Discoidin I and Discoidin II Are Localized Differently in Developing *Dictyostelium discoideum*

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ABSTRACT   The distribution of discoidin I and discoidin II, developmentally regulated lectins in *Dictyostelium discoideum*, was determined immunohistochemically at various stages of development. Discoidin I was first prominent as focal clumps in aggregating cells, then accumulated on the surface of aggregates and around them. Discoidin II became prominent later and ultimately localized in what appear to be prespore vesicles. The results indicate that discoidin I and discoidin II have different and possibly multiple functions.

As cellular slime molds differentiate, they synthesize carbohydrate-binding proteins generally referred to as lectins (1, 16). *Dictyostelium discoideum* contains two lectins—discoidin I (dI) and discoidin II (dII)—distinguishable by isoelectric point, subunit molecular weight, and affinity for a range of saccharides (10, 18). Most work has focused on the more abundant lectin, dI, which may play a role in cell-cell adhesion (5). This is supported by evidence that a mutant with a presumed defect in the carbohydrate-binding site of dI shows both impaired cell-cell adhesion and abortive differentiation (15, 17). Despite these findings, the role of developmentally regulated lectins in *D. discoideum* is far from clear.

In search of further clues to the function of these lectins, we raised antibodies to highly purified preparations of dI and dII, and used them for immunohistochemical localization. The distributions of the two lectins were markedly different and changed strikingly with development, suggesting that dI and dII have different and possibly multiple functions.

MATERIALS AND METHODS

Preparation of Antigens and Antisera: Discoidin was purified by affinity chromatography (18). DI and dII were separated by a procedure which gives greater purity and higher yield than that described previously (10). The lectins were bound to an *N*-acetyl-galactosamine conjugated agarose column (Selectin 6, Pierce Chemical Co., Rockford, IL) and selectively eluted with a galactose gradient, as will be published in detail elsewhere. DI and dII preparations for immunization and adsorption were determined to be pure and uncontaminated with the other isolectin by Coomassie Blue staining of polyacrylamide gels after electrophoresis in SDS.

Initial immunization in rabbits or mice was carried out by subcutaneous injection of antigen in complete Freund's adjuvant. Repeated boosts were given by subcutaneous injection of antigen in incomplete Freund's adjuvant. Serum was collected ~7 d after the final boost.

Rabbit antispore serum, generously provided by Dr. James Morrissey (University of California, San Diego), was raised against spores of *Dictyostelium mucoroides* and adsorbed with vegetative *D. discoideum* cells. This serum is believed to react primarily with spore coat and pre-spore vesicle antigens (22; J. Morrissey, personal communication).

Estimation of Immunological Cross-reaction: Initial evaluation of the reaction of rabbit antidiscoidin I (anti-dI) and antidiscoidin II (anti-dII) with dI and dII was done by studying the binding of biotinylated immunoglobulins to lectins adsorbed to polystyrene wells. Immunoglobulin (IgG) was prepared by column chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. IgG was biotinylated with the *N*-hydroxysuccinimide ester of biotin (Calbiochem/Behring Corp., La Jolla, CA) as described by Heggeness and Ash (12). Following a dialysis against 75 mM Na_2HPO_4/KH_2PO_4, 75 mM NaCl, pH 7.2 (PBS), the biotinylated IgG was stored frozen at 15 mg/ml.

A series of dilutions of biotinylated IgG were reacted with polystyrene wells (Immulon 1 Removawells, Dynatech Laboratories, Alexandria, VA) that had been saturated with dI or dII by preincubation for 1 h with 150 μl of a 40 μg/ml lectin solution in PBS containing 0.3 M galactose. Before reaction with biotinylated IgG, the wells were washed four times with 400 μl of PBS containing 0.3 M galactose, 0.2% BSA (RIA grade, Sigma Chemical Co., St. Louis, MO). They were then incubated with 150 μl of biotin-conjugated IgG diluted to various concentrations with PBS containing 0.3 M galactose, 0.2% BSA. After four washes with this diluent, the wells were incubated for 30 min with 150 μl of avidin-conjugated peroxidase (ABC vectastain standard kit, Vector Laboratories, Burlingame, CA) prepared according to the manufacturer's instructions and then diluted tenfold in PBS. Wells were then washed six times with PBS containing 0.3 M galactose, 0.2% BSA before addition of 200 μl of 0.1% *o*-phenylenediamine (Bionetics Laboratory Products, Kensington, MD) in 1 M sodium citrate, pH 4.5, with 0.02% hydrogen peroxide. The reaction was allowed to proceed for 5 min and then stopped by the addition of 50 μl of saturated sodium fluoride. Results were recorded spectrophotometrically at 410 nm.

