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DISCOIDIN I-MEMBRANE INTERACTIONS

III. INTERACTION OF DISCOIDIN I WITH LIVING *DICTYOSTELIUM DISCOIDEUM* CELLS

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We have examined the interaction of exogenous ^{125}I-discoidin I with living wild type (NC-4) *Dictyostelium discoideum* cells and the subcellular localization of endogenous discoidin I during development. The living cells bind ^{125}I-discoidin I relatively rapidly, with an apparent steady state being reached within 40 min at 23°C. The rate of association is reduced to a similar extent by lowering the temperature to 4°C and by glutaraldehyde fixation of the cells. The level of steady-state binding is not developmentally regulated. Living cells do not degrade significant amounts of the ^{125}I-discoidin I during incubations at 23°C. Differentiated cells bind the ^{125}I-discoidin I via both the carbohydrate or C site receptors and the ionic or I site receptors, first identified on the corresponding glutaraldehyde-fixed cells (Bartles, J.R. and Frazier, W.A. (1982) Biochim. Biophys. Acta 687, 121–128). Depending on the concentration of ^{125}I-discoidin I, 50% or more of the binding to the 8.5-h differentiated cells is via the I sites. Binding exhibits apparent positive cooperactivity with respect to discoidin I. This causes the binding to appear nonsaturable, with a capacity for more than 10^7 discoidin I tetramers per cell. Independent verification of the nonsaturability of the interaction was obtained from experiments using diazotized [^{125}I]iodosulfanilic acid to radiolabel the surfaces of cells treated with increasing concentrations of unlabeled discoidin I. The susceptibility of the surface radiolabeled discoidin I to various extractive treatments of the cellular particulate fraction suggests that as much as 10% is very tightly associated with cellular membranes. Dissociation experiments also suggest the existence of an irreversible component of binding, the magnitude of which increases with time of association. While the 8.5-h differentiated NC-4 cells exhibit maximal developmental cohesiveness and contain about $5 \cdot 10^6$ discoidin I tetramers per cell, cell surface labeling and radioimmunoassay indicate that they display only $1.3 \cdot 10^3$ tetramers per cell on their surface and only $6 \cdot 10^3$ tetramers per cell in their surrounding extracellular medium.

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Abbreviations: 17 mM P_i, 17 mM Na_2HPO_4-KH_2PO_4/100 U penicillin G per ml/100 μg streptomycin sulfate per ml (pH 6.3); PP_i sucrose, 40 mM $NaPP_i$/10% (w/v) sucrose/3 mM NaN_3, adjusted to pH 8.0 with HCl; 150 mM NaCl/P_i, 58 mM Na_2HPO_4/18 mM KH_2PO_4/75 mM NaCl/3 mM NaN_3 (pH 7.2); albumin, bovine serum albumin; SDS, sodium dodecyl sulfate.

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Introduction

In a previous paper [1], we demonstrated that the endogenous lectin of *Dictyostelium discoideum*, discoidin I, can bind to fixed *D. discoideum* cells by two different mechanisms. One mechanism involves binding to the carbohydrate or C sites, developmentally regulated carbohydrate-containing receptors. The other mechanism involves an electrostatic interaction between discoidin I and the ionic or I sites. The I sites are believed to represent negatively charged lipids [1,2].

In order to assess the relative importance of the two modes of discoidin I binding to the function of the lectin in developmental processes, such as cell cohesion, we have examined the interaction of discoidin I with living *D. discoideum* cells under physiological buffer conditions (17 mM P_i, pH 6.3). We have prefaced this binding study with an analysis of the subcellular distribution of endogenous discoidin I during development and under the conditions of the binding assay.

Materials and Methods

Materials. [^{125}I]Iodosulfanilic acid (> 2000 Ci/mmol) was obtained as a labeling kit (NEX-121) from New England Nuclear (Boston, MA). Normal chicken serum was from Gibco (Grand Island, NY). Crude insoluble protein A (P-5642) was from Sigma (St. Louis, MO). Emulphogene BC-720 was from GAF (Melrose Park, IL). Complete and incomplete Freund's adjuvants and *Bordetella pertussis* antigen were from Difco (Detroit, MI). All other materials were obtained from the sources designated previously [1,2]. Discoidin I was isolated, radioiodinated and prepared for use in experiments as described previously [1]. The wild type (NC-4) *D. discoideum* cells were grown on suspensions of *Escherichia coli* strain B/r and then allowed to differentiate in suspension as described previously [1].

Preparation of subcellular fractions for radioimmunoassay. After the indicated times of suspension differentiation, the NC-4 cells were harvested by centrifugation (800 × *g*, 3 min, 23°C). The extracellular medium was made 3 mM in NaN_3 and 0.2 mM in phenylmethylsulfonyl fluoride and centrifuged (48000 × *g*, 15 min, 4°C) to remove cellular debris. The cells were washed with 1 volume of 17 mM P_i by centrifugation (800 × *g*, 3 min, 4°C) and quickly frozen in a solid CO_2-isopropranol bath at 10^8 cells/ml in PP_i/sucrose. The cells were lysed by two cycles of freezing and thawing in the presence of 0.4 mM phenylmethylsulfonyl fluoride. The soluble fraction was prepared by microfuge centrifugation (13000 × *g*, 5 min, 4°C) of the lysate. The extracellular medium and soluble fraction samples were stored at −70°C and recentrifuged (13000 × *g*, 5 min, 4°C) prior to radioimmunoassay.

