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An analysis of discoidin I binding sites in *Dictyostelium discoideum* (NC4)

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Vegetative wild-type (strain NC4) *D. discoideum* cells and cells at the 10h stage of development (aggregation) were harvested in the presence of 0.5 M-galactose to remove any endogenous discoidin I already bound to the cell surface, and fixed with glutaraldehyde. Affinity-purified ^{125}I-labelled discoidin I bound to these fixed cells in a specific manner, $\geqslant$95% of binding being inhibited by 0.5 M-galactose. Binding of ^{125}I-labelled discoidin I was essentially complete in 90min at 22°C. Based on specific radioactivity measurements, vegetative (0h) *D. discoideum* (NC4) cells bind approx. 8.4×10^5 discoidin I tetramers/cell and aggregated (10h) cells bind 5.1×10^5 discoidin I tetramers/cell, each exhibiting apparent positive co-operativity of binding with highest limiting affinity constants (K_a) of approx. 1×10^7 and $2 \times 10^7 M^{-1}$, respectively. *Klebsiella aerogenes*, the food source used for growth of *D. discoideum* NC4 amoebae, also binds ^{125}I-labelled discoidin I and this is >99% inhibited by 0.5 M-galactose. However, at the levels of bacterial contamination present, >97% of ^{125}I-labelled discoidin I binding to *D. discoideum* cell preparations was to the cells themselves. Confirmation of the number of discoidin I tetramers bound per *D. discoideum* cell was obtained by elution of bound ^{125}I-labelled discoidin I followed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and then quantification by scanning of stained discoidin I bands.

In several developmental systems, specific cell-surface molecules have been suggested as possible mediators of cell–cell adhesion, but the detailed molecular characterization of these components is lacking. One of the more promising systems for the study of cell cohesion is the cellular slime mould, *Dictyostelium discoideum*. Upon starvation, the free-living amoebae of this organism become cohesive and aggregate to form multicellular complexes which then undergo further development to form fruiting bodies. The entire developmental sequence is complete within about 24 h.

Beug *et al.* (1973) have identified two independent systems of cell cohesion in *D. discoideum*, one involving antigens present on the surface of both vegetative and aggregation-competent cells and designated 'contact sites B' (CsB), the other involving antigens present only on aggregation-competent, cohesive cells and referred to as 'contact sites A' (CsA). Current evidence strongly suggests a role for CsA, a glycoprotein with a molecular weight of 80000–90000 (Müller *et al.*, 1979), in mediating cell cohesion during aggregation (Müller & Gerisch, 1978). Although several possible models have been formulated (Müller & Gerisch, 1978), the detailed mechanism of cell cohesion via CsA remains to be elucidated.

Abbreviations used: Cs, contact sites; SDS, sodium dodecyl sulphate.

In addition to CsA, two lectin-like proteins, discoidin I and discoidin II, which specifically bind galactose and structurally related sugar residues, are also accumulated during aggregation (Frazier *et al.*, 1975). Evidence which has been presented in support of a role for at least discoidin I in aggregative-phase cell cohesion includes its rapid accumulation on the surface of wild-type (strain NC4) *D. discoideum* cells concomitant with the acquisition of cohesiveness (Chang *et al.*, 1975; Siu *et al.*, 1976), the ability of purified discoidin I to agglutinate fixed *D. discoideum* cells (Reitherman *et al.*, 1975), and the observation that a mutant (HJR-1), which produces a non-functional discoidin I, also fails to become cohesive (Ray *et al.*, 1979; Shinnick & Lerner, 1980). It remains to be shown that this mutant carries a lesion in the structural gene for discoidin I rather than in a distinct gene, the expression of which is essential for the activation of an unknown developmentally-required function and, coincidentally, discoidin I function. Opposing a

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direct involvement of discoidin in cell cohesion are the findings that low concentrations of discoidin (Rosen *et al.*, 1973) do not enhance cohesion and high concentrations of discoidin (Rosen *et al.*, 1973), monovalent anti-discoidin Fab fragments (Springer & Barondes, 1980) and galactose (Rosen *et al.*, 1973) fail to inhibit the cohesion of *D. discoideum* cells. Nevertheless, galactose does delay the expression of CsA-mediated (but not CsB-mediated) cell cohesion (Marin *et al.*, 1980) and therefore discoidin may play a regulatory role rather than a direct role in cell cohesion. On either model of discoidin involvement, discoidin is presumed to function by binding to a cell-surface receptor(s) bearing galactose, or a structurally similar sugar residue, as the interacting ligand.

Direct evidence for cell-surface receptors for discoidin I has been provided by Reitherman *et al.* (1975) and Bartles & Frazier (1980). Both groups of workers quantified the number of discoidin I receptors based on the amount of exogenously-added discoidin I which bound to *D. discoideum* cells fixed with glutaraldehyde at various stages of aggregation. However, aggregating *D. discoideum* cells recently have been shown to have endogenous discoidin already bound to the cell surface in a form which is elutable with *N*-acetylgalactosamine and therefore bound to cell-surface receptors (Springer *et al.*, 1980). Previous studies concerned with quantifying discoidin I receptors during development therefore, presumably, have titrated only those receptors not already masked by endogenous discoidin I. In the work reported in the present paper, we re-investigated the number and binding affinity of discoidin I receptors on *D. discoideum* (NC4) cells during aggregation, using cells which were washed with galactose prior to fixation in order to ensure that all potential discoidin receptors were available for detection by discoidin I binding. Discoidin I receptors were assayed by direct measurement of bound ^{125}I-labelled discoidin I, analogous to the assay of Bartles & Frazier (1980). Our data support the earlier estimates of Reitherman *et al.*, (1975) for discoidin I receptor numbers during aggregation but, unlike these investigators, we find no evidence of a marked increase in receptor affinity during this phase of development.