To estimate immunological cross reaction of various crude antisera used for immunohistochemical localization of dI and dII, we tested their inhibitory effects on the binding of biotinylated anti-dI IgG to dI and biotinylated anti-dII IgG to dII. For these experiments, polystyrene wells saturated with dI or dII were prepared as just described, then incubated for 30 min with 150 μl of antiserum at various dilutions in PBS containing 0.3 M galactose, 0.2% BSA. After washing four times with the same buffer, the wells were incubated for another 30 min with 150 μl of a 50 μg/ml solution of the appropriate biotinylated IgG then washed, and binding of the biotinylated IgG was determined as just described.

Adsorption of the Antisera with Immobilized Lectins: Isolectin conjugated columns for adsorption of antisera were prepared with cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscata-

way, NJ) according to the manufacturer's instructions. Rabbit anti-dI serum was adsorbed by repeated passage of 1 ml of a 1/100 dilution in PBS over a 2-ml column conjugated with 200 μg of dI, and rabbit anti-dII serum was adsorbed in the same manner with a column conjugated with dII. Similar adsorption of rabbit anti-dI serum with a column conjugated with discoidin II and of rabbit anti-dII serum with a column conjugated with dI did not substantially inactivate these antisera as judged by immunohistochemical methods.

Growth and Differentiation of D. discoideum:
D. discoideum cells (strain NC-4) were grown for 48 h on nutrient agar in association with *Klebsiella aerogenes* by procedures like those described by Sussman (21) as detailed previously (4). Amoebae were scraped from the agar and washed five times in 10 vol cold water then resuspended in water to 2 × 10⁸/ml. 1 ml of this
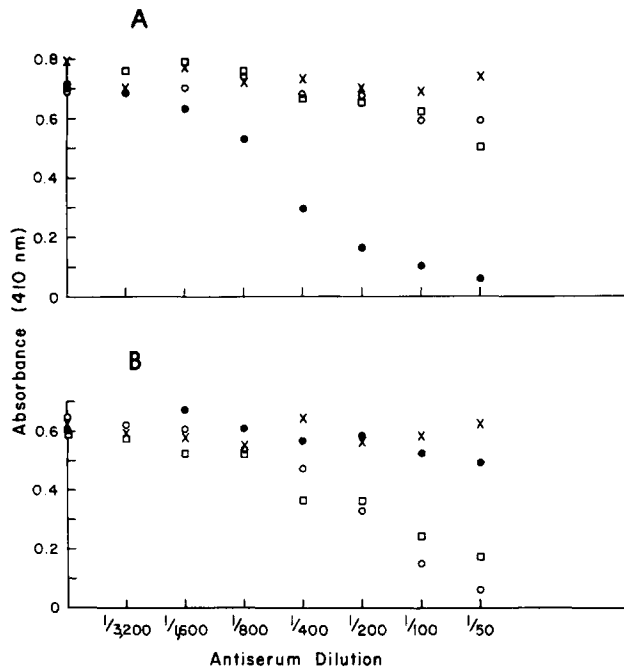
FIGURE 1   Relative binding of four antisera to (*A*) dI and (*B*) dII. Polystyrene wells were saturated with either dI or dII, then reacted with a series of dilutions of the four antisera indicated on the figure. After washing, the discoidin I wells were reacted with biotinylated anti-dI IgG and the discoidin II wells were reacted with biotinylated anti-dII IgG, and the amount of biotinylated IgG bound to the wells was determined with avidin-conjugated peroxidase, as described in Materials and Methods. ×, rabbit anti-sp. ○, rabbit anti-dII. ●, rabbit anti-dI. □, mouse anti-dII.

suspension was spread on a layer of 2% Noble agar (Difco Laboratories, Detroit, MI) in glass-distilled water in a 100-×-20-mm petri dish. Dishes were placed, uncovered, in a box and covered with a layer of aluminum foil that was punctured at one end with several small holes. The box was placed under a light source and amoebae were allowed to differentiate at room temperature.