Preparation and characterization of antiserum. Antiserum specific for discoidin I was raised in rabbits by a modification of the procedure of Vaitukaitis et al. [3]. For the first immunization, a 0.6 mg/ml solution of discoidin I in 0.15 M NaCl was emulsified with an equal volume of complete Freund's adjuvant, and the mixture was injected intradermally at 12 to 15 different sites (approx. 0.15 ml/site) on the back of the rabbit. A solution of *Bordetella pertussis* antigen was simultaneously injected intradermally at three to five different sites (approx. 0.15 ml/site) on the back of the rabbit. For the second immunization one month later, a 0.6 mg/ml solution of discoidin I in 0.15 M NaCl was emulsified with an equal volume of incomplete Freund's adjuvant, and the mixture was injected intradermally at five to seven different sites (approx. 0.15 ml/site) on the back of the rabbit. The rabbits were bled one month later from the ear vein, and the antiserum was stored at −20°C. The antiserum was judged to be specific for disocidin I by double immunodiffusion and immunoprecipitation. 25 μl of a 10^5-fold (v/v) dilution of the antiserum could routinely immunoprecipitate 50% of the 0.25 ng of ^{125}I-discoidin I included in the standard radioimmunoassay (see below).

Radioimmunoassay for discoidin I. 75 μl of the solution to be assayed or an appropriate standard solution containing 1 ng/ml to 1 μg/ml of unlabeled discoidin I was mixed with 75 μl of a 3.3 ng/ml solution of ^{125}I-discoidin I (1.5 to 2.0 · 10^4 cpm) in 150 mM NaCl/P_i containing 2 mg/ml of albumin. 25 μl of a 10^5-fold (v/v) dilution of antiserum into normal chicken serum was added, and the samples were incubated at 4°C. After 15 h, 100 μl of a 5% (w/v) suspension of crude insolu-

ble protein A in 150 mM NaCl/P_i was added, and the samples were incubated at 4°C for 4 to 6 h. The suspensions were centrifuged (1500 × *g*, 5 min, 4°C). The pelleted radioactivity was determined on the ^{125}I channel of a Beckman 300 γ counter. The amount of discoidin I in an unknown sample was determined from a standard curve of pelleted radioactivity versus the logarithm of the concentration of unlabeled discoidin I in the added sample. The standard curve was linear for concentrations between 10 ng/ml and 1 μg/ml.

Binding assay. After 0 h (vegetative cells) or 8.5 h of suspension differentiation, the NC-4 cells were harvested and washed twice with 1 vol. of 23°C 17 mM P_i containing 1 mg/ml of albumin by centrifugation (800 × *g*, 3 min, 23°C). The washed cells were incubated at $(0–1.6) \cdot 10^7$ cells/ml in albumin pre-equilibrated Falcon tubes with $(0.7–1.7) \cdot 10^6$ cpm/ml (9 to 46 ng/ml) of ^{125}I-discoidin I and the indicated final concentrations of binding inhibitors or unlabeled discoidin I all in 17 mM P_i containing 1 mg/ml of albumin. The tubes were shaken at approx. 380 strokes/min on a New Brunswick model R-2 reciprocating shaker for the indicated time intervals, and then 200- to 300-μl aliquots were removed and either: (i) washed twice by centrifugation (800 × *g*, 3 min, 4°C) with 5 ml of 4°C 17 mM P_i containing 1 mg/ml of albumin (centrifugation method); or (ii) delivered onto prewashed 25-mm diameter 0.8-μm polycarbonate filters (Nuclepore) and washed four times within 30 s with 1-ml washes of 4°C 17 mM P_i containing 1 mg/ml of albumin by vacuum filtration (filtration method).

For dissociation experiments, 200-μl aliquots of the incubation mixture were removed to determine initial binding (see above). The remainder was centrifuged (800 × *g*, 3 min, 23°C) to remove unbound ^{125}I-discoidin I, the cells were diluted to one-fiftieth their initial (incubation mixture) concentration, and the suspension was periodically agitated. At the indicated times after dilution, 10-ml aliquots were assayed for binding by the centrifugation or filtration method as described above.

Cell bound radioactivity was determined by counting tubes or filters on the ^{125}I channel of a Beckman 300 γ counter. The binding data represent the means of three or four determinations, which typically varied by less than ±5% from their reported means. In all cases, the data have been corrected for the binding to tubes or filters observed in parallel incubations in the absence of cells (typically 1–5% of the added cpm). The centrifugation and filtration methods gave similar results.

Cell surface labeling. 1 mCi of [^{125}I]iodosulfanilic acid (> 2000 Ci/mmol) was diazotized at 4°C as described by the manufacturer approx. 10 min before use and was diluted to a concentration of 3.0 mCi/ml in 4°C 20 mM NaP_i, pH 7.5.