Experimental

Preparation and radio-iodination of discoidin I

D. discoideum AX3 amoebae were grown in HL5 medium supplemented with 86mM-glucose, as described by Watts & Ashworth (1970), to a final cell density of 6.0×10^6–8.0×10^6 cells/ml, and then a mixture of discoidin I and discoidin II was isolated by affinity chromatography on Sepharose 4B as described by Frazier *et al.* (1975). The purified discoidin was dialysed against 0.1M-NaCl/0.3M-galactose/0.05M-sodium phosphate buffer, pH7.0, and then radio-iodinated by using immobilized lactoperoxidase and glucose oxidase (purchased as Enzymobead Radio-iodination Reagent from Bio-Rad). Typically the reaction mixture contained 1mCi of carrier-free ^{125}I, 1mg of discoidin, 0.2ml of Enzymobeads, 0.01M-glucose and 0.2M-galactose in a final volume of 1ml. After incubation at room temperature for 1h, the Enzymobeads were removed by low-speed centrifugation and the supernatant was dialysed against 0.15M-NaCl/0.1% NaN_3/0.01M-sodium phosphate buffer, pH7.4, at 4°C. The radio-iodinated discoidin was dialysed against a total of five 1-litre batches of this buffer, changed every 1h, and finally against 1 litre of 0.075M-NaCl/0.075M-sodium phosphate+potassium phosphate buffer, pH7.2 (phosphate-buffered saline). This extensive dialysis protocol removed the bulk of unincorporated ^{125}I and galactose. Active discoidin I was purified by binding to formalinized sheep erythrocytes prepared according to Butler (1963); under appropriate conditions, discoidin II fails to bind to erythrocytes from this species (Simpson *et al.*, 1974). Typically, 5mg of radio-iodinated discoidin was mixed with 3.5ml packed cell volume of sheep erythrocytes in 8ml (final volume) of phosphate-buffered saline. Discoidin binding was allowed to occur by incubation at 22°C for 30min on a gyrator shaker set at 140rev./min. After incubation, the erythrocytes were removed by low-speed centrifugation and the supernatant incubated with additional batches of sheep erythrocytes until no further radio-iodinated protein was adsorbed from the supernatant and the supernatant was depleted of discoidin I activity as monitored by haemagglutination of sheep erythrocytes (Simpson *et al.*, 1974). The erythrocyte pellets were washed four times with five vol. of phosphate-buffered saline to remove unbound, contaminating discoidin II, and then active discoidin I was eluted by resuspension of the sheep-erythrocyte pellet in an equal volume of 0.5M-galactose/5mM-*N*-acetylgalactosamine in phosphate-buffered saline and incubation at 22°C for 30min, shaking at 140rev./min. The cells were removed by centrifugation and eluted twice more with the galactose/*N*-acetylgalactosamine mixture. The eluted discoidin I was centrifuged at 25000g for 20min to remove any residual particulate material and then dialysed extensively against phosphate-buffered saline at 4°C to decrease the galactose concentration to less than 50nM. The specific radioactivity of the purified discoidin I varied between 5.35×10^4c.p.m./μg and 2.5×10^5c.p.m./μg (four preparations). After determination of the specific radioactivity of the purified discoidin I and confirmation of its purity by SDS/polyacrylamide-gel electrophoresis (see the

Results section), bovine serum albumin was added to 1 mg/ml and aliquots were frozen in liquid N_2 and then stored at −70°C.

Preparation of fixed D. discoideum cells

D. discoideum (NC4) cells were grown in association with *Klebsiella aerogenes* on SM agar plates (Sussman, 1966) and harvested when the bacterial lawn was just clearing. Contaminating bacteria were removed by repeated centrifugation at 300 *g* for 5 min and then the amoebae were dispensed onto Millipore filters at 5×10^7 cells/filter as described by Sussman (1966). Development was allowed to occur in a humid atmosphere at 22°C. Cells were harvested in phosphate-buffered saline containing 0.5 M-galactose either prior to development (0 h) or after aggregates had formed (10 h). Cell clumps were disaggregated by very gentle trituration, the single cells were washed twice by centrifugation in phosphate-buffered saline containing 0.5 M-galactose, and then resuspended at 10^8 cells/ml in this buffer. Glutaraldehyde was added to 1% (v/v) and the cell suspension was mixed gently by inversion by attachment to a disc set at an angle of 45° and rotating at 17 rev./min for 30 min at room temperature. The fixed cells were harvested by centrifugation (850 *g* for 5 min) and unreacted glutaraldehyde was blocked by resuspension of the cells in 10 mM-glycine in phosphate-buffered saline, and incubation at room temperature for 15 min, mixing by inversion as before. Next, the cells were washed three times with phosphate-buffered saline by centrifugation, dialysed overnight against 100 vol. of that buffer, and stored at 4°C as a suspension (10^8 cells/ml). The galactose concentration in the final suspension of fixed cells was less than 50 nM. The fixed cells were used for receptor binding assays within 2 weeks, although they are reportedly stable for at least 1 year (Bartles & Frazier, 1980).

Preparation of fixed K. aerogenes

K. aerogenes was grown in HL5 medium supplemented with 86 mM-glucose, final concentration, overnight at 37°C in shaking culture. The bacteria were harvested by centrifugation (16 500 *g* for 15 min), washed with 100 vol. of phosphate-buffered saline, and then fixed with 1% (v/v) glutaraldehyde at a concentration of 8×10^{10} bacteria/ml as described above for the fixation of *D. discoideum* cells. After blocking unreacted glutaraldehyde with an excess of glycine, the fixed bacteria were washed five times with phosphate-buffered saline by centrifugation and stored at 4°C as 10^{10} bacteria/ml suspension.