Cell Preparation for Immunohistochemistry:
At the desired stages of differentiation selected areas of agar containing aggregates were cut out and floated on fixative in culture dishes. In most cases we used Carnoy's fixative (glacial acetic acid, chloroform, absolute ethyl alcohol, 1:3:6 by volume) at room temperature for 48-96 h as used by Gregg (11). The fixed aggregates were scraped from the agar and allowed to settle in 1.5-ml microfuge tubes containing 30% sucrose in PBS for 1 h at room temperature. In some cases we used the periodate-lysine-paraformaldehyde (PLP) fixative containing 0.01 M periodate and 2% paraformaldehyde developed by McLean and Nakane (14) for 1 h at room temperature. The fixed aggregates were then allowed to settle in 1.5-ml microfuge tubes overnight at 4°C in PLP that had been diluted tenfold in PBS containing 30% sucrose. Aggregates fixed by either method settled to the bottom of the microfuge tube without centrifugation. The supernatant was aspirated and one drop of O.C.T. compound (Miles Laboratories, Napeville, IL) was added to the tube and mixed with the slime molds. The pellet was then quick frozen and mounted on a microtome chuck using O.C.T. compound. 1- to 2-μm frozen sections were cut at −35°C in an H/I Bright Cryostat (Hacker Instruments, Inc., Fairfield, NJ) fitted for sectioning with glass knives with an LKB "Ralph" knife adapter (LKB Instruments, Bromma, Sweden). Sections were picked up on slides that had been cleaned then dipped while warm in a mixture of 0.5% gelatin and 0.05% chromic potassium sulfate heated to 50°C. Sections on slides were stored at −20°C before staining.

Immunofluorescence Staining:
Slides were washed before staining for 1 h in two changes of PBS containing 2.5% normal goat serum (PBS + GS). PBS + GS was used throughout the staining procedure for washes and as a diluent for antisera.

In most experiments, primary antibody binding was done with a mixture of appropriate dilutions of a rabbit antiserum to one antigen and a mouse antiserum to another antigen, as indicated in the text. Slides were incubated with these antisera for 30 min at room temperature in a moist atmosphere. For each experiment, control slides were incubated with an appropriate mixture of normal rabbit and mouse sera or adsorbed immune sera. Slides were washed for 30 min in two changes of PBS + GS, then transferred to a staining jar containing a mixture of either fluorescein conjugated goat-anti-mouse IgG and rhodamine-conjugated goat-anti-rabbit IgG, or fluorescein-conjugated goat-anti-rabbit IgG and rhodamine-conjugated goat-anti-mouse IgG. The combination used was chosen to facilitate visualization and photography of both bound primary antibodies. Conjugated antisera were obtained from N. L. Cappel Laboratories, Inc. (Cochranville, PA), and were diluted 1:100 in PBS + GS in all experiments. After two 15-min washes, slides were drained and mounted with coverslips using 90% glycerol.

Microscopy:
Slides were examined with a Leitz Dialux epifluorescence microscope using a Leitz × 40 (NA 1.3) oil immersion lens. Photomicrographs were taken with a Wild MPS 45 camera and Kodak Tri-X film. Both exposures and printing of experimental and control slides were done under identical conditions.
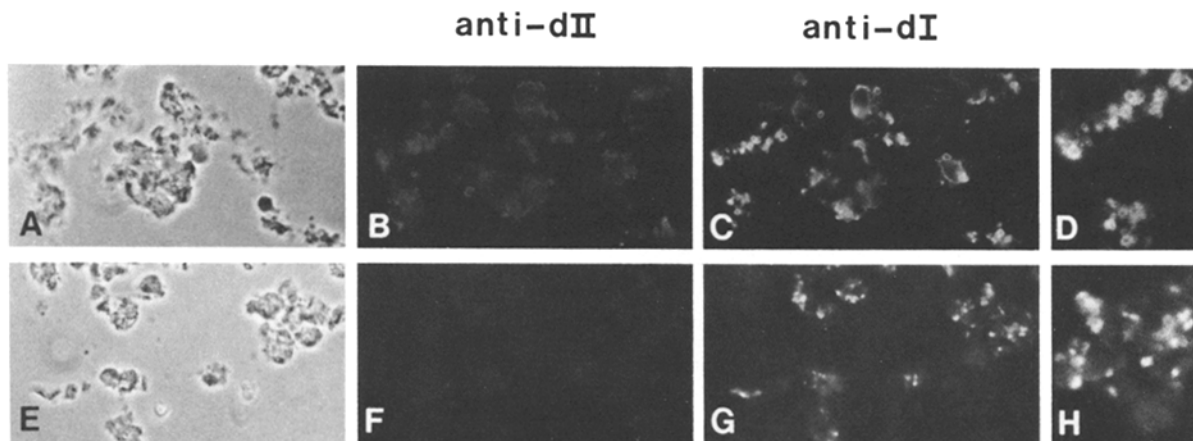
FIGURE 2   Immunohistochemical localization of discoidin I and discoidin II in sections of cells fixed at the early aggregating stage with either PLP (*A−D*) or Carnoy's fixative (*E−H*). The sections were reacted with a mixture containing a 1:100 dilution of rabbit anti-dI and a 1:50 dilution of mouse anti-dII, and bound antibody was visualized with fluorescein-conjugated goat-anti-mouse IgG and rhodamine-conjugated goat-anti-rabbit IgG. *D* and *H* are enlargements of portions of *C* and *G*, respectively. × 1,400, except *D* and *H*, which are × 2,800.