The 8.5-h differentiated NC-4 cells were harvested, washed and incubated at 10^7 cells/ml with increasing concentrations of unlabeled discoidin I (0 to 100 μg/ml) as described for the binding assay (see above). After 40 min of shaking at 23°C, 3-ml aliquots of the incubation mixtures were washed twice by centrifugation (800 × *g*, 3 min, 4°C) with 15 ml of 4°C 17 mM P_i containing 1 mg/ml of albumin. The cells were transferred to fresh Falcon tubes and washed twice by centrifugation (800 × *g*, 3 min, 4°C) with 15 ml of 4°C 20 mM NaP_i, pH 7.5. The cells were resuspended to $3 \cdot 10^7$ cells/ml (1 ml) in 4°C 20 mM NaP_i, pH 7.5; 50 μl of a freshly diazotized 3.0 mCi/ml solution of [^{125}I]iodosulfanilic acid in 4°C 20 mM NaP_i, pH 7.5, was added; and the suspensions were shaken at approx. 380 strokes/min on a New Brunswick model R-2 reciprocating shaker at 4°C. After 30 min, the cells were washed 4 times with 15 ml of the 4°C 20 mM NaP_i, pH 7.5, by centrifugation (800 × *g*, 3 min, 4°C); transferred to fresh Falcon tubes, washed twice more with 15 ml of 4°C 20 mM NaP_i, pH 7.5, by centrifugation (800 × *g*, 3 min, 4°C); and then resuspended to $3 \cdot 10^7$ cells/ml in 4°C PP_i/sucrose. 100-μl aliquots of these suspensions were prepared for electrophoresis (see below), and the remainders were quickly frozen in a dry ice-isopropanol bath and stored at −70°C.

For direct comparison, the binding assay (see above) was performed in parallel to the cell surface labeling experiment on quadruplicate 300-μl samples containing $4.2 \cdot 10^5$ cpm (6.8 ng) of ^{125}I-discoidin I in addition to the indicated final concentrations of unlabeled disocidin I, both in the presence and absence of cells. These samples were subjected to all of the additional washes, transfers

and incubations used in the cell surface labeling procedure.

The surface labeled cells were lysed at $3 \cdot 10^7$ cells/ml by two cycles of freezing and thawing in the presence of 0.4 mM phenylmethylsulfonyl fluoride. The membranes were pelleted and washed with 2 volumes of 4°C PP_i/sucrose by centrifugation (48000 × *g*, 45 min, 4°C). The washed membranes from $3 \cdot 10^6$ cells were extracted for 30 min with 200 µl of PP_i/sucrose containing the indicated components at 23°, and the extracts were collected by centrifugation (48000 × *g*, 45 min, 4°C). Samples of the lysates, soluble fractions, washed membranes and membrane extracts were prepared for electrophoresis (see below).

Other methods. In preparation for eletrophoresis, samples were diluted at least 4-fold (v/v) into 85°C 50 mM Tris-HCl/4 M urea/3 mM NaN_3/1% (w/v) SDS (pH 6.8) containing 5% (v/v) 2-mercaptoethanol and heated at 85°C for 10 min. Electrophoresis was performed on 1-mm thick 10, 15 or 7.5 to 15% gradient polyacrylamide-SDS slab gels with 3% stacking gels at a constant 100 to 150 V [4]. The gels were dried with heating under vacuum, with or without prior fixation and staining with Coomassie blue in 25% (v/v) isopropanol, 10% (v/v) acetic acid; and autoradiographed at −70°C on Kodak XR-5 film using Dupont Cronex lightning plus intensifying screens. The molecular weight markers used were: phosphorylase *a* (92000), ovalbumin (43000), discoidin I (26000), myoglobin (17000), and cytochrome *c* (12000).

The concentration of purified discoidin I was estimated by absorbance at 280 nm; a 0.1 mg/ml solution of discoidin I has an A_{280} of 0.3. Total extracellular protein was quantitated colorimetrically [5], using albumin as a standard, on samples of extracellular medium that had been dialyzed against distilled water containing 0.2% (v/v) toluene at 4°C, concentrated 60-fold (v/v) by lyophilization, and dissolved in 50 mM Tris-HCl/4 M urea/3 mM NaN_3/1% (w/v) SDS (pH 6.8).

Results

Before examining the interaction of exogenous ^{125}I-discoidin I with living *D. discoideum* cells, it was necessary to determine the location of endogenous discoidin I during differentiation and the selected conditions of the binding assay. When differentiating in suspension, these NC-4 cells routinely acquired developmental (i.e., 10 mM EDTA-resistant, see Ref. 6) cohesiveness within about 6 h and displayed maximal developmental cohesiveness by about 8 h, by which time they had typically formed tight aggregates when differentiating in parallel on a surface. As is shown in Fig. 1A (△), soluble discoidin I (all cell-associated discoidin I which was soluble upon cell lysis in PP_i/sucrose) accounted for greater than 99% of the total cellular discoidin I throughout the first 12.5 h of suspension differentiation. Despite the release of large amounts of protein into the extracellular medium during suspension differentiation (Fig. 1A, dashed line), extracellular discoidin I was not detected until after 7.5 h (Fig. 1B, ●),

