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Developmentally Regulated, Carbohydrate-Binding Protein in *Dictyostelium discoideum*

(cellular slime mold/morphogenesis/intercellular recognition/lectin)

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ABSTRACT **A carbohydrate-binding protein assayed by its ability to agglutinate formalinized sheep erythrocytes is synthesized between 3 and 9 hr after *Dictyostelium discoideum* cells are deprived of food, as the cells become cohesive. Agglutination of erythrocytes by this protein was inhibited by *N*-acetyl-D-galactosamine, D-galactose, and L-fucose, but other monosaccharides had little or no effect. The protein bound completely to Sepharose 4B, and was isolated in highly purified form by elution with D-galactose. It appears to be present on the surface of cohesive but not vegetative slime-mold cells. The possibility that this protein may mediate intercellular adhesion in *Dictyostelium* is considered.**

The life cycle of the cellular slime mold *Dictyostelium discoideum* has two distinct phases: a nonsocial vegetative state in which separate amoebae feed on bacteria and divide by fission every few hours, and a social phase initiated by a period of starvation, in which the free-living amoebae aggregate to form a multicellular structure before fruiting body formation (1, 2). Under standard culture conditions, the entire life cycle takes 24 hr. The cells develop several properties during the aggregation phase of differentiation. These include the abilities to respond to and to relay chemotactic signals and the capacity to form stable intercellular contacts once chemotaxis has brought the cells into proximity with one another (2).

Previous work has demonstrated that a protein factor assayed by its activity in agglutinating formalinized sheep erythrocytes can be extracted in increasing amounts from *D. discoideum* cells as they differentiate after removal of food (3). In a 12-hr period over which the cells become progressively more cohesive, the specific activity of the agglutination factor in cell extracts increases by over 400-fold (3). In this report, we show that the appearance of the agglutination factor in *D. discoideum* is closely correlated with development of cell cohesiveness, as measured by a newly devised quantitative assay. We also show that the factor is a carbohydrate-binding protein with a high degree of specificity. We present preliminary evidence that the factor is detectable on the surface of cohesive slime-mold cells.

MATERIALS AND METHODS

Culture Methods. Starting cultures of *Aerobacter aerogenes* and *Dictyostelium discoideum* strains NC-4 (haploid) and A3 were kindly provided by William F. Loomis, Jr. *D. discoideum* NC-4 cells were grown for 40–44 hr at 22° on nutrient agar in association with *Aerobacter aerogenes* as described by Sussman (4). The vegetative amoebae were separated from bacteria by differential centrifugation, washed several times in water at 4°, and suspended in water at a concentration of 2×10^8 cells per ml. The cells were distributed on Millipore filters (10^8 cells per filter) supported by pads saturated in PDF buffer consisting of 40 mM potassium phosphate buffer (pH 6.5) containing per liter 1.5 g of KCl, 0.61 g of $MgSO_4$, and 0.5 g of streptomycin sulfate (4, 9). The Millipore filters and pads were, respectively, catalogue nos. AABP04700 and AP100-4700. The cells were then kept in a moist atmosphere at 22°. At intervals cells were harvested from the filter supports and assayed for cohesiveness; extracts were assayed for agglutination activity.

D. discoideum strain A3 cells (mutant derived from NC-4) were grown in axenic culture (5). Growth-phase cells (vegetative) were tested for cohesiveness and extracts were assayed for agglutination activity.