RESULTS

Specificity of Antisera

Rabbit antisera were raised against highly purified preparations of dI and dII, and a mouse antiserum was raised against dII. The antisera raised against one lectin showed very minor reaction with the other lectin in immunodiffusion tests. To further evaluate cross-reaction we bound dI or dII to plastic wells, prepared biotinylated IgG from rabbit anti-dI and rabbit anti-dII, and compared the binding of each IgG to each lectin using avidin-peroxidase as described in Materials and Methods. With dII about 40 times more anti-dII bound and with dI
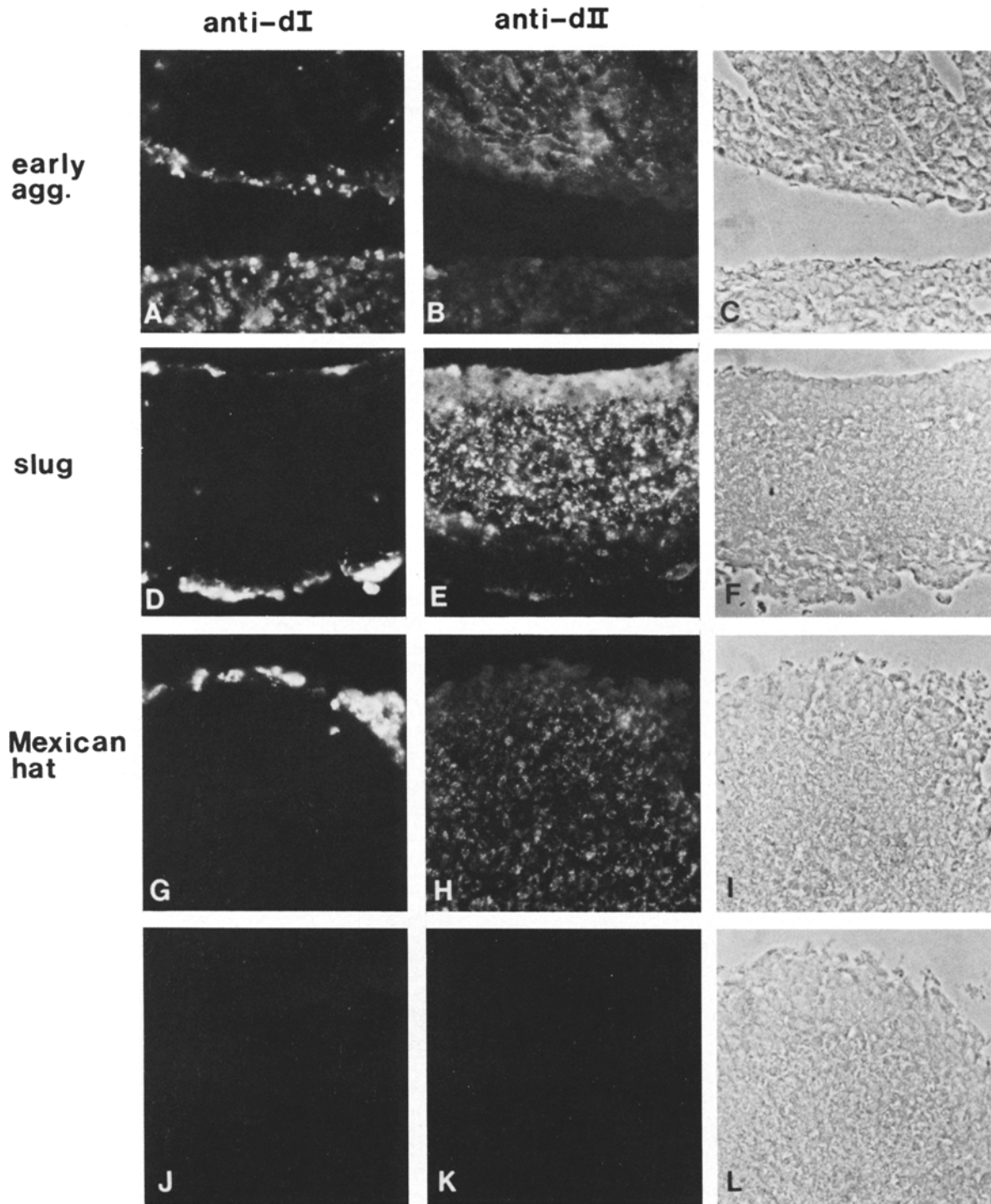

