Identification of endogenous binding proteins for the lectin discoidin-I in *Dictyostelium discoideum*

(discoidin column/glycoproteins/hemagglutination/cohesion assay)

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ABSTRACT   Recent biochemical and genetic evidence has
shown that the endogenous lectin discoidin-I is involved in inter-
cellular adhesion during development of the cellular slime mold
Dictyostelium discoideum. We have prepared discoidin-I affinity
columns and used them to isolate the lectin receptors. By using the
cell surface radioiodination method, 11 discoidin-I binding pro-
teins were identified in wild-type NC4 cells by gel electropho-
resis, with apparent molecular weights of 95,000, 85,000, 78,000,
72,000, 60,000, 33,000, 31,000, 28,000, 25,000, 21,000, and 15,000.
Only three (gp33, gp31, and gp28) were under developmental reg-
ulation. The amount of gp31 increased 8- to 10-fold during aggre-
gation, and it was the predominant discoidin-I binding protein
synthesized at the aggregation stage. Discoidin-I binding proteins
derived from aggregation stage cells were potent inhibitors of dis-
coidin-I in a hemagglutination assay *in vitro*. The same prepara-
tion was found to promote aggregation of cells bearing discoidin-
I on the surface, suggesting a multivalent interaction.

The amoebae of *Dictyostelium discoideum* are capable of dif-
ferentiating from a free-living vegetative state to an organized
multicellular structure (1). Removal of nutrients from the
growth medium serves as the trigger for cells to embark on a
differentiation pathway. A chemotactic relay system with cyclic
AMP as the signal molecule is established at the initial phase
of development, which is followed by the formation of stable
EDTA-resistant aggregates. The intercellular cohesiveness of
these cells is species specific and much evidence has been ac-
cumulated that it is mediated by specific cell surface molecules
(2–4). Two endogenous lectins, discoidins I and II, have been
isolated from *D. discoideum* (5). Because their temporal expres-
sion corresponds closely with the acquisition of cellular cohe-
siveness in developing cells, it has been suggested that they play
a role in cell–cell adhesion (3). Recently, it was conclusively
demonstrated by genetic means that the lectin discoidin-I is an
essential component of the cell adhesion system (6, 7). It has
also been shown that there are specific surface receptors for
discoidin-I and that they may be glycoproteins (8, 9).

In the present report, we identify the endogenous glycopro-
tein receptors for discoidin-I, a carbohydrate binding protein
that is involved in mediating cell–cell adhesion in *D. discoi-
deum*. By means of chromatography on affinity columns, we
have found that a restricted, albeit heterogeneous, population
of discoidin-I binding proteins is maintained on the cell surface
throughout development. The expression of a few of these gly-
coproteins appears to be under developmental regulation.
These endogenous discoidin-I binding proteins are also potent
inhibitors of the hemagglutination activity of the lectin, and they
appear to have an aggregation-enhancing effect on differen-
tiated cells.

MATERIAL AND METHODS

Cell Culture. NC4 cells were cultured on nutrient agar plates
in association with *Klebsiella aerogenes* for 36–40 hr. Cells were
washed free of bacteria and deposited on filter paper for de-
velopment (10). Aggregation began at about 5 hr. Amoebae that
had developed for 3 hr were defined as preaggregation cells,
those developed for 7–10 hr as aggregation stage cells. Cells of
the axenic strain A3 were grown in HL-5 medium (4) and de-
veloped on filter paper.

Labeling of Cells with [³⁵S]Methionine. [³⁵S]Methionine was
obtained from New England Nuclear (specific activity ≈550 μCi/
mmol; 1 Ci = 3.7 × 10¹⁰ becquerels). To label cells metabol-
ically, a 4-cm-diameter filter disc with 10⁸ cells was put directly
on top of 80 μl of PDF buffer (10) containing 60 μCi of
[³⁵S]methionine. Cells were labeled for 2 hr and then washed
twice in 17 mM potassium phosphate (pH 6.5). The cell pellet
was stored at −70°C.