Cohesiveness Assay. Based on a procedure developed by Gerisch (6), we used roller-tube culturing to promote aggregation of cohesive slime-mold cells by passively bringing them into contact with one another independently of their chemotactic system. Particle-size analysis by a Coulter Electronic Particle Counter was used to determine the degree of aggregation. The procedure was as follows: (*i*) Harvested cells were washed twice in water at 4° and suspended in EDTA–phosphate buffer [16.7 mM Na_2HPO_4–KH_2PO_4, 10 mM EDTA (pH 6.2)]. This buffer is slightly modified from one described by Gerisch (6). (*ii*) The suspension was dispersed into single cells by repeated pipetting through a fine-tipped pipette. The cell concentration was adjusted to 5×10^5 per ml. (*iii*) 5 ml of this suspension was added to a 17 × 150-mm glass test tube. The tube was rolled about its long axis at 20 revolutions per min for 30 min at room temperature (23°). (*iv*) The contents of each tube were diluted 10-fold with the EDTA–phosphate buffer. A size spectrum of the particles was determined with a Coulter Counter (model ZBI) using a 200-μm aperture, with 1/aperture current fixed at $^1/_{16}$ and the upper and lower thresholds set at 50 and 100. Multiple readings were taken at each setting of the 1/amplification dial from $^1/_{16}$ to $^1/_{64}$. A comparison was made of the size spectra of cells that had been rolled and cells kept for 30 min in EDTA–phosphate buffer and dispersed into single cells just before reading. Readings were taken on three replicate tubes for both aggregated cells and dispersed cells. The ratio of the average particle volume of the cell aggregates in the rolled tubes to the average single cell volume was computed. This ratio, which is equivalent to the mean number of cells per clump, is taken as an index of the cohesiveness of the cells. It is designated as the "s value." The standard error of the mean for each "s value" was calculated.

Erythrocyte Agglutination Assay has been described (3). Briefly, the method was as follows: (*i*) Soluble crude extracts were prepared by homogenization of washed slime-mold cells

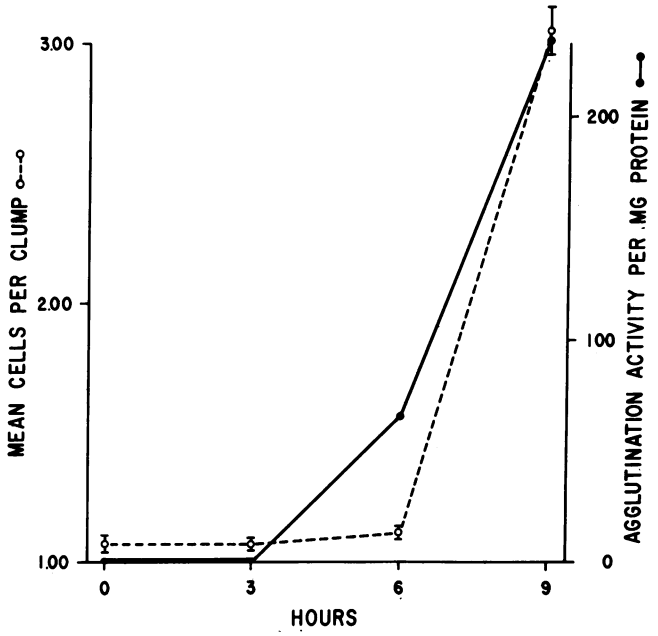

FIG. 1. Specific activity of agglutination factor and cell cohesiveness in differentiating *D. discoideum*. Development was initiated by washing growth phase NC-4 cells free of bacteria and distributing them on Millipore filters and supports. At intervals after plating, cells were assayed for cohesiveness (*dashed line*) and extracts of the cells were assayed for agglutination activity (*solid line*). Index of cohesiveness is the mean number of cells per clump formed after 30 min of roller tube culturing. *Vertical bars* denote two standard errors of the mean.

in 0.15 M NaCl at 4° and centrifugation of the homogenates at 150,000 × *g* for 45 min. (*ii*) A 2-fold dilution series of the supernatant was made in 0.15 M NaCl, and 0.5 ml of each dilution was mixed with 2 ml of 75 mM NaCl, 75 mM Na_2HPO_4–KH_2PO_4 (pH 6.4). To each tube was added 0.5 ml of a 2.5% suspension of formalinized sheep erythrocytes. [Formalinized cells were prepared from erythrocytes by the method of Butler (7). These cells were stable at 4° for several months.] (*iii*) The tubes were kept at room temperature for 10 min and then centrifuged at 500 × *g* for 5 min. The supernatants were discarded. The cells were suspended in 1 ml per tube of 1% normal rabbit serum in 0.15 M NaCl, centrifuged again, and suspended in 0.5 ml per tube of the same solution. (Bovine-serum albumin, 0.6 mg per ml in 0.15 M NaCl can be substituted for the 1% normal rabbit serum.) (*iv*) Agglutination of treated erythrocytes was measured in Microtiter "V" plates (Cooke Engineering). 25 μl of each cell suspension was added to each of triplicate wells containing 25 μl of 0.15 M NaCl. The plate was covered with transparent tape, shaken, and kept at room temperature for at least $1^1/_2$ hr before the agglutination patterns were evaluated. (*v*) The agglutination patterns were comparable in appearance to these produced by antibody agglutination of erythrocytes. The agglutination endpoint was taken as the first dilution of the extract at which the treated cells settled into a clearly circumscribed dot at the bottom of the plate well. The agglutination activity of an extract is defined as the reciprocal of the endpoint dilution. Thus, if an extract has to be diluted 1:32 before an endpoint is attained, the agglutination activity would be 32 units per 0.5 ml (0.5 ml is the standard volume assayed) or 64 units per ml. Specific agglutination activity is defined as the agglutination activity per mg of protein. Protein concentration was determined by the method of Lowry (8) with bovine-serum albumin as a standard.