Discoidin I receptor binding assay

Assays for discoidin I binding to glutaraldehyde-fixed *D. discoideum* NC4 cells were carried out in siliconized glass test-tubes by a modification of the method of Phillips *et al.* (1974). The test incubation mixtures routinely contained 10^7 fixed *D. discoideum* NC4 cells, 1 mg of bovine serum albumin/ml, and various amounts of ^{125}I-labelled discoidin I in 0.5 ml (final volume) of phosphate-buffered saline. Binding of ^{125}I-labelled discoidin I to fixed cells was allowed to occur for 90 min at 22°C with shaking at 140 rev./min on a gyratory shaker. After this incubation, each cell suspension was transferred to a 1.5 ml vol. polypropylene conical tube (Sarstedt U.K.) pre-soaked overnight in 0.5% bovine serum albumin to saturate non-specific protein-binding sites, and the cells were pelleted by centrifugation (14 000 *g* for 1 min) in a microfuge. The supernatant was retained for the determination of unbound lectin. The cell pellet was resuspended in 50 μl of phosphate-buffered saline and gently layered onto 250 μl of 5% (w/v) serum albumin in phosphate-buffered saline in a 0.4 ml vol. polypropylene conical tube (Sarstedt U.K.) The 1.5 ml vol. polypropylene tube was washed out with another 50 μl of phosphate-buffered saline and then this was also layered onto the 5% albumin cushion. The cells were washed by centrifuging them through the 5% albumin cushion at 9000 *g* for 1 min, using a Sorvall HB-4 rotor, and then the cell pellet was recovered by cutting off the tip of the tube with a hot scalpel-blade. The amount of ^{125}I-labelled discoidin I bound to each cell pellet was determined by counting for radioactivity in an Intertechnique gamma counter. Control experiments showed that the 5% albumin immediately above the pellet contained negligible amounts of radioactivity. Binding assays for discoidin I receptor were routinely carried out in duplicate, together with duplicate controls for non-specific binding in which 0.5 M-galactose was present in the original incubation mixture and in all subsequent solutions, including the 5% albumin cushion used to wash the cells.

Assays for discoidin I receptors on *K. aerogenes* were performed according to the standard albumin-cushion protocol described above for *D. discoideum* cells except that 3.35×10^8 glutaraldehyde-fixed *K. aerogenes* cells replaced the *D. discoideum* cells in each 0.5 ml incubation mixture. Control experiments showed that all the bacteria were pelleted under the centrifugation conditions routinely used in the assay.

Recovery and electrophoretic analysis of bound ^{125}I-labelled discoidin I

Cell pellets were recovered from tube tips by resuspension in phosphate-buffered saline, pH 7.2, using a siliconized Pasteur pipette. The pooled cells were washed 5 times by centrifugation, each time with a 100-fold vol. excess of phosphate-buffered saline, to remove albumin from the 5% albumin cushion. Finally, ^{125}I-labelled discoidin I was eluted

by incubating the washed cell pellet with an equal volume of 0.5 M-galactose in phosphate-buffered saline (60 min at 22°C with occasional mixing) and the cells were then removed by centrifugation (16500 **g** for 5 min). The elution with galactose was repeated, the eluates pooled, and then counted for radioactivity in an Intertechnique gamma counter. The discoidin I content of the eluate was calculated from a knowledge of the eluent radioactivity and the specific radioactivity of the ^{125}I-labelled discoidin I. Eluted ^{125}I-labelled discoidin I was prepared for gel electrophoresis by precipitation with trichloroacetic acid, using calf thymus DNA as co-precipitant, followed by washing of the precipitated discoidin I with acetone to remove trichloroacetic acid and then dissolution in 1% (w/v) SDS/2% (v/v) 2-mercapto-ethanol/10% (v/v) glycerol/0.005% Bromophenol Blue/0.05 M-Tris/HCl, pH 6.8. Known aliquots of the eluted discoidin I were denatured by heating at 100°C for 3 min and then loaded onto a 6–18% (w/v) linear gradient polyacrylamide slab gel prepared with the SDS/discontinuous buffer system described by Laemmli (1970). Standard amounts of authentic unlabelled discoidin I, purified by affinity chromatography on sheep erythrocytes (see above), were loaded into adjacent wells of the slab gel. After electrophoresis (30 mA constant current, 5 h) the slab gel was stained with 0.1% (w/v) Coomassie Blue R250 in methanol/water/acetic acid (5:5:2 by vol.) and destained in propan-1-ol/acetic acid/water (5:4:31, by vol.). Stained discoidin I bands were quantified by scanning at 550 nm using a Gilford spectrophotometer fitted with a gel-scanning attachment.

Results

^{125}I-labelled discoidin I, purified by affinity chromatography on fixed sheep erythrocytes, contained no detectable discoidin II as determined by SDS/polyacrylamide-gel electrophoresis followed by staining for protein (Fig. 1*c*) or autoradiography (Fig. 1*e*). Since the affinity chromatography step was performed after the radio-iodination, all of the ^{125}I-labelled discoidin I must be functionally active. The molecular weight of the discoidin I polypeptide was estimated as 27500 by comparison with polypeptides of known molecular weight co-electrophoresed on the same polyacrylamide slab gel (Fig. 1*a*). Sucrose-density-gradient centrifugation of the ^{125}I-labelled discoidin I indicated that more than 95% was present in the form of tetramers (I. C. Madley, M. J. Cook & B. D. Hames; unpublished work). Based on the discoidin I subunit molecular weight of 27500, the molecular weight of the native discoidin I tetramer is estimated at 110000.