Radioiodination of the Cell Surface. The lactoperoxidase
method was used to label cell surface proteins as described (4).

Radioiodination of Proteins. Soluble proteins were labeled
with ¹²⁵I by the chloramine-T method (11). Asialofetuin [pre-
pared according to Lotan et al. (12) from type IV calf serum fe-
tuin (Sigma)] was radioiodinated at 200 μg/ml in 25 mM
Tris·HCl (pH 7.2) containing 0.15 M NaCl to an activity of 5–10
× 10⁴ cpm/μg protein. Unreacted ¹²⁵I was removed by gel fil-
tration on a 1 × 10 cm Sephadex G-25 column.

Preparation of Discoidin. Discoidin was isolated according
to the method of Frazier et al. (5) from axenically grown cells
of strain A3 at their late exponential growth phase. Soluble pro-
teins were charged onto a 1000-ml column of Sepharose 4B,
which was eluted with 0.3 M D-galactose after washing. Ap-
proximately 0.8% of the total starting protein was eluted with
galactose, and fractions consisting primarily of discoidin-I
(≈95%) were pooled.

Preparation of Discoidin-I Bio-Gel Columns. The method
described by Ternynck and Avrameas (13) was followed with
some modifications. Bio-Gel P-300, 200 mesh (from Bio-Rad)
was swollen in H₂O overnight. Four milliliters of hydrated gel
was activated by suspension in 5 vol of 0.1 M sodium phosphate
buffer (pH 7.0) containing 6% (wt/vol) glutaraldehyde and in-
cubation overnight at 37°C. The gel was then washed in a sin-
tered glass funnel with 300 ml of H₂O, equilibrated with a buffer
containing 75 mM NaCl, 0.3 M galactose, and 75 mM sodium
phosphate (pH 7.4) and packed by centrifugation. It was re-
suspended in 20 ml of discoidin-I solution containing 2–3 mg
of protein in 75 mM NaCl, 0.3 M galactose, and 75 mM sodium
phosphate (pH 7.4). The tube containing the activated beads
and discoidin-I was rotated end-over-end overnight at room

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temperature. The gel was washed three times with the same buffer solution. The extent of coupling was usually between 70% and 80%. Rotation of the discoidin-Bio-Gel beads end-over-end for 3 hr at room temperature in 20 ml of 0.1 M lysine/0.3 M galactose, titrated to pH 7.4, blocked free active aldehyde groups. Although the Schiff base links between protein and the matrix were already stable at this stage, reduction with NaBH₄ was carried out as suggested (12). The affinity gel was stored in 25 mM Tris·HCl (pH 7.2)/75 mM NaCl/0.3 M galactose/1 mM EDTA/0.02% NaN₃.

Affinity Chromatography. Labeled cells, stored as pellets, were thawed in 4 ml of 25 mM Tris·HCl (pH 7.1)/75 mM NaCl/75 mM KI and centrifuged at 20,000 rpm for 20 min in a Beckman Ti 40 rotor. The supernatant was discarded and the crude particulate pellet was solubilized with 100 μl of 10% (wt/vol) Triton X-100 for 10 min on ice. The volume was adjusted to 4 ml with the same buffer solution containing phenylmethylsulfonyl fluoride. The detergent suspension was centrifuged at 40,000 rpm for 1 hr in the same rotor. Usually 90% of the radioactivity in the pellet was solubilized. The supernatant was then incubated with 3–4 ml of discoidin-Bio-Gel beads for 2 hr in the cold with mild agitation, and the column material was transferred to a 10-ml column and washed at a flow rate of 0.4 ml/min with at least 10 column vol of the same buffer containing 0.05% Triton X-100. Then the flow rate was reduced to 0.2 ml/min and the column was eluted first with the same buffer containing 0.15 M D-mannose and 1 mM EDTA, followed by elution with 0.3 M D-galactose. Fractions of the radioactive peak eluted by galactose were combined, dialyzed for 24 hr against several changes of H₂O, lyophilized, and prepared for gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gradient slab gels (5–15% acrylamide) were prepared according to the method of Laemmli (14). Molecular weight standards were: β-galactosidase (130,000), phosphorylase a (92,000), bovine serum albumin (68,000), immunoglobulin G heavy chain (52,000), ovalbumin (45,000), deoxyribonuclease I (32,000), chymotrypsinogen (25,000), myoglobin (18,000), and cytochrome c (12,000). Electrophoresis was performed as described (4). For ¹²⁵I-labeled proteins, autoradiography was carried out at −70°C on a pre-exposed film with CaWO₄ intensifying screen according to Laskey and Mills (15). ³⁵S-Labeled proteins were detected by fluorography using the procedure of Bonner and Laskey (16).