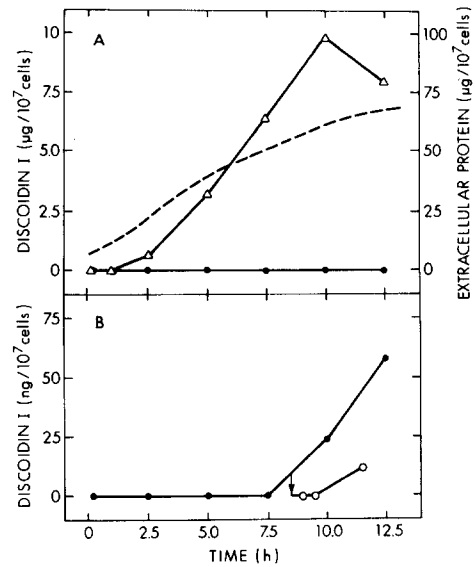

Fig. 1. Localization of endogenous discoidin I during differentiation. The NC-4 cells were allowed to differentiate in suspension at 23°C for the indicated time intervals, and the amount of discoidin I in the extracellular medium and the soluble fraction was determined by radioimmunoassay as described in Materials and Methods. (A) Cell-associated discoidin I rendered soluble after cell lysis in PP_i/sucrose (△), discoidin I in the extracellular medium (●), total extracellular protein (dashed line). (B) Discoidin I in the extracellular medium plotted on an expanded scale (●), discoidin I in the extracellular medium after washing cells with 17 mM P_i containing 1 mg/ml of albumin at 8.5 h and then continuing suspension differentiation in 17 mM P_i containing 1 mg/ml of albumin (○).

and even then amounted to less than 1% of total cellular discoidin I. In contrast, the axenic strain A3 *D. discoideum* cells often exhibited extracellular discoidin I levels of greater than 1 μg/10^7 cells after only 5 or 6 h of suspension differentiation (not shown). The time course of appearance of extracellular discoidin I (Fig. 1B, ●) was quite different from that of total extracellular protein (Fig. 1A, dashed line), suggesting that the discoidin I does not become externalized by cell lysis or a nonspecific secretion process. Interestingly, at no time during the first 12.5 h of suspension differentiation was any discoidin I detected in a 1% (v/v) Emulphogene BC-720 extract (30 min, 23°C) of the PP_i/sucrose-washed membrane pellets (data not shown).

We selected 8.5-h differentiated cells for binding assays. These cells displayed maximal developmental cohesiveness (data not shown), had maximal levels of soluble discoidin I (Fig. 1A, △), were just beginning to externalize discoidin I (Fig. 1B, ●), and showed both I and C site receptors when fixed [1]. When 8.5-h differentiated cells were washed with albumin-containing buffer, the reappearance of extracellular discoidin I did not occur for at least 1 h (Fig. 1B, ○). Thus, there should have been little or no competition between exogenous ^{125}I-discoidin I and endogenous extracellular discoidin I under these circumstances.

Association experiments

Fig. 2 shows the time course for the binding of ^{125}I-discoidin I to living, 8.5-h differentiated NC-4 cells at 23°C (●) and 4°C (○). Binding occurred relatively rapidly at 23°C, reaching apparent steady-state levels after 35 to 40 min, and gradually declined thereafter. The binding occurred much more slowly at 4°C and approached the 23°C steady-state level after about 2 h. In fact, the binding time course at 4°C was similar to those observed previously for binding to the corresponding fixed cells at 23°C or 4°C [1] and to negatively charged phospholipid vesicles at 23°C [2].

Vegetative and 8.5-h differentiated cells exhibited a similar cell concentration dependence of steady-state (40 min) binding. Steady-state binding increased with cell concentration, but did not saturate up to 1.6 · 10^7 cells/ml.

To assure that the ^{125}I-discoidin I remained

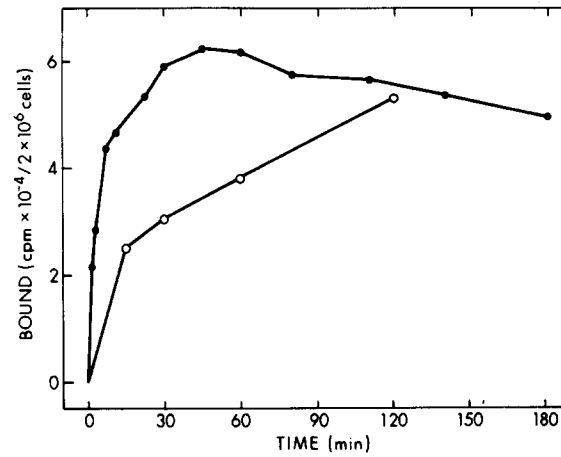

Fig. 2. Association time courses at 23°C and 4°C. At the indicated times, the binding assay was performed by the filtration method (see Materials and Methods) on 200-μl samples that were prepared and incubated at 23°C (●) or 4°C (○) and contained 2 · 10^6 8.5-h differentiated NC-4 cells and 2.1 · 10^5 (5.7 ng) of ^{125}I-discoidin I.

intact during the incubation with living cells at 23°C, samples of bound and free ^{125}I-discoidin I were collected at various times during the association experiment shown in Fig. 2 (●) and analyzed on a SDS-polyacrylamide gel. An autoradiogram of this gel showed that virtually all of both the cell-bound and free radioactivity remained as intact 26 kDa discoidin I subunits throughout the first 160 min of the incubation.