Preliminary experiments to examine the binding of ^{125}I-labelled discoidin I to glutaraldehyde-fixed *D. discoideum* cells utilized a binding assay analogous to that previously used to assay concanavalin A receptors in *D. discoideum* (Geltosky *et al.*, 1976); after incubation of 2×10^6 cells with ^{125}I-labelled discoidin I, the cells were pelleted by centrifugation in 1.5 ml-vol. polypropylene conical tubes (pre-soaked overnight in 0.5% bovine serum albumin in phosphate-buffered saline), washed three times with phosphate-buffered saline and then counted for radioactivity. Control experiments showed that less than 10% of bound ^{125}I-labelled discoidin I was

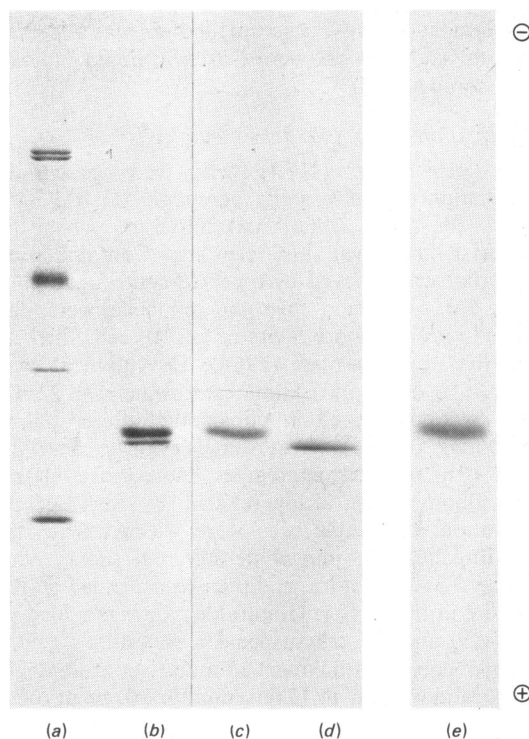

Fig. 1. *SDS/polyacrylamide-gel electrophoretic analysis of purified ^{125}I-labelled discoidin I*

(*a*) Molecular weight marker polypeptides: RNA polymerase β' and β subunits (mol. wts. 165000 and 155000 respectively), bovine serum albumin (68000), RNA polymerase α subunit (39000), and soya bean trypsin inhibitor (21500). (*b*) The mixture of unlabelled discoidin I and II as isolated by affinity chromatography on Sepharose 4B from *D. discoideum* AX3 cells (see the Experimental section). The lectins are shown after staining with Coomassie Blue R250. (*c*) ^{125}I-labelled discoidin I purified by affinity chromatography on sheep erythrocytes, stained with Coomassie Blue R250. (*d*) Purified discoidin II run as a marker polypeptide. (*e*) Autoradiograph of the ^{125}I-labelled discoidin I in track (*c*).

dissociated during this washing procedure. However, duplicate samples often revealed significant variability and hence a second assay was devised in which the cells were initially pelleted to remove the majority of unbound lectin, resuspended in phosphate-buffered saline and then centrifuged through a cushion of 5% (w/v) albumin in phosphate-buffered saline (see the Experimental section). This assay proved to be considerably superior to the assay based on repeated washing, with routinely less than 5% variability between duplicates, and was therefore used for all the work described here.

Binding of ^{125}I-labelled discoidin I (at 1 μg/ml) to 10^7 glutaraldehyde-fixed *D. discoideum* NC4 cells reached equilibrium after 60 min at 22°C and approx. 87% of this binding was inhibited by the presence of 0.3 M-galactose during the incubation (Fig. 2). The amount of ^{125}I-labelled discoidin I binding at time 0 represents that which occurs during the time taken to process the sample in the binding assay. The amount of non-specific binding at all time points was unaffected by the blocking agent used after glutaraldehyde fixation; glycine, ethanolamine and ethylenediamine all produced similar results (I. C. Madley & B. D. Hames, unpublished work). When the galactose concentration was raised to 0.5 M the non-specific binding was decreased to 5% or less. That ^{125}I-labelled discoidin I binding which could be inhibited by 0.5 M-galactose was linear with cell concentration over the entire range of 2×10^6–2×10^7 cells per assay (Fig. 3). As a result of these preliminary experiments, all subsequent analyses of ^{125}I-labelled discoidin I binding were carried out with 10^7 glutaraldehyde fixed cells per assay (with cells which were washed with 0.5 M-galactose prior to fixation and blocked with glycine after fixation) and incubated for 90 min at 22°C. Specific binding of discoidin I was defined as that which could be inhibited by 0.5 M-galactose.

Estimates of the number and affinity of discoidin I receptors on the surface of vegetative and aggregated *D. discoideum* NC4 cells were obtained from saturation experiments. Both vegetative (0 h) (Fig. 4*a*) and aggregated (10 h) cells (Fig. 4*b*) bound similar amounts of ^{125}I-labelled discoidin I and in both cases the binding of discoidin I was $\geqslant$95% inhibited by 0.5 M-galactose. Scatchard plot analysis of the saturation curve data for 10 h cells (Fig. 5) showed apparent positive co-operativity of ^{125}I-labelled discoidin I binding and, given a discoidin I

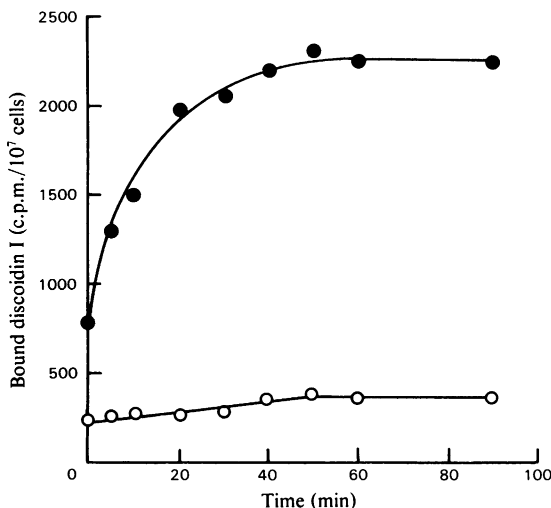

Fig. 2. *Time course of ^{125}I-labelled discoidin I binding to fixed D. discoideum cells*

