Discoidin I, an Endogenous Lectin, Is Externalized from *Dictyostelium discoideum* in Multilamellar Bodies

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ABSTRACT Discoidin I, a soluble lectin synthesized by aggregating *Dictyostelium discoideum* and implicated in their adhesion to the substratum, is localized in multilamellar bodies both intracellularly and upon externalization. These structures also contain a glycoconjugate that binds discoidin I. The multilamellar bodies apparently serve to package the lectin for externalization, and may then gradually release it to function extracellularly.

As the cellular slime mold *Dictyostelium discoideum* differentiates from a vegetative to an aggregating stage, it synthesizes discoidin I, a lectin that binds glycoconjugates that contain *N*-acetylgalactosamine or galactose (1, 2). The lectin has recently been implicated in cell–substratum adhesion (3) by a mechanism like that used in fibronectin-mediated adhesion in higher organisms. The sequence arg-gly-asp-his found in discoidin I (4) is apparently responsible for its binding to the cell surface of *D. discoideum* in the same way that the sequence arg-gly-asp-ser binds fibronectin to fibroblasts (5). Another domain of discoidin I contains its carbohydrate binding site. This site apparently binds the lectin to extracellular materials, since discoidin I (6) and a complementary glycoconjugate (7) have been immunohistochemically located in a matrix around aggregating cells.

Three genes that direct the synthesis of variants of discoidin I have been identified (4). Based on the amino acid composition inferred from the nucleotide sequence of these genes, no form of discoidin I contains a terminal signal peptide (4), which suggests that it is synthesized as a soluble cytoplasmic protein. Given these findings, we sought to determine how discoidin I leaves the cell. We show here that discoidin I and a complementary glycoconjugate ligand are concentrated intracellularly in multilamellar bodies and are externalized together in this structure. The released materials may participate in the construction of an extracellular matrix.

MATERIALS AND METHODS

Growth and Differentiation of D. discoideum: *D. discoideum* strain NC-4 was grown on a lawn of *Klebsiella pneumoniae* on agar until bacteria were depleted and the cells showed signs of aggregation. At this stage, the cells have begun to synthesize discoidin I. Amebae were scraped from the agar and harvested by centrifugation at 200 *g* for 5 min in cold glass-distilled water. The pellet was washed three times in 10 vol of cold water, then resuspended to 2×10^8 cells/ml. 1 ml of this suspension was spread on a layer of 2% noble agar (Difco Laboratories, Inc., Detroit, MI) in glass-distilled water in a 100 × 20-mm petri dish and kept uncovered. The amebae were allowed to differentiate further at room temperature for 2 h before fixation.

Antibodies and Immunological Procedures: Antiserum was raised against highly purified discoidin I and was affinity-purified on a column of immobilized lectin (6). All immunohistochemistry was done with affinity-purified antibody that had been cross-absorbed on immobilized discoidin II, as described previously (7). For controls, we used identical concentrations of either preimmune IgG or affinity-purified antibody raised against discoidin II that had been absorbed on a column of discoidin I to remove any cross-reactive immunoglobulin.

Polyacrylamide gel electrophoresis and immunoblotting with peroxidase-conjugated second antibody were performed as described previously (7). Quantitative immunoassay for discoidin I was also described previously (7). All assays were conducted in detergent and galactose (7) to disrupt membranes and ensure solubilization of the lectin.

FIXATION AND EMBEDDING: We harvested the amebae by rinsing the surface of the noble agar plates with 5 vol of fixative solution. The cells were fixed for 1 h at room temperature in freshly prepared 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4. After fixation, cells were collected by centrifugation and transferred to 1.8-ml microfuge tubes for further processing. The cells were then washed for 10 min in buffer alone and postfixed for 1 h at room temperature in 1% OsO_4 in 0.1 M sodium phosphate pH 7.4. After another 10-min buffer wash, samples were dehydrated in ethanol and embedded in Epon 812 by standard procedures. In pilot experiments, several other primary fixatives were tested to determine the best protocol for immunohistochemistry. Some samples were fixed for 1 h at room temperature in periodate-lysine-paraformaldehyde fixative solutions (8) that contained 2% paraformaldehyde and either 0.05 or 0.1 M sodium-*m*-periodate, since we previously found that such fixatives were useful for fluorescence immunohistochemical localization of discoidin I (6). We also evaluated fixation for 1 h at room temperature in 3% paraformaldehyde, 0.1% glutaraldehyde, in 0.1 M sodium phosphate buffer pH 7.4. We reduced the glutaraldehyde concentration in an attempt to increase preservation of the antigenicity of discoidin I, which is diminished by this fixative (7). These alternative procedures were tested both with and without postfixation with 1% OsO_4, but were rejected because of poorer preservation of morphology, weaker staining with antibody, or both.