FIGURE 3   Immunohistochemical localization of dI and dII in early aggregates (A–C), slugs (D–F), and Mexican hats (G–I). Sections of Carnoy's-fixed aggregates were stained with mixtures of 1:100 dilutions of rabbit anti-dI and mouse anti-dII, and bound antibody was visualized with rhodamine-conjugated goat-anti-rabbit IgG and fluorescein-conjugated goat-anti-mouse IgG. Sections of aggregates at the Mexican hat stage were also reacted with rabbit anti-dI adsorbed with dI immobilized on beads (J) or mouse anti-dII adsorbed with dII immobilized on beads (K), and bound antibody was then visualized with fluorescent second antibody. C, F, and I are phase-contrast photographs of the section stained with anti-dI and anti-dII. L is a section like those used in J and K. × 1,280.

about 20 times more anti-dI bound.

We then evaluated the four whole sera which were employed for immunohistochemistry in a competitive binding assay against biotinylated rabbit anti-dI and anti-dII (Fig. 1). Rabbit and mouse anti-dII and rabbit anti-dI showed considerable specificity but some cross reaction. A rabbit antiserum raised against spore antigens (anti-sp) showed no significant binding to either lectin. We conclude that the antisera are sufficiently specific for immunohistochemical studies, which was supported by the results of the immunohistochemistry shown below. To test this further we adsorbed antisera with either dI or dII immobilized on agarose beads in critical experiments. In all cases the specificity of the antisera was verified.

Choice of Fixatives

In pilot studies we attempted to fix *D. discoideum* at various stages with Carnoy's fixative, PLP fixative, cold methanol or various mixtures of glutaraldehyde and paraformaldehyde. Carnoy's fixative preserved intact aggregates, as observed by Gregg (11). This fixative permitted the study of overall distribution of antigens in the aggregate, although it did not preserve cellular detail as well as the other fixatives. PLP and cold methanol were useful for studying individual aggregating cells, but these fixatives failed to preserve the integrity of aggregates when examined as frozen sections. In contrast with the others, fixatives containing glutaraldehyde markedly diminished antibody binding.

Localization of Discoidin I

dI was readily detected in cells as they streamed into aggregates (Fig. 2). The cells had variable amounts of antigen, which tended to be localized in clumps and at the cell periphery. This was apparent with both Carnoy's and PLP fixatives. With the latter, the focal accumulations had a ringlike structure (Fig. 2 *C* and *D*) as if the lectin were associated with large vesicles or blebs, whereas, with Carnoy's fixative, the antigenic clumps tended to be more solid, although ringlike structures were sometimes seen (Fig. 2 *G* and *H*).

Because of the focal distribution of dI it is difficult to interpret the paucity of the lectin in some cells at this stage. It is possible that other serial sections of such cells might show more dI although our impression from examining serial sections is that some cells are truly deficient in this lectin. Whether such cells are at an earlier or later stage of development when dI is no longer prominent, or whether some cells may never express much dI, cannot be determined, because cell differentiation is not completely synchronous.

dI was also focally accumulated in cells at a slightly later stage (aggregate in the lower part of Fig. 3*A*). In the more mature aggregate in the upper part of Fig. 3*A*, dI was confined to cells at the aggregate's periphery. The remaining cells contained little or no dI (Fig. 3*A*) but had considerable dII (Fig. 3*B*). At later stages of development, dI was confined to amorphous material associated with the surface of slugs and other late aggregating forms, as well as with material shed from the aggregates (Fig. 3*D* and *G*). dI staining could be completely adsorbed by dI coupled to beads at this stage (Fig. 3*J*) and at all others (not shown). Some dI was also found around stalk cells as they formed this structure (Fig. 4*A*). However, the intensity of staining of dI at these sites was much less than was found in association with material at the surface of an aggregate (left part of Fig. 4*A*). dI was never found in spore cells.