D. discoideum NC4 cells at the 10 h stage of development were harvested in the presence of 0.3 M-galactose/10 mM-*N*-acetylgalactosamine, fixed with glutaraldehyde, and incubated with 1 μg of ^{125}I-labelled discoidin I/ml for various periods of time, from 0 to 90 min, at 22°C. ●, ^{125}I-labelled discoidin I binding that was inhibited by 0.3 M-galactose (i.e. total c.p.m. bound in the absence of galactose minus total c.p.m. bound in the presence of 0.3 M-galactose); ○, ^{125}I-labelled discoidin I binding that was not inhibited by 0.3 M-galactose.

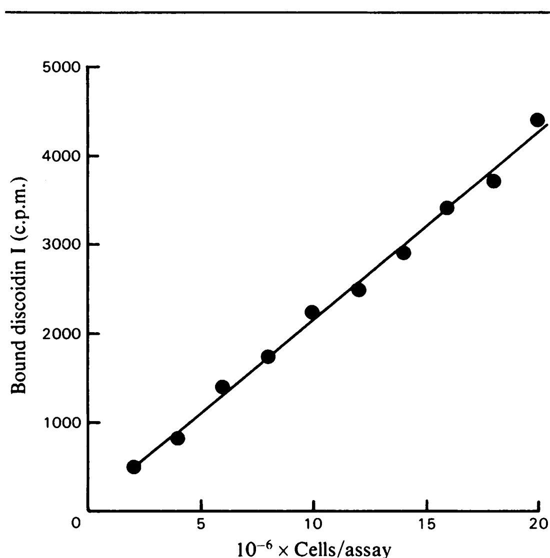

Fig. 3. *Linearity of ^{125}I-labelled discoidin I binding with cell number*

D. discoideum NC4 cells at the 10 h stage of development were harvested and fixed in the presence of 0.5 M-galactose and assayed for ^{125}I-labelled discoidin I binding sites by the standard albumin-cushion assay (see the Experimental section). All incubations were with 1 μg of ^{125}I-labelled discoidin I for 90 min at 22°C. The curve shown shows binding of ^{125}I-labelled discoidin I that was inhibited by 0.5 M-galactose.

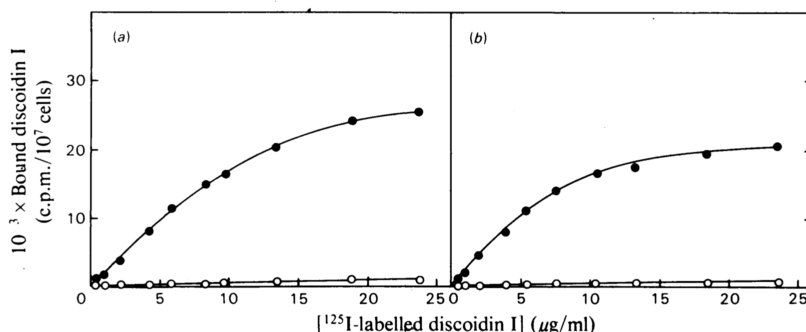

Fig. 4. *Saturation curves for* ^{125}*I-labelled discoidin binding to D. discoideum*

(*a*) Vegetative *D. discoideum* NC4 cells. The cells, harvested and fixed in the presence of 0.5 M-galactose, were incubated with various concentrations of ^{125}I-labelled discoidin I at 22°C for 90 min and then binding was determined by the standard albumin-cushion assay (see the Experimental section). ●, ^{125}I-labelled discoidin I binding that was inhibited by 0.5 M-galactose; ○, binding that was not inhibited by 0.5 M-galactose. The specific radioactivity of ^{125}I-labelled discoidin I was 2.68×10^4 c.p.m./µg. (*b*) Aggregation-phase *D. discoideum* NC4 cells. The assay conditions for ^{125}I-labelled discoidin I binding were as described for vegetative cells in (*a*) above except that the specific radioactivity of the ^{125}I-labelled discoidin I at the time of the experiment was 2.80×10^4 c.p.m./µg. ●, Binding that was inhibited by 0.5 M-galactose; ○, binding that was not inhibited by 0.5 M-galactose.

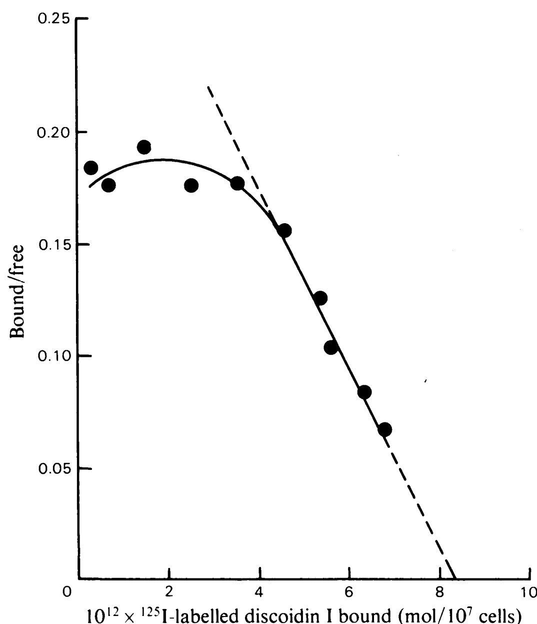

Fig. 5. *Scatchard plot analysis of* ^{125}*I-labelled discoidin I binding to D. discoideum*

The Scatchard plot analysis is for the data of discoidin I binding inhibited by 0.5 M-galactose given in Fig. 4(*b*) using aggregated (10 h) *D. discoideum* NC4 cells.

tetramer molecular weight of 110000 (see above), indicated 5.1×10^5 discoidin I tetramer binding sites per cell with a highest apparent affinity constant (K_a) of 2.03×10^7 M^{-1}. Analysis of ^{125}I-labelled discoidin I binding to vegetative (0 h) *D. discoideum* cells gave similar Scatchard plots showing apparent co-operativity of binding and an estimated 8.4×10^5 discoidin I tetramer binding sites per cell with a K_a of approx. 1.0×10^7 M^{-1}.