The accuracy of the assay is limited because of the 2-fold dilution procedure used (3). If it is assumed that the transition to an agglutination endpoint is a sharp threshold phenomenon, then a well-defined error range can be assigned. Thus if the endpoint dilution were 1:32, the range of possible values for the agglutination activity would be 32–64 units per ml. In an analogous manner, a range can be defined for specific agglutination activity.

RESULTS

Developmental Regulation of Agglutination Factor in D. discoideum. After cells were plated on Millipore filters in the absence of food, the cohesiveness of *D. discoideum* NC-4 cells and the specific agglutination activities of extracts of these cells increased after a lag of at least 3 hr (Fig. 1). At 0 and 3 hr, the cells showed no cohesiveness and cell extracts had no detectable agglutination activity. Cohesiveness and agglutination activity were both detectable 6 hr after plating and both had risen strikingly at 9 hr. Our measurements of the temporal development of cohesiveness in *D. discoideum* are qualitatively consistent with the observations of Gerisch (6). He found that in roller-tube culturing in the presence of EDTA, vegetative cells were not cohesive, but cells deprived of food for 9 hr formed large clumps.

In contrast to vegetative cells of the wild-type NC-4, axenically grown vegetative cells of the mutant strain A3 were

TABLE 1. *Effects of monosaccharides and disaccharides on agglutination of formalinized sheep erythrocytes by D. discoideum factor*

Sugar	Agglutination activity (units/ml) in 0.15 M sugar	Concentration for 50% inhibition of agglutination (mM)
No sugar	256	—
N-Acetyl-D-galactosamine	0	2
6-Deoxy-L-galactose (L-fucose)	16	9
D-Galactose	32	19
D-Galactosamine	32	19
Lactose	32	19
Sucrose	128	150
D-Mannose	128	150
D-Glucose	256	>150
N-Acetyl-D-glucosamine	256	>150
N-Acetyl-D-mannosamine	256	>150

Vegetative A3 cells were extracted in an EDTA salt buffer (24) consisting of 75 mM NaCl–75 mM KCl–1 mM EDTA–15 mM Tris·HCl (pH 7.3). Formalinized cells treated with extract by the standard assay procedure were added (25 μl per well) to the "V" plates, each containing 25 μl of either 0.15 M NaCl or sugar in 0.15 M NaCl. The degree of inhibition of agglutination activity produced by each sugar at a final concentration of 0.15 M was determined. In addition, the concentration of sugar that produced a 50% reduction in agglutination activity was determined. Comparable results were obtained with agglutination factor from differentiated NC-4 cells.

TABLE 2. *Agglutination of erythrocytes by intact D. discoideum cells*

Inhibitor	Maximum dilution of slime-mold cells that induced erythrocyte agglutination	
	0-hr cells	12-hr cells
None	1/2	1/32
GlcNAc	Undiluted	1/16
GalNAc	Undiluted	Undiluted