We examined the effects of I and C site binding inhibitors [1] on the level of steady-state binding. 50 mM *N*-acetyl-D-galactosamine inhibited steady-state binding by about 50%, while 50 mM *N*-acetyl-D-glucosamine had no effect. Since the amount of inhibition observed with 50 mM *N*-acetyl-D-galactosamine was always greater than the approx. 25% inhibition expected for an electrostatic (I site) interaction alone [2], this suggested that the discoidin I was binding to C site receptors on these living cells. A similar conclusion can be drawn from the effects of these inhibitors on dissociation (see below). The 50 mM *N*-acetyl-D-galactosamine typically inhibited binding to vegetative NC-4 cells by less than 15%, consistent with their lower numbers of C site receptors [1]. Interestingly, 100 μg/ml of heparin inhibited binding to differentiated cells by about 80%, suggesting that it prevented binding

to the C sites as well as to the I sites. Support for this notion comes from the fact that little further inhibition (2–6%) resulted when 50 mM *N*-acetyl-D-galactosamine was included with the 100 μg/ml of heparin.

Dissociation experiments

The living NC-4 cells were incubated with ^{125}I-discoidin I for 40 min at 23°C. The unbound ^{125}I-discoidin I was removed by centrifugation, and the cells were resuspended to one-fiftieth their initial (incubation mixture) concentration. At various times, aliquots of this diluted cell suspension were removed and assayed for ^{125}I-discoidin I binding. With dilution alone the majority of the bound ^{125}I-discoidin I (70–90%) dissociated very slowly ($t_{1/2} > 15$ h). Dissociation occurred to a much greater extent when the dilution was performed in the presence of 10 mM *N*-acetyl-D-galactosamine (Table I), implying a multivalent interaction with the C sites [7]. No further enhancement in the extent of dissociation was observed when the concentration of *N*-acetyl-D-galactosamine was increased from 10 mM to 50 mM. The enhancement of dissociation was sugar-specific because 10 mM *N*-acetyl-D-glucosamine had no effect (Table I).

Table I shows that 100 μg/ml of heparin also enhanced the extent of dissociation by as much as 40%. The combination of 10 mM *N*-acetyl-D-galactosamine plus 100 μg/ml heparin gave a greater enhancement of dissociation than either inhibitor alone, suggesting that discoidin I was multivalently bound to both I and C sites of living cells at steady state [7]. When the time of association was increased from 40 min to 120 min, the percent of the initially bound ^{125}I-discoidin I that could be dissociated with dilution into *N*-acetyl-D-galactosamine or heparin (or both) was decreased (Table I). This suggested that, with time at 23°C, the mode of association of the ^{125}I-discoidin I was altered to one which was not as readily reversed.

TABLE I

EFFECTS OF BINDING INHIBITORS ON DISSOCIATION

The binding assay was performed at 23°C on samples containing 10^7 cells/ml 8.5-h differentiated NC-4 cells and $1.2 \cdot 10^6$ cpm/ml (23 ng/ml) of ^{125}I-discoidin I. After 40 min and 120 min of association, aliquots were removed to determine initial binding by the centrifugation method, the remainder of the samples were centrifuged to remove unbound ^{125}I-discoidin I, the cells were diluted to one-fiftieth their initial concentration in 17 mM P_i containing 1 mg/ml of albumin and the indicated final concentrations of inhibitors. The amount of ^{125}I-discoidin I remaining bound after 45 min of dissociation was determined by the centrifugation method and is presented as a percentage of the initial binding ($4.7 \cdot 10^4$ cpm/$3 \cdot 10^6$ cells for the 40 min association, $4.2 \cdot 10^4$ cpm/$3 \cdot 10^6$ cells for the 120 min association) (see Material and Methods).

Inhibitor	Bound (% of initial)	
	40 min association	120 min association
None	72	74
10 mM *N*-acetyl-D-glucosamine	76	75
10 mM *N*-acetyl-D-galactosamine	44	50
100 μg/ml heparin	49	62
10 mM *N*-acetyl-D-glucosamine + 100 μg/ml heparin	51	62
10 mM *N*-acetyl-D-galactosamine + 100 μg/ml heparin	27	40

Discoidin I concentration dependence

The discoidin I concentration dependence of binding to living cells was determined by including increasing amounts of unlabeled discoidin I in the binding assay with a constant amount of ^{125}I-discoidin I. Fig. 3A shows that unlabeled discoidin I did not compete with tracer for steady-state binding, but instead appeared to enhance tracer binding. This apparent positive cooperative binding behavior is the same as that observed previously for the binding of ^{125}I-discoidin I to the I sites of fixed *D. discoideum* cells [1] and to negatively charged phospholipid vesicles [2]. This positive cooperativity causes the binding to appear nonsaturable with respect to discoidin I, with a binding capacity of greater than 10^7 discoidin I tetramers/cell (Fig. 3B). It also precludes estimation of the affinity of the interaction.