K. aerogenes, used as food source for the *D. discoideum* amoebae, also binds ^{125}I-labelled discoidin I in a form which is >99% inhibited by 0.5 M-galactose (Fig. 6), and therefore it is important to rule out contaminating bacteria as a major source of ^{125}I-labelled discoidin I binding to *D. discoideum* NC4 cell preparations. Contaminating bacteria were quantified both before cell fixation by plating on SM agar at 37°C and then by counting the bacterial colonies that grew up, and after fixation by microscopic examination. At the levels of bacterial contamination observed in the saturation experiments shown in Figs. 4(*a*) and 4(*b*) (bacterium/cell ratios of approx. 0.1 and 0.4, respectively), and correcting for the specific radioactivities of the ^{125}I-labelled discoidin I used, at least 99% and 97% of the binding to the 0 h and 10 h fixed *D. discoideum* cell preparations, respectively, at 25 µg/ml discoidin I represented binding to the *D. discoideum* cells themselves.

Finally, in order to check directly the amount of ^{125}I-labelled discoidin I bound to the *D. discoideum* cells at saturation, in some experiments the cell pellets corresponding to the 10, 15, 20 and 25 µg of ^{125}I-labelled discoidin I/ml incubation mixtures (Figs. 4*a* and 4*b*) were pooled separately for 0 h and 10 h developmental stage cells, washed free of most of the contaminating albumin resulting from the 5% (w/v)

albumin cushion used in the receptor assay, and the bound ^{125}I-labelled discoidin I was eluted with 0.5 M-galactose (see the Experimental section). The amount of ^{125}I-labelled discoidin I eluted was calculated from a measurement of its radioactivity and a knowledge of the specific radioactivity of the ^{125}I-labelled discoidin I before the binding assay. Known aliquots of the eluted ^{125}I-labelled discoidin I were then analysed by SDS/polyacrylamide slab gel electrophoresis (Figs. 7*b* and 7*c*) together with known amounts of authentic unlabelled discoidin I run as standards in parallel tracks (Figs. 7*d*–7*f*). Scanning of the discoidin I standard tracks after staining with Coomassie Blue R-250 showed that discoidin I staining with this dye is linear to at least 4 µg (Fig. 7*g*). The eluted ^{125}I-labelled discoidin I was quantified by scanning the stained bands and then by reference to Fig. 7(*g*) as the calibration curve. Estimates of the amounts of ^{125}I-labelled discoidin I eluted from vegetative (0 h) or aggregated (10 h) cells, as judged by the scanning of Coomassie Blue-stained gel bands, agreed closely with the amounts estimated from specific radioactivity measurements (Table 1).

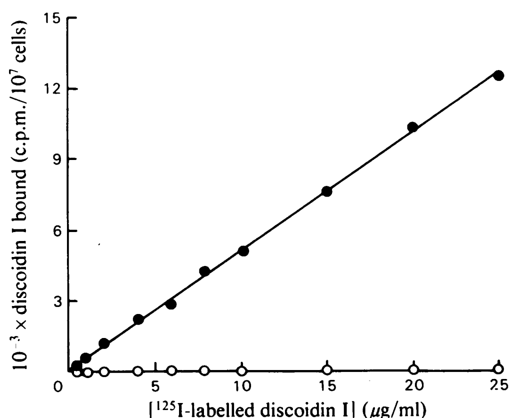

Fig. 6. *Binding of ^{125}I-labelled discoidin I to K. aerogenes*
Binding to *K. aerogenes* was tested using the standard albumin-cushion assay (described in the Experimental section) with 3.35×10^8 bacteria per assay and ^{125}I-labelled discoidin I at a specific radioactivity of 1.9×10^5 c.p.m./µg at the time of the experiment. ●, Binding that was inhibited by 0.5 M-galactose; ○, binding that was not inhibited by 0.5 M-galactose.

Discussion

Cell-surface receptors for discoidin I were initially detected by Reitherman *et al.* (1975) using a receptor assay based on the difference in lectin concentration between added discoidin I and that remaining unbound to *D. discoideum* (NC4) cells, the discoidin I concentration being assayed by