Localization of Discoidin II

dII was barely detectable by immunohistochemical methods in the very early aggregating forms where dI was already prominent (Fig. 2). In early aggregates, dII was apparently present in some cells rich in dI but tended to have a more amorphous distribution (lower aggregate in Fig. 3*B*). At least some anti-dII staining of this aggregate is truly identifying dII and is not due to spill of fluorescence from the dI staining nor to immunological cross reaction. This conclusion is based on experience with sections stained not only by the double label procedure as in Fig. 3 but also by single labeling with mouse anti-dII or rabbit anti-dII, and either fluorescein or rhodamine conjugated second antibodies. Although these studies suggest that some cells may simultaneously contain dI and dII, the major finding is that when one lectin is prominent in a cell the other is not.

With further development, dII became more prominent. Because this was not previously observed after starvation of *D. discoideum* in suspension, (10) we considered that the immunohistochemical results could be due to redistribution rather than additional synthesis of this lectin. To evaluate this we measured dII in crude cellular extracts at several stages of development with a quantitative immunoassay and by gel electrophoresis after purification by affinity chromatography. These studies confirmed the marked increase in dII late in development. The late appearance of dII may not have been detected previously (10) since the cells in those studies were starved in suspension for 12 h. Additional time as well as appropriate differentiation on a solid surface may be necessary to fully induce the synthesis of dII.

As dII accumulated it became more discretely localized. One pattern is seen in the upper aggregate of Fig. 3 *B* where some dII is punctate and some of it appears organized in arrays which may be around the periphery of cells. A wavy layered
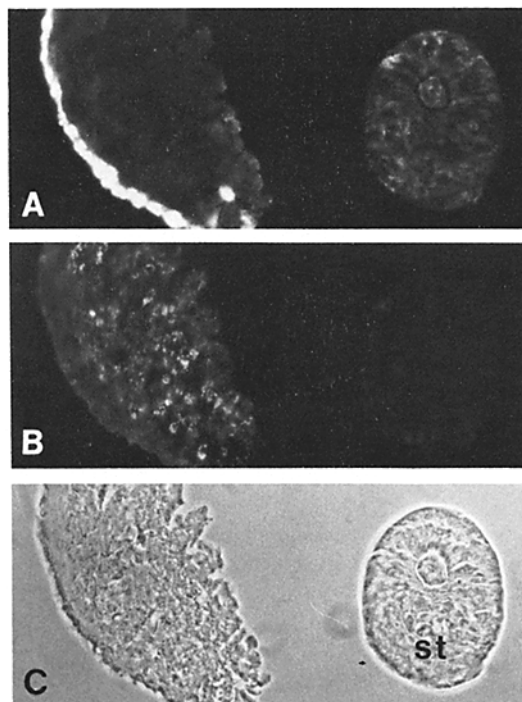

FIGURE 4   Immunohistochemical localization of dI (*A*) and dII (*B*) in late and culminating aggregates. A cross section through a developing stalk (*st*) is shown. Staining was as in Fig. 3. × 1080.

distribution (Fig. 5 *A*) was also frequently observed in aggregates at this stage. This pattern was never observed in slugs or thereafter. At the slug stage, dII tended to be punctate within a large population of cells located centrally (Fig. 3 *E*) and concentrated in the posterior part of the slug. This distribution is typical of pre-spore cells (13, 22). Material on the lower surface of the slug, which was rich in dI (Fig. 3 *D*), contained little or no dII (Fig. 3 *E*). dII was sometimes also distributed very diffusely in a band of cells in the upper surface of slugs (Fig. 3 *E*). These cells may be members of the pre-stalk cell population, although they are not confined to the slug tip where such cells tend to be. The rind of cells that have little dII in the left part of Fig. 4 *B* are probably members of this same population. At the later Mexican hat stage, dII was found only in a punctate central distribution (Fig. 3 *H*), characteristic of pre-spore cells. This lectin was absent in developing stalk (right part of Fig. 4). Specificity of staining with anti-dII was confirmed by adsorption with dII, as exemplified in Fig. 3 *K*.