Erythrocyte Agglutination Assay. A 1:2 dilution series of purified discoidin-I was mixed with an equal volume of a 2% suspension of formalin-treated sheep erythrocytes in microtiter "V" plates (17). The samples were evaluated after 1 hr. The endpoint dilution was taken as the minimal concentration of discoidin-I required for hemagglutination, usually 1.25 μg/ml.

Cohesion Assay. Intercellular cohesiveness was quantitated by the roller-tube method (18). Cells were counted on a hemocytometer to estimate the percentage of reaggregated cells.

RESULTS

Isolation of Discoidin-I Binding Proteins by Affinity Chromatography. Discoidin-I was conjugated to Bio-Gel beads as described in *Material and Methods*. The binding capacity of the discoidin-Bio-Gel column was determined by its ability to retain ¹²⁵I-labeled asialofetuin, a glycoprotein with terminal galactose residues. It was found that the discoidin column specifically retained asialofetuin, although the capacity was low, with approximately 0.4 μg of asialofetuin bound per 2 mg of immobilized discoidin-I. Because asialofetuin is not a physiological receptor, the capacity of the column for endogenous receptors may be higher.

To isolate discoidin-I binding proteins by affinity column chromatography, cells were routinely lysed in 1% Triton X-100, at a ratio of approximately 5 mg of detergent per mg of cell protein for optimal solubilization. Fig. 1 shows the elution profile of such a supernatant prepared from 8-hr NC4 cells, which were labeled with [³⁵S]methionine for 2 hr prior to solubilization. Most of the radioactivity was eluted in the initial wash with Tris/saline buffer. To demonstrate that the retention of glycoproteins in the column was due to specific carbohydrate–protein interaction, the column was first eluted with 0.15 M D-mannose, which is a poor competitor for discoidin-ligand binding. No additional radioactivity was eluted. However, a sharp radioactive peak was obtained when the column was eluted with 0.3 M D-galactose, which is a potent competitor for the carbohydrate binding sites on discoidin-I (3). The discoidin-I binding proteins together contained approximately 0.1% of the total input radioactivity. Essentially the same elution profile was obtained in experiments with cells at different developmental stages or labeled by different methods.