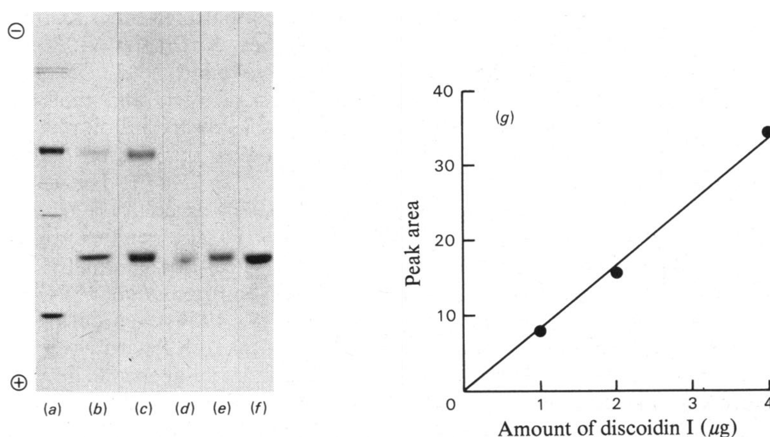

Fig. 7. *Quantification of eluted ^{125}I-labelled discoidin I*
Discoidin I samples were analysed on a 6–18% (w/v) linear gradient polyacrylamide slab gel and then stained with Coomassie Blue R250. (*a*) Molecular weight marker polypeptides; details as given for Fig. 1(*a*). (*b*), (*c*) Representative tracks of ^{125}I-labelled discoidin I eluted with 0.5 M-galactose from aggregated (10 h) and vegetative (0 h) *D. discoideum* NC4 cells, respectively, after use in the ^{125}I-labelled discoidin I binding assay. Track (*b*) was loaded with 1.73 µg of eluted ^{125}I-labelled discoidin I and track (*c*) was loaded with 2.16 µg of eluted ^{125}I-labelled discoidin I as determined from specific radioactivity measurements. The high molecular weight band in tracks (*b*) and (*c*) which comigrates with the marker bovine serum albumin in track (*a*) represents trace contamination with albumin from the binding assay. This band is not radioactive and therefore contains no discoidin. (*d*), (*e*) (*f*) show tracks loaded with 1,2 and 4 µg of unlabelled discoidin I respectively. (*g*) shows the standard curve of peak area versus µg of discoidin I obtained by scanning tracks (*d*), (*e*) and (*f*) and used for quantification of eluted ^{125}I-labelled discoidin I. Peak area was determined by cutting out each peak from the scans, weighing it (mg), and multiplying by the band width (cm).

Table 1. *Quantification of bound* ^{125}*I-labelled discoidin I in D discoideum (NC4)*

Source of bound ^{125}I-labelled discoidin I	Amount (μg) of eluted discoidin I quantified by: Specific radioactivity	Coomassie Blue staining
0 h cells		
Exp. I	1.54	1.65
Exp. II	1.28	1.45
	2.16	2.60
10 h cells		
Exp. I	1.73	1.85

haemagglutination of rabbit erythrocytes. Whereas the number of discoidin I receptors (defined as discoidin I tetramer binding sites) was found to change only slightly during aggregation, from 3×10^5 receptors per vegetative (0 h development) cell to 5×10^5 receptors per aggregating (9 h development) cell, the binding affinity of these receptors increased markedly over this time period from $5 \times 10^7 \text{M}^{-1}$ to 10^9M^{-1}, suggestive of either an activation of pre-existing receptors or an acquisition of new, high-affinity receptors with a potential role in initiating cell–cell adhesion via discoidin I–discoidin I receptor interactions. Recently, Bartles & Frazier (1980) have repeated the analysis of discoidin I receptors using an assay which involves the binding of ^{125}I-labelled discoidin I to glutaraldehyde-fixed cells followed by direct determination of bound ^{125}I-labelled discoidin I by counting the cell pellet for radioactivity, and have obtained data which disagree markedly with the data of Reitherman *et al.* (1975). Thus, Bartles & Frazier (1980) detected only 5×10^3 discoidin I receptors per vegetative cell, increasing to a maximum 2.4×10^4–3.0×10^4 receptors per cell at 15 h development, that is, an approx. 5-fold increase in the number of discoidin I receptors during the aggregation phase. Moreover, the limiting apparent affinity of the discoidin I receptors was 3×10^9 M^{-1} even in vegetative cells and did not change during aggregation. Bartles & Frazier (1980) have pointed out that the receptor assay used by Reitherman *et al.* (1975) involves determination of discoidin I concentration using the haemagglutination activity of this lectin which is now known to be unstable and dependent on co-purified lipid (Bartles *et al.*, 1979). Nevertheless, the work reported in the present paper, quantifying discoidin I receptors by direct measurement of ^{125}I-labelled discoidin I bound to *D. discoideum* NC4 cells, analagous to the assay used by Bartles & Frazier (1980), is in broad agreement with the data of Reitherman *et al.* (1975) on the number of discoidin I receptors per cell; that is, we find approx. 8.4×10^5 receptors per vegetative cell and 5.1×10^5 receptors per aggregated (10 h) cell. These numbers appear to be accurate since they have been confirmed by elution of bound discoidin I and direct quantification by Coomassie Blue staining. Furthermore, unlike the earlier study of Reitherman *et al.* (1975), we have ruled out the possibility that a significant fraction of this binding is due to contaminating bacteria. The number of cell-surface receptors for endogenous lectin has also been quantified in *D. purpureum* (Springer *et al.*, 1980). In this species, each aggregated cell bound 3.6×10^5 molecules of purpurin when exposed to saturating amounts of this lectin, a figure in close agreement with the number of discoidin I tetramers we find will bind to each aggregated *D. discoideum* NC4 cell. The binding of discoidin I to *D. discoideum* cells shows apparent positive co-operativity. This observation has also been made by Bartles & Frazier (1980) who have fully discussed the possible interpretations.

