffer for 4 hours. Cells were harvested by centrifugation and the supernatant (conditioned medium) was frozen at -20°C until immediately prior to use. For treatment with conditioned medium, 1ml of sample containing 1.8mg protein in DOC-Tris buffer was mixed with 1ml of 0.5M sodium acetate (pH 6.1) and 1ml of conditioned medium. The suspension was incubated at 30°C with shaking for periods up to 5 hours. Control samples were treated in the same manner except that starvation buffer was substituted for conditioned medium. At the end of the incubation period, 1M Tris-Cl buffer (pH 9.0) was added dropwise until the precipitate in the sample dissolved. The solution was then dialyzed overnight against DOC-Tris buffer.

RESULTS. Initial experiments were concerned with developing conditions for extraction of enzyme activities from the membrane fraction. Alkaline phosphatase, 5'-nucleotidase, and cAMP phosphodiesterase were chosen for study since previous investigations had shown that these activities were present in plasma membranes of D. discoideum (4,18). Extraction with 2% DOC, as described in Materials and Methods, solublized 90% of the total membrane protein and over 90% of the nucleotidase, alkaline phosphatase, and phosphodiesterase activities. None of these activities were detected in the insoluble residue.

The DOC-extracts from vegetative, pre-aggregation (4-hour), and aggregation competent (16-hour) membranes were then subjected to affinity chromatography on a concanavalin A-Sepharose column. In each experiment there was at least a 15-fold excess of concanavalin A present in the column in comparison to the amount of protein which was applied to the column. The A_{280} elution profile was identical whether vegetative or developmental membrane extracts were used, and in each case approximately 15% of the protein was recovered in the bound fraction. Polyacrylamide gel electrophoresis (19) of the bound fractions produced 10 major bands in the range from 30-140,000 apparent molecular weight. In aggrement with the results of Geltosky et al. (10), we observed that the majority of these bands were conserved during this developmental period.

When the abilities of the alkaline phosphatase, 5'-nucleotidase, and cAMP phosphodiesterase activities to bind to the column were examined at early stages of development, significant differences were observed. The results presented in Table I reveal that while over 90% of each of these activities are bound from vegetative extracts, as the cells acquire aggregation competence there is a progressive decrease in the percentage of total activity bound for each of the enzymes. Total (bound + unbound) alkaline phosphatase and 5'-nucleotidase activities remained relatively constant during this period of development, but the level of bound activity decreased while that in the unbound fraction increased. In contrast to these results, the total cAMP

TABLE I

BINDING OF MEMBRANE-ASSOCIATED ENZYMES TO CONCANAVALIN A-SEPHAROSE[a]

	VEGETATIVE (0-hour)		PRE-AGGREGATION (4-hour)		AGGREGATION COMPETENT (16-hour)	
	Unbound	Bound	Unbound	Bound	Unbound	Bound
Alkaline Phosphatase	3.1	34.8 (92%)[b]	7.8	26.5 (77%)	15.1	19.7 (57%)
5'-Nucleotidase	0.3	11.9 (98%)	2.0	7.6 (79%)	5.6	4.2 (43%)
cAMP Phosphodiesterase	0	6.5 (100%)	5.6	10.5 (65%)	23.0	18.0 (44%)

[a]All values are expressed as total units per fraction where one unit equals one μmole/hour at 30°C for alkaline phosphatase and 5'-nucleotidase, and one unit equals one nmole/hour for phosphodiesterase.

[b]Numbers in parentheses refer to the percentage of the total activity which is recovered in the bound fraction.

phosphodiesterase activity increased markedly during this period of development, a result previously reported (4,20). Phosphodiesterase activity increased in both the bound and unbound fractions although the percentage of the total activity which could be bound decreased. Re-passage of unbound fractions through freshly prepared columns resulted in no additional binding of the activities, indicating that the columns were not overloaded. Repeating the assays in the presence of 50mM αMG or in the presence of concanavalin A at concentrations up to 300μg per ml had no effect on the activities measured. Thus, the solublized enzyme activities are not affected by the presence of concanavalin A.