Characterization of Discoidin-I Binding Proteins. The ³⁵S-labeled proteins that were specifically eluted by D-galactose in Fig. 1 were analyzed by gel electrophoresis and the profiles are shown in Fig. 2. Although a broad spectrum of cellular proteins was labeled by [³⁵S]methionine, only five distinctive discoidin-I binding protein bands were detectable by fluorography. Their apparent molecular weights were 95,000, 60,000, 31,000, 28,000 and 21,000. Although the band at the 31,000 molecular weight region was relatively broad, shorter exposures of the gel showed only a single band. Co-electrophoresis with discoidin-I showed that gp31 (glycoprotein of molecular weight 31,000) was a polypeptide distinct from discoidin-I, which consistently migrated ahead of gp31 and had an apparent molecular weight of 29,000 in the Laemmli gel system. When the amount of radioactivity associated with each binding protein was estimated by densitometer tracings, gp31 was found to constitute about 90% of the total radioactivity eluted by galactose. The data indicate that gp31 was either very rich in methionine or synthesized in much larger quantities than the other binding proteins during cell aggregation.
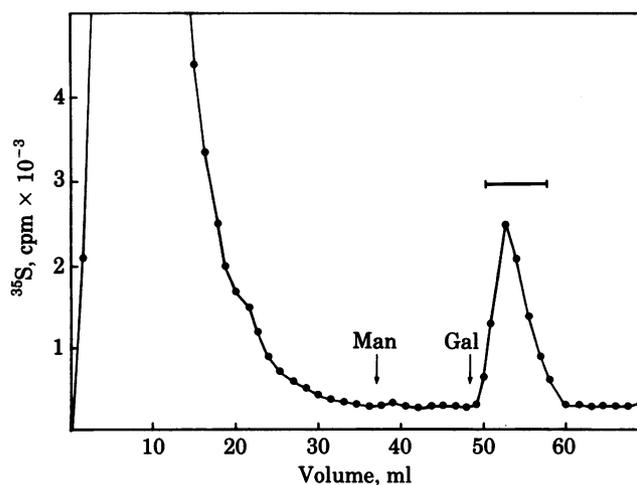

FIG. 1. Affinity chromatography of [³⁵S]methionine-labeled extract of 8-hr NC4 cells in a discoidin-Bio-Gel column. Cells were labeled with [³⁵S]methionine at 60 μCi per 10⁸ cells for 2 hr and then solubilized in 0.25% Triton X-100 containing phenylmethylsulfonyl fluoride at 100 μg/ml. Inclusion of 0.15 M D-mannose and 0.3 M D-galactose in the elution buffer is indicated by arrows. The radioactive peak eluted with 0.3 M D-galactose constituted approximately 0.1% of the input radioactivity.

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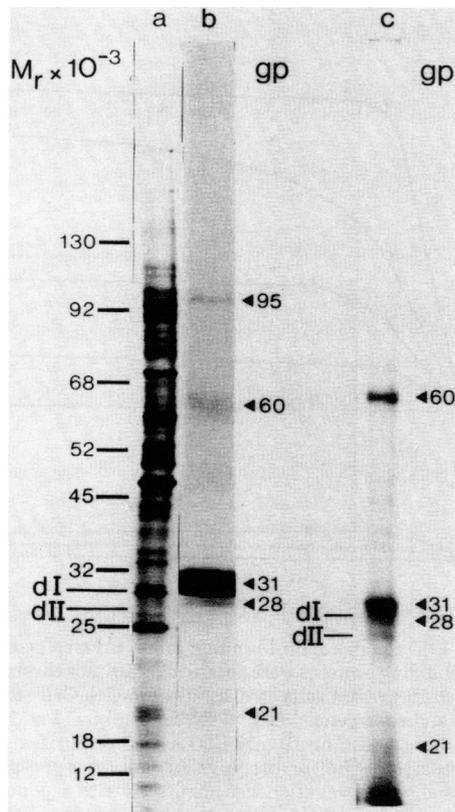*Proc. Natl. Acad. Sci. USA 78 (1981)*   2117

FIG. 2. Gel profiles of discoidin-I binding proteins from 8-hr NC4 cells. Lanes a and b represent fluorograms of gel electrophoretic profiles of the two radioactive peaks obtained in Fig. 1. Lane a, ^{35}S-labeled material not bound to the discoidin-Bio-Gel column. Lane b, ^{35}S-labeled proteins retained on the column and eluted with 0.3 M D-galactose. In another experiment, crude membranes from unlabeled 10-hr NC4 cells were solubilized and chromatographed on a discoidin column. Material eluted with 0.3 M galactose was radioiodinated by using the chloramine-T method (11). Lane c, autoradiogram of ^{125}I-labeled discoidin-I binding proteins. Molecular weight markers are indicated on the left and dI and dII denote the positions of discoidin-I and discoidin-II. Molecular weights ($\times$ 10^{-3}) of the discoidin binding glycoproteins (gp) are indicated on the right.