The localization and punctate distribution of dII raised the possibility that it was associated with pre-spore vesicles. To evaluate this we compared the distribution of staining with rabbit anti-sp. and mouse anti-dII (Fig. 5). The former antiserum is known to react with pre-spore vesicles and with material on the surface of developing spores, and this was confirmed in our studies, (Fig. 5 *E* and *H*). At the tight aggregate stage, anti-dII staining was very prominent whereas anti-sp staining was relatively sparse. The two antigens showed some overlap but marked differences (Fig. 5 *A* and *B*). However, in the slugs, where anti-sp staining was much more prominent, the staining patterns of anti-dII and anti-sp were often superimposable (Fig. 5 *D* and *E*). This suggests that, at this stage, much of the discoidin II is associated with pre-spore vesicles. The congruence of discoidin II with antigens stained by anti-sp did not persist with further development. Some overlap of anti-dII and anti-sp staining was seen around the surface of developing spores (Fig. 5 *G* and *H*), but many spore surfaces which stained with anti-sp did not stain with anti-dII. The surfaces of more mature spores also stained with anti-sp, but there was no significant staining with anti-dII either within spore cells or on their surfaces (not shown).
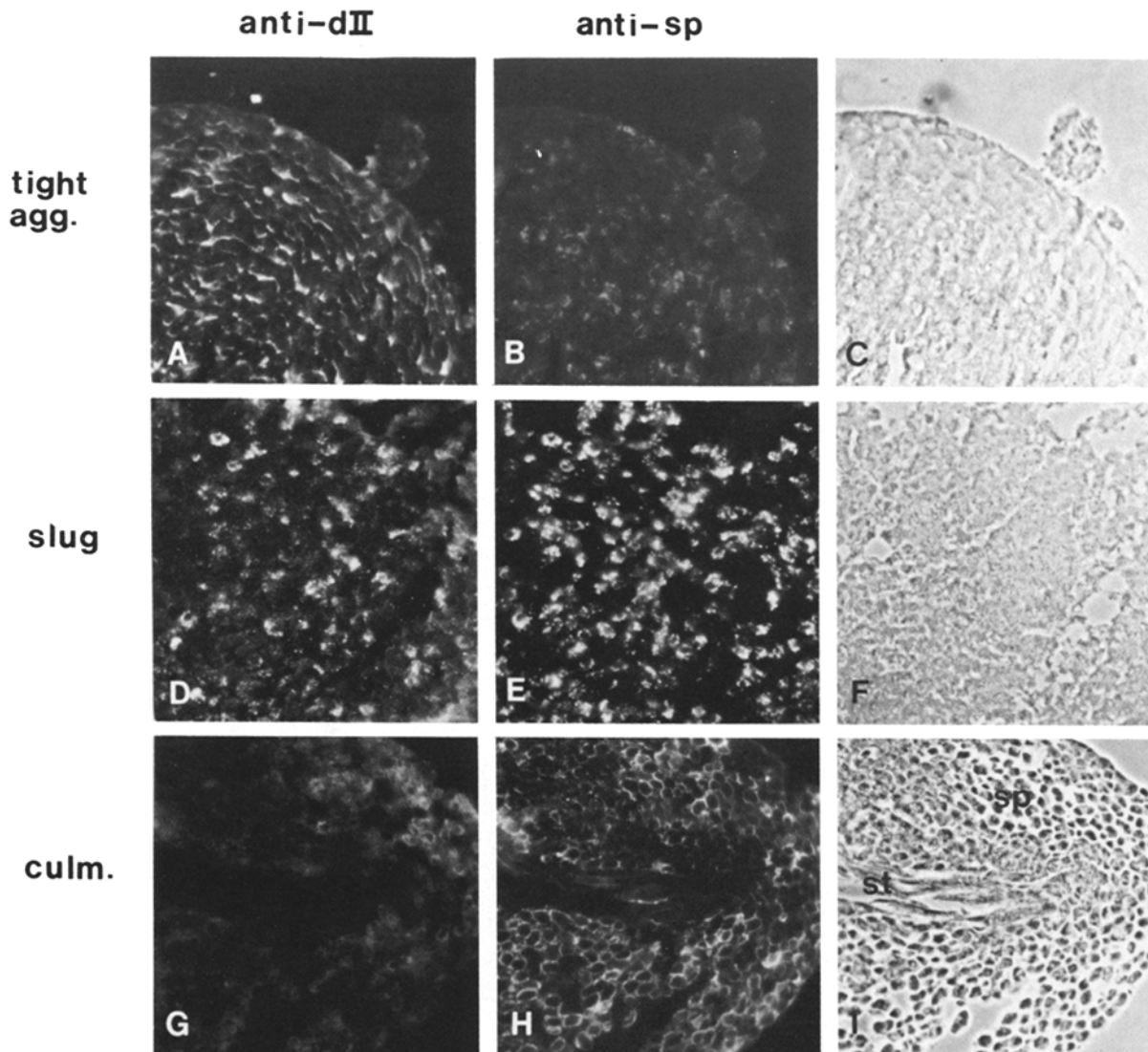