The effects of periodate oxidation on the ability of the enzymes to bind to the affinity column were also examined. When the concanavalin A-bound fraction isolated from a vegetative membrane extract was subjected to periodate oxidation and then passed through a second affinity column, 43% of the 5'-nucleotidase, 84% of the alkaline phosphatase, and 95% of the cAMP phosphodiesterase activity was recovered in the bound fraction. In the control samples, greater than 90% of each activity was recovered in the bound fraction. Thus the portions of the enzymes which mediate binding to the affinity column, presumably carbohydrate chains, appear to be chemically different in their sensitivity to periodate oxidation.

Ashworth and Quance have reported that the enzymes α-mannosidase, α-glucosidase, β-N-acetylglucosaminidase, and β-glucosidase are secreted by D. discoideum when true exponential growth ceases (21). In agreement with their results, we have detected each of these activities in the conditioned medium of developing cells and have found that each enzyme is active at pH 6.1 in this medium. Since both alkaline phosphatase and 5'-nucleotidase are plasma membrane marker enzymes for D. discoideum (18) and may be associated with the cell surface (22-24), one mechanism to account for the observed changes in binding to the affinity column (Table I) would be for the secreted glycosidases to remove carbohydrate residues from the surface of developing cells.

TABLE II

EFFECTS OF INCUBATION WITH CONDITIONED MEDIUM ON BINDING TO CONCANAVALIN A-SEPHAROSE[a]

	% of Total Activity Recovered in Bound Fraction	
Incubation time(hrs.)	Alkaline Phosphatase	5'-Nucleotidase
0	100	100
2	68	53
5	43	24

[a]Aliquots of the concanavalin A-bound fraction isolated from vegetative membrane extracts were incubated with conditioned medium for the indicated periods of time as described in Materials and Methods. The incubation products were subsequently passed through a concanavalin A-Sepharose column to determine the percentage of each enzyme activity which could be bound by the column.

We thus examined the effects of incubation with conditioned medium on the ability of the solubilized nucleotidase and alkaline phosphatase to bind to the affinity column (Table II). Since cAMP phosphodiesterase and a phosphodiesterase inhibitor are both present in the conditioned medium (25), the effects on cAMP phosphodiesterase binding were not examined. Neither alkaline phosphatase nor 5'-nucleotidase activity was detectable in the conditioned medium. The results presented in Table II reveal that incubation with conditioned medium yields a progressive decrease in the ability of each activity to bind to the column. Control incubations in the absence of conditioned medium yielded 85-90% recovery of each activity in the bound fraction. While glycosidase activities could account for this result, other factors present in conditioned medium may also alter the ability of the 5'-nucleotidase and alkaline phosphatase activities to bind to a concanavalin A-Sepharose affinity column. The differential effects of periodate oxidation and treatment with conditioned medium on the abilities of 5'-nucleotidase and alkaline phosphatase to bind to the affinity column lend further support to the contention

of Lee et al. (24) that these activities are associated with two separate enzymes.

DISCUSSION. We have attempted to investigate the question of whether the interaction between concanavalin A and specific membrane components is altered during the period when developing cells acquire aggregation competence (11) by using the binding of specific enzyme activities to a concanavalin A-Sepharose affinity column as an assay. When various enzyme activities present in the plasma membrane-enriched fraction were examined for their ability to bind to the affinity column, clear differences were evident during the developmental period. The results presented show a progressive decrease in the percentage of total alkaline phosphatase, 5'-nucleotidase, and cAMP phosphodiesterase activity which could bind to the affinity column as the cells acquired aggregation competence.

One explanation for the ability of the enzymes to bind to the column is that they are glycoproteins. Since alkaline phosphatase (23,24), 5'-nucleotidase (22), and cAMP phosphodiesterase (4) may be associated with the cell surface, and D. discoideum cells are known to secrete glycosidases when cell growth ceases (21), it seems possible that the extracellular glycosidases may alter the ability of these enzymes to bind to the column. While our results are consistent with this hypothesis, further studies with purified glycosidases are clearly necessary.

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