Because only a limited number of binding proteins were detected by metabolic labeling with [^{35}S]methionine, eluates from the affinity column were labeled with ^{125}I in order to detect any additional protein bands. Fig. 2, lane c shows that essentially the same proteins were labeled and no additional bands were detected by this method.

Discoidin-I Binding Proteins on Cell Surface. Because discoidin-I is present on the cell surface and presumably binds to glycoconjugates on adjacent cells, discoidin-I binding proteins should also be present on the cell surface. To identify the surface receptors, intact cells were radioiodinated by the lactoperoxidase method (4). Labeled cells were solubilized in Triton X-100 and the supernatant was passed through a discoidin-I column. Material eluted specifically by galactose was subjected to gel electrophoresis (Fig. 3). A comparison of the autoradiograms in lanes c and f showed that the majority of discoidin-I binding proteins were conserved between the preaggregation and midaggregation stages. Proteins with apparent molecular weights of 95,000, 85,000, 78,000, 72,000, 60,000, 31,000, 25,000, 21,000, and 15,000 appeared to be common to both stages of development. All of the discoidin-I binding proteins, with the exception of gp21, were minor surface components as detected by radioiodination.

Out of 11 identified discoidin-I binding proteins, gp33 was present on 3-hr cells but not on 10-hr cells, while gp28 appeared only at the aggregation stage. Again the binding protein gp31 showed an approximate 10-fold increase in its relative labeling intensity between 3 and 10 hr of development. This protein was the major discoidin-I binding protein synthesized during this period (Fig. 2, lane b). gp21 also showed a 2- to 3-fold increase in its relative labeling intensity.

Discoidin-I Binding Proteins in A3 Cells. Because several recent reports (19, 20) have failed to detect discoidin binding proteins in A3 cells, similar experiments were performed using axenically grown A3 cells to resolve this discrepancy. A3 cells were allowed to develop on filter pads, and vegetative and aggregated cells were radioiodinated and solubilized for affinity chromatography. The radioactivity in the receptor fraction recovered from 15-hr cells was about 5 times higher than that from vegetative cells. Autoradiograms of the binding proteins are shown in Fig. 4. Only three discoidin-I binding protein bands—with apparent molecular weights of about 95,000, 28,000, and 21,000—were detected. gp95 of A3 cells showed an approximate 3-fold increase in its labeling intensity in differentiated cells. The major developmentally regulated discoidin-I binding protein gp31 found on NC4 cells was not detected on A3 cells.

Inhibition of Hemagglutination Activity of Discoidin-I by Receptors. To investigate the degree of affinity between discoidin-I and its putative receptors, the ability of proteins eluted specifically from the discoidin-Bio-Gel column to compete for the binding of discoidin-I in an *in vitro* hemagglutination assay was tested. The minimal concentration of discoidin-I binding
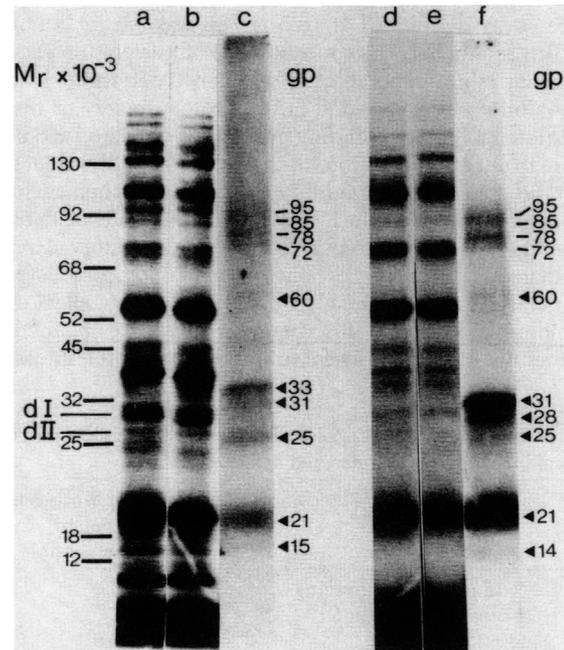