FIGURE 5   Immunohistochemical staining with mouse anti-dII and rabbit anti-sp of a tight aggregate (*A* –*C*), a slug (*D* –*F*) and a culminating aggregate (*G* –*I*). Sections of Carnoy's-fixed aggregates were stained with a mixture of 1:100 dilutions of mouse anti-dII and rabbit anti-sp, then with fluorescein-conjugated goat-anti-mouse IgG and rhodamine-conjugated goat-anti-rabbit IgG. In the section through a culminating aggregate the discrete dark cells are developing spore cells (*sp*), and an area of developing stalk is labeled (*st*). × 1,280.

DISCUSSION

The results demonstrate that dI and dII are localized differently in developing *D. discoideum* and that their distribution changes with development. Localization of the lectins during aggregate formation is summarized in Fig. 6. Although these studies localize the lectins they do not clearly reveal their function.

dI is prominent relatively early in development and is concentrated in clumps, which could be large vesicles. This localization is probably a prelude to the externalization of dI because, later, it is outside the aggregate. An extracellular location is consistent with previous studies which showed that some slime mold lectin is on the surface of aggregating cells (9, 20) and that much more can be externalized (19, 20).

The immunohistochemical studies neither decisively support nor refute a role for dI in cell-cell adhesion (5). In aggregating cells this lectin is apparently associated with large vesicles and surface membranes. Surface lectin could play a role in cell adhesion, and vesicular lectin could be externalized for this purpose. The fact that the lectin is later extruded from the aggregate might mean that it is no longer needed for its intercellular function and is discarded. Alternatively, the true function of dI might be to organize material around the aggregate or in slime trails. It is also possible that dI functions in both ways. Other studies of lectin distribution have suggested that a lectin may play more than one role (1). This may be the case with an endogenous chicken lectin that undergoes marked changes in distribution in developing muscle (2) and is also found at various locations in other tissues (6, 8).

The localization of dII differs markedly from that of dI. It is first detected by faint diffuse staining in aggregating cells which may simultaneously contain some dI. However, the cells in which dII becomes prominent are devoid of dI. In early aggregates dII is transiently concentrated near the periphery of the cells, either inside or outside of the plasma membrane, and does not show the punctate distribution that will shortly become prominent. Were it external at this stage of development, it could play a role in organizing cellular interactions, but its precise cellular localization cannot be determined from these studies. Later, it shows a punctate intracellular distribution

Streams

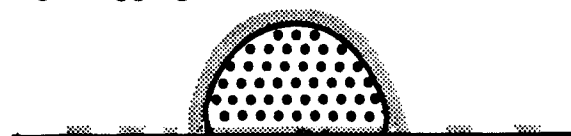### Early Aggregates

Tight Aggregates

FIGURE 6 Schematic summary of the predominant distribution of dI (stippled) and dII (large dots) during aggregate formation.

frequently coincident with anti-sp staining. Although association of these two antigens with pre-spore vesicles seems clear, they do not stay together. Some dII may be transiently present on the surface of maturing spore cells but the lectin is absent from mature spores. In contrast, antigens staining with anti-sp are prominent on the surface of developing and mature spore cells. Since anti-sp stains many substances it is possible that some are lost from the spores along with dII.

One interpretation of these results is that dII is externalized along with glycoconjugates which form the spore coat. Since it is not found on mature spores it is probably shed or degraded. The finding that polysaccharides from fruiting bodies bind well to dII (unpublished) is consistent with the hypothesis that dII is associated with them in pre-spore vesicles and is secreted with them. A similar interaction of an endogenous lectin with large glycoconjugates in secretory vesicles and luminal mucin of chicken intestine has been proposed (7).

Although these studies have not identified the function of the discoidins, they support the proposal that endogenous lectins are involved in externalization of glycoconjugates and/ or their subsequent extracellular organization (3). This seems reasonable since glycoconjugates, presumed to be the native ligands for lectins, are concentrated on cell surfaces and extracellularly.

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