The nonsaturable nature of the discoidin I-cell interaction was verified using cell surface labeling. The cells were incubated with increasing amounts of unlabeled discoidin I, washed several times by centrifugation, and then surface labeled with diazotized [^{125}I]iodosulfanilic acid. The surface-labeled cells were electrophoresed on a SDS-polyacrylamide gel, and the gel was autoradio-

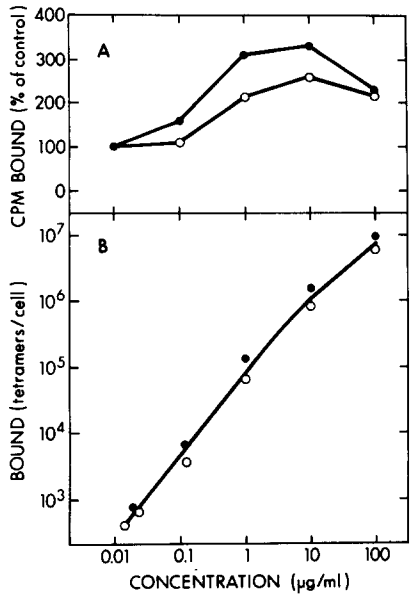

Fig. 3. Discoidin I concentration dependence of binding. The binding assay was performed in the presence of increasing concentrations of unlabeled discoidin I for 40 min at 23°C on 200-μl samples containing 10^7 cells/ml 8.5-h differentiated NC-4 cells and $1.4 \cdot 10^5$ cpm (4.7 ng) of ^{125}I-discoidin I by the filtration method (○) or on 300-μl samples containing 10^7 cells/ml 8.5-h differentiated NC-4 cells and $4.2 \cdot 10^5$ cpm (6.8 ng) of ^{125}I-discoidin I by a modification of the centrifugation method (●), in parallel to the cell surface labeling experiment shown in Fig. 4 (see Materials and Methods). (A) The cpm bound in the presence of the indicated concentration of unlabeled discoidin I is plotted as the % of the control cpm bound in the absence of unlabeled discoidin I (○, $7.6 \cdot 10^3$ cpm/$2 \cdot 10^6$ cells; ●, $2.9 \cdot 10^4$ cpm/$3 \cdot 10^6$ cells). (B) the data in A have been replotted to show the number of discoidin I tetramers bound per cell as a function of the total discoidin I concentration.

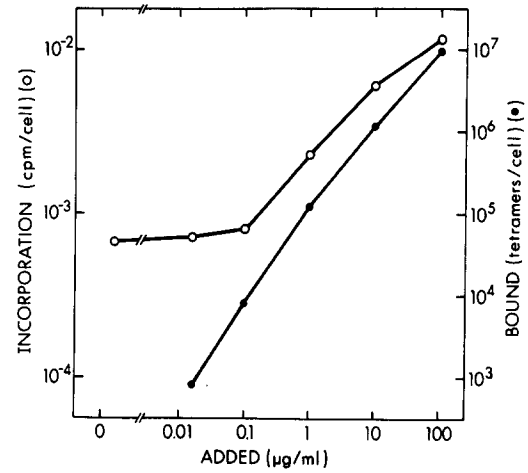

Fig. 4. Discoidin I binding assayed by surface radiolabeling. The 8.5-h differentiated NC-4 cells were incubated at 10^7 cells/ml with the concentrations of unlabeled discoidin I indicated for 40 min at 23°C. The cells were washed at 4°C and surface labeled at $3 \cdot 10^7$ cells/ml with 0.14 mCi/ml of freshly diazotized [^{125}I]iodosulfanilic acid in 20 mM NaP_i, pH 7.5, for 30 min at 4°C. Samples containing $1.5 \cdot 10^4$ cells were electrophoresed on a 7.5 to 15% gradient SDS-polyacrylamide gel. The gel was fixed and stained with Coomassie blue and dried. The 26 kDa discoidin I subunit bands were cut from the dried gel and counted directly. The amount of radioactivity per cell incorporated into the discoidin I band by surface labeling (○) and the corresponding number of discoidin I tetramers bound per cell in the binding assay performed in parallel (●) (see Fig. 3, ●) are plotted versus the total concentration of added discoidin I.

graphed. The cells which were incubated with higher concentrations of unlabeled discoidin I showed correspondingly more radioactivity incorporated into the 26 kDa discoidin I subunit band on the gel. In fact, at added concentrations of unlabeled discoidin I of 1 μg/ml or higher, discoidin I was clearly the major cell surface-labeled component. The amount of radioactivity in the discoidin I bands was determined by counting gel slices. Fig. 4 indicates that, over three orders of magnitude in unlabeled discoidin I concentration (from 0.1 to 100 μg/ml), the incorporation of radioactivity into discoidin I showed the same type of nonsaturability as the ^{125}I-discoidin binding determined in parallel incubations. With the assumption that incorporation was proportional to cell surface concentration under these reagent-limiting conditions, the unlabeled discoidin I appeared to bind to these cells in the nonsaturable fashion predicted from the tracer binding studies (Fig. 3). The fact that discoidin I becomes the major cell surface-labeled component with high unlabeled discoidin I inputs is consistent with the prediction that large numbers of unlabeled discoidin molecules are binding to these cells.