FIG. 3. Discoidin-I binding proteins on the surface of 3-hr and 10-hr NC4 cells. Cells from the 3-hr and 10-hr stages were radioiodinated by the lactoperoxidase method and then solubilized in 0.25% Triton X-100. The 10,000 $\times$ *g* supernatant was equilibrated for 2 hr with discoidin-Bio-Gel. After washing with 10 vol of the Tris/saline buffer (pH 7.1), the column was eluted with 5 vol of 0.15 M D-mannose followed by elution with 0.3 M D-galactose. Lanes a, b, and c represent material from 3-hr preaggregation stage cells, and lanes d, e, and f represent material from 10-hr aggregation stage cells. Lanes a and d, radioiodinated cell surface proteins solubilized by Triton X-100; lanes b and e, soluble material not bound to the discoidin-Bio-Gel column; lanes c and f, proteins retained on the column and eluted by 0.3 M D-galactose. Figure labeling conventions are as in Fig. 2.
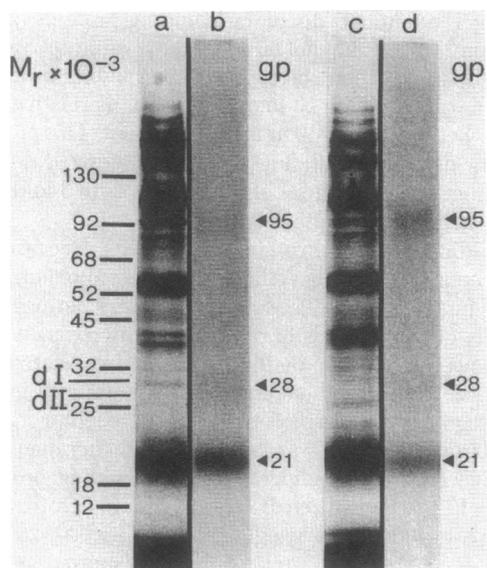
FIG. 4. Discoidin-I binding proteins on the surface of 0-hr and 15-hr A3 cells. Surface radioiodinated A3 cells were solubilized in Triton X-100 and the supernatant was passed over a discoidin-Bio-Gel column as described for Fig. 3. Material from 0-hr cells was run in lanes a and b, and from 15-hr cells in lanes c and d. Lanes a and c, radioiodinated 0-hr and 15-hr whole cells; lanes b and d, proteins that were retained on the discoidin column and eluted with 0.3 M D-galactose. Figure labeling conventions are as in Fig. 2.

proteins required to inhibit completely the hemagglutination mediated by discoidin-I at 1.25 μg/ml was 0.6 μg/ml, 1/6th to 1/7th as much as the concentration of asialofetuin required for similar inhibition (Table 1). In order to determine whether the inhibitory effect resided in the protein moiety or the carbohydrate moiety, the binding proteins were heated at 85°C for 20 min to denature the protein, then cooled rapidly. Heat treatment did not affect their inhibitory effect on the hemagglutination activity of discoidin-I. However, periodate oxidation of discoidin-I binding proteins reduced their inhibitory activity to less than 1/8th (Table 1). Controls containing periodate pre-neutralized with ethylene glycol did not have any effect on the discoidin-I binding proteins. Similar results were obtained with asialofetuin. These results confirm the idea that the interaction

Table 1. Inhibition of discoidin-I mediated hemagglutination by discoidin-I binding proteins and asialofetuin

Inhibitor	Minimal concentration for complete inhibition, μg/ml
Discoidin-I binding proteins	0.6
Heat-treated discoidin-I binding proteins	0.6
NaIO$_4$-treated discoidin-I binding proteins	4.8
Asialofetuin	4.0
NaIO$_4$-treated asialofetuin	32.0