NC-4 cells deprived of food for 12 hr and vegetative NC-4 cells were grown. Slime-mold cells were dispersed by pipetting and were suspended in 0.15 M NaCl to a final concentration of 2×10^7 cells per ml. A 2-fold dilution series of each suspension was prepared in 0.15 M NaCl. One volume of each dilution was mixed with one volume of formalinized sheep erythrocytes (8×10^8 cells per ml in 2% normal rabbit serum in 0.15 M NaCl). 25 μl of the mixed cells were added to "V" plate wells containing 25 μl of 0.15 M NaCl, or 0.3 M *N*-acetylgalactosamine (GalNAc) in 0.15 M NaCl, or 0.3 M *N*-acetylglucosamine (GlcNAc) in 0.15 M NaCl. The plate was covered and then briefly agitated. After 1 hr, the patterns were evaluated for agglutination. The maximum dilution of slime-mold cells at which strong erythrocyte agglutination patterns occurred was determined.

cohesive and had high levels of agglutination factor. The specific agglutination activity in vegetative A3 cell extracts was about 5–10 times higher than in extracts of 9 hr-differentiated NC-4 cells. The cohesiveness of vegetative A3 cells is not as great as that of 9 hr NC-4 cells. A3 cells harvested while in log phase (2.5×10^6 cells per ml) had a cohesiveness value of 1.97 ± 0.04. The premature appearance of agglutination factor in these cells is not surprising since several proteins that normally develop in wild-type cells only after food is removed are present in growth-phase axenic cells (9).

The protein nature of the agglutination factor was indicated by the observations that it was heat-labile and nondialyzable, and that its activity was destroyed by preincubation with trypsin (3). Appearance of the factor in NC-4 cells during differentiation appears to be due to *de novo* protein synthesis, since addition of cycloheximide (0.5 mg/ml) to the Millipore support pad at time zero completely blocked development of both cohesiveness ($s = 1.02 \pm 0.01$) and agglutination activity at 9 hr.

Effects of Simple Sugars on the Agglutination of Erythrocytes by D. discoideum Factor. Several monosaccharides inhibited agglutination of formalinized sheep erythrocytes by *D. discoideum* factor (Table 1). Of those tested, *N*-acetyl-D-galactosamine was the most active in inhibiting agglutination produced by factors derived from either differentiated NC-4 cells or vegetative A3 cells. L-Fucose (6-deoxy-L-galactose), D-galactose, lactose, and D-galactosamine also showed considerable inhibitory activity, whereas other sugars had little or no effect (Table 1). These inhibition studies clearly demonstrate a resemblance between the agglutination factor and lectins, a class of specific carbohydrate-binding proteins of unknown function, isolated from a wide variety of plant and animal sources, which agglutinate erythrocytes and other cell types (10). Preliminary studies indicate that a glycopeptide fraction derived by exhaustive Pronase digestion of slime-mold homogenate contains a far more potent inhibitor, on a molar basis, than any of the monosaccharides studied.

Purification by Affinity Chromatography. The carbohydrate-binding character of the agglutination factor was further indicated by the fact that Sepharose 4B, a linear polymer of alternating D-galactose and 3,6-anhydro-L-galactose, can be used to purify the factor by affinity chromatography. Upon application of a crude slime-mold extract to a Sepharose 4B column, we found that none of the activity was eluted when the column was washed with three column volumes of buffer. However, all the activity was recovered by elution of the washed column with 0.3 M D-galactose. Upon polyacrylamide-gel electrophoresis, only two protein bands with similar mobilities were found when we used up to 200 μg of protein per gel (Fig. 2*a*). Upon dissociation with Na dodecyl sulfate and reduction with 2-mercaptoethanol, a single protein band was found (Fig. 2*b*), but with application of 200 μg of protein, a second more rapidly migrating band was observed which contained about 6% of the Coomassie blue staining material.

Agglutination of Erythrocytes by Intact Slime-Mold Cells. Because of the correlation between synthesis of this protein and development of cohesiveness, we sought to determine if the protein was detectable on the cell surface. To do this we reacted intact slime-mold cells with formalinized sheep erythrocytes. This approach was based on the finding that viruses and bacteria can agglutinate erythrocytes (11, 12) by interaction of surface factors on the microbes with receptors on erythrocyte membranes (11, 13).