A major difference between the study reported here plus that of Reitherman *et al.* (1975), and that of Bartles & Frazier (1980), is the strain of *D. discoideum* used. *D. discoideum* strain NC4 (used in the present work and that of Reitherman *et al.*, 1975) lacks discoidin I during vegetative growth and accumulates this lectin only during aggregation (Rosen *et al.*, 1973; Frazier *et al.*, 1975; Ma & Firtel, 1978). In contrast, *D. discoideum* AX3, when grown in axenic culture (as used by Bartles & Frazier, 1980), accumulates discoidin both within the cell (Simpson *et al.*, 1974) and on the cell surface (Siu *et al.*, 1976) even during vegetative growth. *D. discoideum* AX3, grown axenically, is therefore known to be aberrant in the developmental accumulation of discoidin. Now, Springer *et al.* (1980) have recently shown for *D. purpureum* that 1.1×10^5 molecules of purpurin are bound to the surface of each aggregated cell even in the absence of exogenously added purpurin. More than 90% of this cell-surface located purpurin appears to be bound to cell-surface lectin receptors, since it can be specifically eluted with lactose. Similarly, each aggregated *D. discoideum* NC4 cell was found to have 5.2×10^4 molecules of endogenous discoidin I bound to the cell surface in a form able to be displaced by *N*-acetylgalactosamine. Obviously, the proportion of discoidin receptors that are masked by endogenous

cell-surface discoidin, and hence the number of discoidin receptors still available for detection in a subsequent discoidin I binding assay, will depend on the amount of endogenous cell-surface discoidin in the cell preparation under study. Unfortunately, the relative amount of cell-surface discoidin I in aggregated AX3 cells (grown under axenic conditions), compared with aggregated NC4 cells, has not been measured and therefore the potential influence of receptor masking on previously reported numbers for discoidin I receptors in these cells (Bartles & Frazier, 1980; Reitherman *et al.*, 1975, respectively) cannot be assessed at this time. However, if the endogenous discoidin detected on the surface of vegetative AX3 cells grown axenically (Siu *et al.*, 1976) is actually bound to discoidin receptors, then the data of Bartles & Frazier (1980) for the number of discoidin I receptors, at least on vegetative cells, must be minimal estimates. Receptor masking would not be a problem with vegetative NC4 cells since these lack endogenous discoidin. Finally, irrespective of the number of cell-surface receptors complexed with endogenous discoidin *in vivo*, any cell damage during harvesting can release cytosolic discoidin which may then bind to receptors before cell fixation. Again this would decrease the number of receptors available for assay. Clearly it is important to wash any *D. discoideum* cell preparation with galactose to elute bound lectin prior to fixation, as in the present study, if the true number of potential discoidin I receptors is to be estimated with confidence.

Despite our broad agreement with Reitherman *et al.* (1975) on the number of discoidin I receptors on the surface of vegetative and aggregated *D. discoideum* NC4 cells, and on the affinity of the discoidin I receptors on vegetative cells (Reitherman *et al.*, 1975: $5\times10^{7}\,\text{M}^{-1}$; this work: $1\times10^{7}\,\text{M}^{-1}$) we were unable to detect the marked increase in receptor affinity during aggregation reported by Reitherman *et al.* (1975). Therefore, if discoidin I binds to only one or a few types of receptor and is involved in cell cohesion, our data suggest that changes in receptor affinity are not involved in triggering discoidin I–discoidin I receptor-mediated cell cohesion. Rather, it is the appearance *de novo* of cell-surface discoidin I that initiates this phenomenon. However, it is conceivable that there are multiple types of receptor molecules that are responsible for the majority of ^{125}I-labelled discoidin I binding observed and changes in the number or affinity of a few minor types of receptor molecule, which are possibly those of physiological importance, have gone undetected. This can only be tested by identification and study of the individual discoidin I receptors involved.

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References

Bartles, J. R. & Frazier, W. A. (1980) *J. Biol. Chem.* **255**, 30–38

Bartles, J. R., Pardos, B. T. & Frazier, W. A. (1979) *J. Biol. Chem.* **254**, 3156–3159

Beug, H., Katz, F. E. & Gerisch, G. (1973) *J. Cell Biol.* **56**, 647–658

Butler, W. T. (1963) *J. Immunol.* **90**, 663–668

Chang, C. M., Reitherman, R. W., Rosen, S. D. & Barondes, S. H. (1975) *Exp. Cell Res.* **95**, 136–142

Frazier, W. A., Rosen, S. D., Reitherman, R. W. & Barondes, S. H. (1975) *J. Biol. Chem.* **250**, 7714–7721

Geltosky, J. E., Siu, C. H. & Lerner, R. A. (1976) *Cell* **8**, 391–396

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Ma, G. C. L. & Firtel, R. A. (1978) *J. Biol. Chem.* **253**, 3924–3932

Marin, F. T., Goyette-Boulay, M. & Rothman, F. G. (1980) *Dev. Biol.* **80**, 301–312

Müller, K. & Gerisch, G. (1978) *Nature (London)* **274**, 445–449

Müller, K., Gerisch, G., Fromme, I., Mayer, H. & Tsugita, A. (1979) *Eur. J. Biochem.* **99**, 419–426

Phillips, P. G., Furmanski, P. & Lubin, M. (1974) *Exp. Cell Res.* **86**, 301–308

Ray, J., Shinnick, T. & Lerner, R. A. (1979) *Nature (London)* **279**, 215–221

Reitherman, R. W., Rosen, S. D., Frazier, W. A. & Barondes, S. H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3541–3545

Rosen, S. D., Kafka, J. A., Simpson, D. L. & Barondes, S. H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2554–2557

Shinnick, T. M. & Lerner, R. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4788–4792

Simpson, D. I., Rosen, S. D. & Barondes, S. H. (1974) *Biochemistry* **13**, 3487–3493

Siu, C. H., Lerner, R. A., Ma, G., Firtel, R. A. & Loomis, W. F. (1976) *J. Mol. Biol.* **100**, 157–178

Springer, W. R. & Barondes, S. H. (1980) *J. Cell Biol.* **87**, 703–707

Springer, W. R., Haywood, P. L. & Barondes, S. H. (1980) *J. Cell Biol.* **87**, 682–690

Sussman, M. (1966) *Methods Cell Physiol.* **2**, 397–416

Watts, D. J. & Ashworth, J. M. (1970) *Biochem. J.* **119**, 171–174