The hemagglutination assay used formalin-treated sheep erythrocytes. Discoidin-I binding proteins were isolated from 10-hr NC4 cells. A final concentration of 1.25 μg/ml of discoidin-I was used and various concentrations of the inhibitor were added to the samples, which were evaluated after 1 hr of incubation. Heat denaturation of discoidin-I binding proteins was performed at 85°C for 20 min followed by rapid cooling. Periodate oxidation was carried out at 23°C for 5 hr in the dark, using 40 mM NaIO$_4$ (21). The reaction was stopped with 1% ethylene glycol.
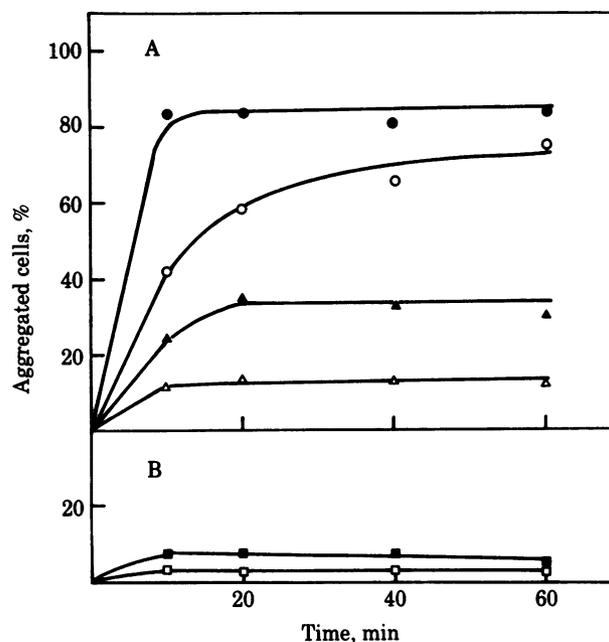

FIG. 5. Effect of discoidin-I binding proteins on cell cohesiveness. Discoidin-I binding proteins were obtained by affinity chromatography of solubilized 10-hr NC4 cells on discoidin-Bio-Gel. Cell samples were dissociated and resuspended at 2 × 10^6 cells/ml in 17 mM phosphate buffer (pH 6.2) containing 10 mM EDTA. Either purified discoidin-I binding proteins (3 μg/ml) or an equivalent volume of phosphate buffer was added and cell aggregation was monitored with a hemocytometer. (*A*) Aggregation of 9-hr NC4 cells in the presence (●) or absence (○) of discoidin-I binding proteins; aggregation of 6-hr NC4 cells in the presence (▲) or absence (△) of discoidin-I binding proteins. (*B*) Aggregation of WL3 cells in the presence (■) or absence (□) of discoidin-I binding proteins.

between discoidin-I and its receptors involves carbohydrate moieties.

Enhancement of Cell Aggregation by Discoidin-I Binding Proteins. Discoidin-I binding proteins were added to cells in the roller-tube cohesion assay in order to investigate their effect on cell–cell adhesion. The binding protein preparation did not cause any detectable changes in individual cell morphology as determined by phase-contrast microscopy, but a definite enhancement of cell aggregation was observed (Fig. 5). In the presence of discoidin-I binding proteins, cell aggregation was accelerated and 9-hr cells attained maximal reaggregation in 10 min as opposed to 60 min in the control (Fig. 5A). Also, larger aggregates were formed in the presence of discoidin-I binding proteins. Addition of fetuin or immunoglobulin to these cells had no effect on aggregation. At 6 hr of development, when the expression of discoidin-I on the cell surface just became detectable and only 10% of the cells were able to form EDTA-stable contacts, addition of discoidin-I binding proteins almost tripled the number of cells capable of forming aggregates. On the other hand, addition of the putative receptors to a noncohesive mutant WL3, which does not express any detectable surface discoidin (4), did not have any appreciable effect on these cells (Fig. 5B). These data suggest that the enhancement of cell aggregation by discoidin-I binding proteins is dependent on the presence of discoidin-I.