Various concentrations of NC-4 cells deprived of food for 12 hr or vegetative NC-4 cells were mixed with a fixed number of

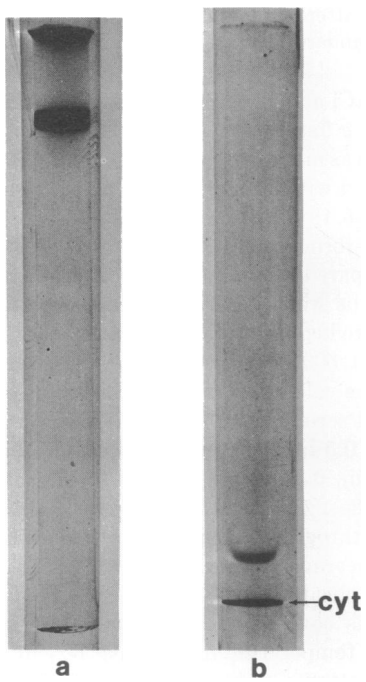

FIG. 2. Electrophoresis of purified protein. Both gels were composed of a 3% polyacrylamide-stacking gel containing 123 mM Tris·HCl (pH 6.8) and a 7.5% polyacrylamide running gel containing 375 mM Tris·HCl (pH 8.8). Intact protein (*a*) was electrophoresed in 0.1 M sodium borate buffer (pH 9.2); protein that had been dissociated (*b*) in 2% Na dodecyl sulfate and reduced with 5% 2-mercaptoethanol was electrophoresed in Tris-glycine buffer (pH 8.4) containing 0.1% Na dodecyl sulfate. The gels were stained with Coomassie blue. Cytochrome *c* (*cyt*) was added to the sample in the Na dodecyl sulfate gel.

formalinized sheep erythrocytes and added to a V-plate. At high cell concentrations both types of cells induced erythrocyte agglutination, but the minimum concentration of cells that induced agglutination was 16-times less for the 12-hr differentiated cells than for vegetative cells (Table 2). To determine if the agglutination was due to a carbohydrate–protein interaction like that found with isolated factor, we studied the effect of monosaccharides. The agglutination induced by 12-hr cells was substantially inhibited by *N*-acetylgalactosamine but not by *N*-acetylglucosamine (Table 2). In contrast, agglutination induced by vegetative cells was not differentially inhibited by these monosaccharides, suggesting that the binding between these cells and the erythrocytes was nonspecific.

Since it was possible that agglutination induced by the 12-hr cells was due to leakage of protein into the medium, we suspended 2×10^7 cells per ml in 0.15 M saline for $1^1/_2$ hour, then tested the medium. It produced only trace agglutination. Association of factor with the surface of the cells was also indicated by microscopic examination of samples removed from the V-plate that contained intact slime-mold cells. These samples showed large numbers of erythrocytes bound to slime-mold cells. These results suggest that the agglutination induced by 12-hr cells was indeed mediated by factor that is present on the surface of the slime-mold cells.

DISCUSSION

These experiments indicate that a protein agglutinin of formalinized sheep erythrocytes is synthesized during development in *D. discoideum* and that its synthesis is correlated with development of cohesiveness of these cells. We have shown that the agglutination factor is a carbohydrate-binding protein with specificity directed towards monosaccharides with a galactose configuration. The factor appears to be present on the surface of cohesive but not vegetative cells.

This evidence raises the possibility that the agglutination factor may be involved in intercellular adhesion. Beug *et al.* have shown that formation of intercellular contacts in *D. discoideum* can be blocked by univalent antibodies directed against discrete cell-surface antigens (14). The developmental appearance of these antigens (15) very closely parallels the appearance of agglutination factor. These antigens may be functionally related or possibly identical to the agglutination factor.

The finding that the agglutination factor is a carbohydrate-binding protein is of particular interest because of evidence implicating carbohydrates in the mechanisms of cellular interactions in various systems, including microbe–host cell interactions (11–13, 16–18), mating reactions of bacteria (19), yeast (20), and *Chlamydomonas* (21), and cell–cell interactions in tissue formation (14, 22, 23).

The above evidence, although consistent with a role for agglutination factor in cell adhesion, is largely circumstantial. Attempts to implicate the agglutination factor directly in cell adhesion have been negative. Thus we have not found conditions under which addition of purified agglutination factor influences the aggregation of cohesive cells in roller-tube culture. Low concentrations of factor have not augmented aggregation and high concentrations have not inhibited. Secondly, we have not been able to demonstrate that the monosaccharides that are most potent in inhibiting erythrocyte agglutination reaction selectively reduce slime-mold aggregation in roller-tube culture. High concentrations of galactose or *N*-acetylgalactosamine do reduce aggregation, but are not more effective than glucose or *N*-acetylglucosamine.