PREPARATION OF COLLOIDAL GOLD–IgG COMPLEXES: Colloidal gold–IgG complexes were prepared as described previously (9). Colloidal gold with a mean diameter of 4 nm was generously provided by C. M. Chang (Scripps Clinic and Research Foundation, La Jolla, CA). Colloidal gold with a mean diameter of 9 nm was purchased from Janssen Pharmaceutical Inc., Life Sciences Products Division (Piscataway, NJ).

PREPARATION OF DISCOIDIN I–IgG COMPLEXES: Highly purified discoidin I, free of discoidin II, was prepared as described previously (10) and conjugated to colloidal gold by a modification of the procedure of Roth (11). To remove the salts in which it is normally stored, which interfere with conjugation, we dialyzed 2 ml discoidin I (25 μg/ml) overnight against 1 liter of water that contained 50 mM galactose and 1% ethylene glycol. The latter ingredients were added to preserve lectin activity and keep it from forming aggregates (10). They did not interfere with the conjugation. The pH of the 9-nm coloidal gold sol was adjusted to 7.0 with 2% KCO_3, and 1 ml was added dropwise with stirring to the dialyzed lectin solution. After 5 min, 1.0 ml of 1% aqueous polyethylene glycol (20,000 mol wt) was added, and the suspension was diluted to 9 ml with 50 mM Tris, 150 mM NaCl pH 7.6 that contained 1% bovine serum albumin (BSA) (RIA grade; Sigma Chemical Co., St. Louis, MO) and 1 mM $CaCl_2$. The discoidin I–gold complex was then sedimented by centrifugation at 60,000 *g* for 20 min. The supernatant was carefully removed, and the sedimented lectin–gold complex was collected and resuspended in 1.0 ml Tris buffer that contained 1% albumin and 1 mM $CaCl_2$, then used for staining.

STAINING AND MICROSCOPY: Thin sections of amebae were cut and picked up on either uncoated or carbon–parlodian coated nickel grids. All solutions were dispensed from syringes fitted with 0.22 μm Millex filters (Millipore Corp., Bedford, MA) immediately before use.

For locating discoidin I, grids that contained thin sections were floated on drops of affinity-purified cross-absorbed antibody by using dilutions that ranged from 5–50 μg/ml in albumin-containing buffer. The buffer used was 50 mM Tris HCl, 150 mM NaCl, pH 7.6, with or without 0.3% BSA (RIA grade; Sigma Chemical Co.), as indicated, for 2 h at room temperature. Control grids were incubated with either an equal concentration of affinity-purified antibody to discoidin II that had been cross-absorbed with discoidin I, or with an equal concentration of normal IgG. Samples were washed three times for 20 min each in albumin-containing buffer, then transferred to drops of colloidal gold–conjugated goat anti-rabbit IgG for 2 h at room temperature. Grids were then rinsed twice for 10 min in albumin-containing buffer, twice for 10 min in buffer, and twice for 5 min in glass-distilled water. All sections were then counterstained with saturated aqueous uranyl acetate, then by lead citrate before they were examined under a Zeiss EM 10 electron microscope (Carl Zeiss, Inc., Thornwood, NY).

To locate glycoconjugate binding sites for discoidin I, we diluted the discoidin I–gold conjugate with an equal volume of either albumin containing buffer, 0.6 M *N*-acetylglucosamine in albumin-containing buffer, or 0.6 M *N*-acetylgalactosamine in albumin-containing buffer. In preliminary experiments, we found that the staining pattern was the same with or without *N*-acetylglucosamine. Therefore, in subsequent experiments the only comparisons made were between samples in *N*-acetylglucosamine (which did not alter binding) or *N*-acetylgalactosamine (which markedly inhibited binding). After grids were incubated for 2–3 h at room temperature with the discoidin I–gold complex, they were washed twice for 20 min in albumin-containing buffer with either 0.3 M *N*-acetylglucosamine or 0.3 M *N*-acetylgalactosamine. Grids were then rinsed twice for 10 min in albumin-containing buffer, once for 5 min in buffer only, and twice for 5 min in glass-distilled water. Counterstaining and microscopy were done as with immunostaining, as described above.