DISCUSSION

The participation of discoidin-I in the process of cell–cell adhesion implicitly depends on the presence of carbohydrate-bearing ligands for this lectin on the surface of developing *D. dis*-

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coideum cells. In this report, we have identified endogenous discoidin-I binding proteins by using affinity column chromatography. In the process of finding an appropriate method for protein immobilization, it became apparent that the common procedure of coupling proteins to CNBr-activated Sepharose beads results in a loss of the lectin's activity *in vitro*. However, a modification of the method of Ternynck and Avrameas (13) has enabled us to obtain functional discoidin affinity columns.

At least 11 discoidin-I binding proteins can be recovered from the affinity column. In comparison, 15–30 surface glycoproteins bind to concanavalin A at the aggregation stage (19, 22). Therefore, the molecules recognized by discoidin-I probably represent a relatively restricted portion of surface molecules. It is also of interest to note that the overall population of these molecules does not change drastically during development, and most of them are probably constitutive components of the plasma membrane. This agrees with our previous observations that only minor alterations in the plasma membrane components seem to accompany major morphogenetic events (23). Of the 11 endogenous discoidin-I binding proteins, only 3—gp33, gp31, and gp28—appear to be under developmental regulation. gp31 is the only binding protein synthesized predominantly during the aggregation period, and the same protein band shows an approximate 10-fold increase in its relative labeling intensity by ^{125}I, indicating that it begins to accumulate on the cell surface at the onset of cell aggregation. Therefore, gp31 may play an important role in the lectin-ligand system responsible for cell–cell adhesion. The other discoidin-I binding proteins are either synthesized at lower rates or have a much lower content of methionine and therefore fail to incorporate the radioactive precursor.

In axenically grown A3 cells, which express discoidin prematurely during vegetative growth (4), only three discoidin-I binding proteins are detectable on the surface of A3 cells, and they appear to be similar to those found on NC4 cells. In A3 cells, gp95 seems to be under developmental regulation and increases during differentiation. Such a variation would not be surprising, because it is known that there are significant biochemical and morphological differences between the axenic mutants and wild-type cells. Because A3 cells are capable of normal development, it appears that, in this strain at least, a more limited repertoire of potential discoidin-I receptors is sufficient for cell–cell adhesion.

The potent inhibitory effect of discoidin-I binding proteins from aggregation stage NC4 cells on discoidin-I-mediated hemagglutination indicates that they are high-affinity ligands for the lectin. Intact terminal carbohydrate residues appear to be essential for discoidin–receptor interactions. It is of interest to note that the same binding protein preparation that inhibits discoidin-I hemagglutination activity also promotes aggregation of discoidin-I-bearing cells in the cohesion assay. A possible explanation is that the receptor glycoproteins are multivalent molecules and therefore capable of crosslinking adjacent cells. Analogous situations have been reported for the aggregation factors in chicken neural retina (24, 25). We have been unable to detect large receptor molecules, such as the proteoglycans found in marine sponges (26, 27). However, it is possible that the receptor glycoproteins aggregate in the detergent-free solution to form large crosslinking entities. In the future, it will be necessary to fractionate the various putative receptor glycoproteins and study them individually.

In addition to discoidin-I, other plasma membrane molecules have been implicated in slime mold cell adhesion. The contact site A glycoprotein with an approximate molecular weight of 80,000 has been shown to be involved in end-to-end cell contacts (28), and gp150 (29) and gp95 (30) are two surface glycoproteins that may be involved in cell–cell adhesion or cell sorting at later stages. Because it is now possible to isolate and identify the discoidin-I binding proteins, further experiments can be carried out to elucidate the relationship between discoidin-I and other molecules involved in cell cohesion.

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