To determine the mode of association of the surface-labeled discoidin I with these living cells, those that were labeled after incubation with 10 μg/ml of unlabeled discoidin I were lysed by freeze-thaw in PP_i/sucrose, and their crude membrane fractions were subjected to a variety of extractive treatments. The various extracts were

electrophoresed on a SDS-polyacrylamide gel, and the gel was sliced and counted after autoradiography. About 80% of the radioactivity associated with the surface-labeled discoidin I band was released into the soluble fraction simply by lysis in PP_i/sucrose. This PP_i/sucrose buffer also completely inhibited the binding of ^{125}I-discoidin I to the C and I sites of fixed *D. discoideum* cells. Interestingly, the 20% of the discoidin I radioactivity remaining with the washed membrane pellet was not extracted by 50 mM *N*-acetyl-D-galactosamine, 0.5 M KI, 10 mM EDTA, 20 mM dithiothreitol, or 1% (v/v) Emulphogene BC-720, but required 1% (w/v) SDS for solubilization. Less than one-half of the radioactivity associated with this nonextractable fraction corresponded to the labeling of endogenous cell surface components as determined by extracting cells surface-labeled after incubation with no exogenous discoidin I. Thus, a fraction of the exogenous unlabeled discoidin I appeared to become very tightly associated with cellular membranes.

Discussion

The data indicate that exogenous discoidin I may demonstrate three modes of association with living *D. discoideum* cells. Two of these modes appear to represent the peripheral association of discoidin I with the cell surface via the I site and the C site receptors first identified on the corresponding fixed cells [1]. The third mode of association has only been observed with living *D. discoideum* cells and appears to represent an irreversible and potentially integral association of exogenous discoidin I with cellular membranes (see below).

In our early binding experiments using living *D. discoideum* cells and their plasma membranes (unpublished data), we found that excess unlabeled discoidin I did not reduce ^{125}I-discoidin I binding, and thus, we deduced there was no 'specific' binding component. The reason for this phenomenon is now clear in light of the unusual discoidin I concentration dependence observed for binding to the living cells. The binding exhibits apparent positive cooperativity and, hence, appears nonsaturable with respect to discoidin I (Fig. 3). This unusual discoidin I concentration dependence appears characteristic for the electrostatic interaction of discoidin I with membranous structures and was observed previously for binding to the I sites of fixed *D. discoideum* cells [1] as well as to negatively charged phospholipid vesicles [2]. In fact, the cell surface labeling experiment reported here (Fig. 4) provides independent and direct verification of the nonsaturability of the electrostatic interaction of discoidin I with membranous structures.

Based on the effects of *N*-acetyl-D-galactosamine and heparin on association and dissociation, the exogenous discoidin I appears to bind to both C and I sites on living *D. discoideum* cells. At low concentrations (10 ng/ml) at least 50% of the cell-associated discoidin I is bound to the I sites, and this fraction must increase dramatically as the concentration of discoidin I is increased because of the different saturability properties for binding to the I and C sites [1]. Since heparin inhibits discoidin I binding to a greater extent than expected for I site inhibition alone, binding to the C sites of living cells may be contingent on a previous I site interaction. Such an ordered binding mechanism was not observed with the corresponding fixed cells, perhaps because binding to the fixed cells exhibits a slower association rate [1]. The fact that the living cell association rate can be reduced by fixation [1], as well as by lowering the temperature to 4°C (Fig. 2), suggests that the binding is dependent on the degree of mobility exhibited by certain membrane components. The binding of discoidin I to negatively charged phospholipid vesicles is almost completely inhibited by lowering the temperature to 4°C [2].

The living cells can bind from 5% to 25% of any added discoidin I per $3 \cdot 10^6$ cells as the concentration of added discoidin I increases from 10 ng/ml to 10 μg/ml. A fraction of this exogenous discoidin I (about 10% with 10 μg/ml of added discoidin I) appears to become very tightly associated with the particulate fraction of these cells. This discoidin I is not dissociated from the membranes by hapten sugars, increased ionic strength, chelating agents, reducing agents or neutral detergents, but requires 1% (w/v) SDS for solubilization. We have often observed a similar resistance to extraction for putative endogenous cell surface discoidin I labeled with diazotized [^{125}I]iodosulfanilic acid or Na ^{125}I and lactoperoxidase (un-

published data). The dissociation data (Table I) also suggest that a fraction of the bound discoidin I is irreversibly cell-associated and that this fraction increases with time of association. This irreversible fraction may represent the internalization of some of the ^{125}I-discoidin I. Fluorescence and electron microscopic analyses of living NC-4 cells treated with rhodamine- and ferritin-labeled discoidin I suggests that some of the exogenous discoidin I does become internalized (Galvin, N.J., and Frazier, W.A., manuscript in preparation). The extraction properties of the tightly cell-associated component are also consistent with cytoskeletal attachment [8].

While our findings concerning the levels and distribution of endogenous discoidin I in the NC-4 cells greatly simplify interpretation of the binding data, they raise certain questions regarding the function of discoidin I as a cell cohesion ligand. The 8.5-h differentiated cells exhibit maximal developmental (10 mM EDTA-resistant) cohesiveness, yet they possess extremely low levels of extracellular and cell surface discoidin I. Fig. 1 demonstrates that while the 8.5-h differentiated NC-4 cells contain $5 \cdot 10^6$ tetramers/cell of 'soluble' discoidin I, slightly less than $6 \cdot 10^3$ tetramers/cell are found in an extracellular location, even prior to the washing step normally included before assaying either binding or cohesiveness.