In view of these findings, the function of the developmentally regulated, carbohydrate-binding protein remains uncertain. Whether or not it participates in intercellular association in *D. discoideum* or in some other developmental process remains to be determined.

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1. Bonner, J. T. (1967) *The Cellular Slime Molds* (Princeton University Press, Princeton, N.J.), 2nd ed.
2. Gerisch, G. (1968) in *Current Topics in Developmental Biology*, eds. Monroy, A. & Moscona, A. (Academic Press, New York), Vol. 3, pp. 157–197; Bonner, J. T. (1971) *Annu. Rev. Microbiol.* **25**, 75–92; Robertson, A. & Cohen, M. H. (1972) *Annu. Rev. Biophys. Bioeng.* **1**, 409–464.
3. Rosen, S. D. (1972) Ph.D. Thesis, Cornell University.
4. Sussman, M. (1966) in *Methods of Cell Physiology*, ed. Prescott, D. (Academic Press, New York), Vol. 2, pp. 397–410.
5. Loomis, W. F., Jr. (1971) *Exp. Cell Res.* **64**, 484–486.
6. Gerisch, G. (1961) *Exp. Cell Res.* **25**, 535–554.
7. Butler, W. T. (1963) *J. Immunol.* **90**, 663–671.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
9. Ashworth, J. M. (1971) *Symp. Soc. Exp. Biol.* **25**, 27–49; Firtel, R. A. & Bonner, J. (1972) *Develop. Biol.* **29**, 85–103.
10. Sharon, N. & Lis, H. (1972) *Science* **177**, 949–959.
11. Burnet, F. M. (1951) *Physiol. Rev.* **31**, 131–150.
12. Neter, E. (1956) *Bacteriol. Rev.* **20**, 166–188.
13. Winzler, R. J. (1970) *Int. Rev. Cytol.* **29**, 77–125.
14. Beug, H., Gerisch, G., Kempff, S., Riedel, V. & Cremer, G. (1970) *Exp. Cell Res.* **63**, 147–158; Beug, H., Gerisch, G. & Muller, E. (1971) *Science* **173**, 742–743.
15. Beug, H., Katz, F. E. & Gerisch, G. (1973) *J. Cell Biol.* **56**, 647–658.
16. Weidel, W., Koch, G. & Bobsch, K. (1954) *Z. Naturforsch.* **9**, 573–579.
17. Gesner, B. M. & Thomas, L. (1966) *Science* **151**, 590–591.
18. Giboa-Garber, N. (1972) *FEBS Lett.* **20**, 242–244.
19. Sneath, P. H. A. & Lederberg, J. (1961) *Proc. Nat. Acad. Sci. USA* **47**, 86–90.
20. Crandall, M. A. & Brock, T. D. (1968) *Science* **161**, 473–475.
21. Wiese, L. (1965) *J. Phycol.* **1**, 46–54; Wiese, L. & Shoemaker, D. W. (1970) *Biol. Bull.* **138**, 88–95; Wiese, L. & Hayward, P. C. (1972) *Amer. J. Bot.* **59**, 530–536.
22. Humphreys, T. (1963) *Develop. Biol.* **8**, 27–47; Moscona, A. A. (1963) *Proc. Nat. Acad. Sci. USA* **49**, 742–474; Margoliash, E., Schenk, J. R., Hargie, M. P., Burokas, S., Richter, W. R., Barlow, G. H. & Moscona, A. A. (1965) *Biochem. Biophys. Res. Commun.* **20**, 383–388.
23. Moscona, A. A. (1962) *J. Cell. Comp. Physiol.* (Suppl. 1) **60**, 56–80; Glaeser, R. M., Richmond, J. E. & Todd, P. W. (1968) *Exp. Cell Res.* **52**, 71–85; Barondes, S. H. (1970) in *The Neurosciences Second Study Program*, ed. Schmitt, F. O. (Rockefeller University Press, New York), pp. 747–760.
24. Takeuchi, I. & Yabuno, K. (1970) *Exp. Cell Res.* **61**, 183–190.