The cell surface labeling experiment shown in Fig. 4 permits estimation of the number of discoidin I tetramers on the surface of the 8.5-h differentiated cells. When the data shown in Fig. 4 are replotted as the logarithm of the number of tetramers bound/cell versus the logarithm of the corresponding fold increase of incorporation into the discoidin I band on the gel, the points fall on a line with a correlation coefficient of 0.98. Extrapolation of this line reveals that only about $1.3 \cdot 10^3$ endogenous discoidin I tetramers are labeled on the surface of an 8.5-h differentiated NC-4 cell not pre-incubated with any unlabeled discoidin I. Implicit in this calculation is the assumption of a common labeling efficiency for the endogenous cell surface discoidin I and the bound exogenous discoidin I. In fact, this calculated value of surface tetramers/cell could even represent an overestimate if the 26 kDa region of the gel contained labeled endogenous components other than discoidin I. The value of $1.3 \cdot 10^3$ surface discoidin I tetramers/cell is entirely consistent with the level of binding expected with $6 \cdot 10^3$ tetramers/cell of extracellular discoidin I (see above). This suggests that endogenous cell surface discoidin I could simply represent secreted discoidin I that subsequently binds to the cells. Certainly, a comparison of the time courses for the appearance of extracellular discoidin I (Fig. 1B, ●) and total extracellular protein (Fig. 1A, dashed line) suggests that discoidin I is externalized by a mechanism, more specific than cell lysis.

While our data agree with the recent data of Springer et al. [9] regarding the amount of total cellular discoidin I (about $5 \cdot 10^6$ molecules/cohesive NC-4 cell), they detected 30 to 40 times more cell surface discoidin I than the $1.3 \cdot 10^3$ tetramers/cell detected by us. This difference could be due to the fact that they employed surface development on pads after differentiating in suspension for up to 16 h. In our hands, the NC-4 cells appear not only to secrete increasing amounts of discoidin I with continued (> 8.5 h) differentiation in suspension (Fig. 1B), but they also require relatively harsh mechanical manipulation to become dissociated from the aggregates formed on a surface. Our data indicate that 5 to 25% of any extracellular discoidin I, whether specifically secreted or released from the cell by mechanical lysis, will be found on the cell surface. Springer et al. [9] showed that treatment of cohesive slime mold cells with IgG directed against the endogenous lectin 'elicits' the appearance of more lectin at the cell surface. If some physiological stimulus, such as stable cell-cell contact, could induce a similar response, then it could readily account for the discrepancies in endogenous cell surface lectin quantitation.

Lerner and coworkers [10,11] have used discoidin I mutants to definitively establish a requirement for functional discoidin I in the acquisition of developmental cellular cohesiveness. Our data suggest that *D. discoideum* cells can exhibit maximal developmental cohesiveness while displaying miniscule amounts of discoidin I on their surfaces or in their surrounding extracellular medium. We have observed that 100 μg/ml of heparin does not inhibit the reaggregation of developmentally cohesive NC-4 cells in the presence or absence of 10 mM EDTA or 1 mg/ml of albumin (unpublished

data), even though it does inhibit discoidin I binding to the I and C sites by as much as 80%. Similarly, it has been quite difficult for others [12–14] to demonstrate a significant effect of antagonists of the endogenous lectins (such as anti-lectin antibody, anti-lectin Fab, hapten sugars, or glycopeptides) on the reaggregation of developmentally cohesive slime mold cells without employing hyperosmotic buffer conditions or metabolic poisons. These antagonists do inhibit the hemagglutination activity of the endogenous lectins under physiological conditions (Refs. 12, 14, unpublished data). Even large amounts of exogenous slime mold lectins exert little effect, if any, on the reaggregation behavior of developmentally cohesive slime mold cells (Ref. 14, unpublished data), unless the cells have been killed by fixation or by heating [15]. Based on these observations, we hypothesize that discoidin I exerts its important developmental influence at an intracellular location rather than as a mechanical or crosslinking component of the cell cohesion apparatus at the cell surface.

We can estimate (Fig. 1 and Ref. 16) that discoidin I is present at a concentration of about 1–2 mg/ml in the cytoplasm of cohesive *D. discoideum* cells. At this high concentration, we suspect that discoidin I could effectively coat the cytoplasmic face of cellular membranes by an electrostatic mechanism, or perhaps even by a more integral hydrophobic mechanism. The lectin pallidin from a related species *Polysphondylium pallidum* has, in fact, been localized to the cytoplasmic face of the endoplasmic reticulum in permeabilized, fixed, cohesive *P. pallidum* cells [17]. This estimated cytoplasmic concentration of discoidin I (1–2 mg/ml) is substantially greater than the threshold concentrations required for phospholipid vesicle agglutination [2]. Thus, discoidin I may be able to agglutinate intracellular membranous structures. This hypothesis of intracellular function is consistent with all available data [18] and suggests new routes of experimentation designed to elucidate the role of endogenous lectins in slime mold development.

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