PREPARATION OF MULTILAMELLAR BODIES: Extracellular multilamellar bodies were isolated from the medium of cells differentiated by gyration in nonnutrient medium for 16 h (12). 100 ml of the cell suspension was centrifuged twice at 1,800 *g* for 5 min to pellet cells and contaminating bacteria, and the supernatant was centrifuged at 10,000 *g* for 20 min to pellet multilamellar bodies. This pellet was resuspended in 8 ml of 50 mM Tris-HCl pH 7.6 in 150 M NaCl and a 1-ml aliquot was pelleted at 100,000 *g* for 1 h and fixed for electron microscopy. To evaluate the mode of association of discoidin I with these structures, we diluted other 1-ml aliquots with 1 ml of the Tris-buffered saline with or without either 0.4 M *N*-acetylgalactosamine or 0.4 M glucose. These samples were briefly sonicated in an ice bath for equal periods and centrifuged for 1 h at 100,000 *g* to separate soluble and particulate fractions that were assayed for discoidin I (7).

RESULTS

Affinity-purified anti-discoidin I bound to multilamellar bodies and other vesicular structures in sections of aggregating *D. discoideum* (Fig. 1). Very little labeling was found in the cytoplasm (Fig. 1), and there was no significant labeling in mitochondria (Fig. 1) or the nucleus. Some discoidin I-containing vesicles did not have any apparent multilamellar structure (Fig. 1). It is possible that they are continuous with multilamellar bodies, and that their multilamellar structure is not preserved. Alternatively, they may contain precursors of the multilamellar bodies, including discoidin I, which they add to these growing structures by fusing with them. Staining was highly specific since comparable concentrations of preimmune immunoglobulin or affinity-purified anti–discoidin II did not significantly stain the cells (Fig. 2).

In many instances, we found discoidin I–containing multilamellar bodies that appeared to be in the process of release (Fig. 3). Some lectin that was not associated with multilamellar bodies was also seen around such cells (Fig. 3). Such lectin might either be free or associated with other materials. Very little labeling of the cell surface was observed under these fixation conditions. This is consistent with the previous finding that only a very small fraction of discoidin I is found associated with the surface of aggregating cells (13), and that this can only be demonstrated by immunohistochemistry with the electron microscope if glutaraldehyde fixation is done for a very short period, i.e., 15–30 s (14). It is also consistent with the failure to detect cell surface discoidin I after more prolonged fixation (15) like that used here, which is necessary to preserve intracellular morphology. Certainly, only a minor portion of externalized discoidin I binds to the cell surface, at least initially, since large amounts of the lectin remain associated with multilamellar bodies even after they have been released (Fig. 4).

Since multilamellar bodies appear to originate from vesicles that have a digestive function (16), we sought to determine if the discoidin I they contain is degraded. This seemed unlikely since most of the lectin in differentiating cells is in these structures, and it can be readily isolated in active form and high yield. Nevertheless, to directly evaluate this, we purified multilamellar bodies released from *D. discoideum* by differential centrifugation. These preparations had very little contamination either with bacteria or with other vesicular structures (Fig. 4). On immunoblots, after polyacrylamide gel electrophoresis of this preparation, one major band stained significantly with affinity-purified anti–discoidin I, and it migrated exactly with authentic discoidin I (Fig. 5), which indicates that the lectin was undegraded. Immunoblots of the bacteria on which the cells had been grown were negative (not shown).

We also assessed the activity of the carbohydrate-binding site of discoidin I in the externalized multilamellar bodies. To solubilize the lectin from bacterially grown cells, it is necessary to vigorously disrupt them in a medium that contains a sugar to which discoidin I binds, since its carbohydrate binding site is apparently occupied by a complementary membrane-associated ligand, which renders the lectin particulate. If the carbohydrate binding site of the lectin were still intact and bound to particulate ligand in extracellular multilamellar bodies, solubilization from these structures would also be expected to require an appropriate sugar. We found that solubilization of lectin from extracellular multilamellar bodies did, indeed, require disruption in the presence of *N*-acetylgalactosamine, to which it binds. After brief sonication, ~70% of the lectin was solubilized in the presence of *N*-acetylgalactosamine, whereas only 5% was solubilized if glucose, a nonbinding sugar, was substituted (Table I). Therefore, the dis-

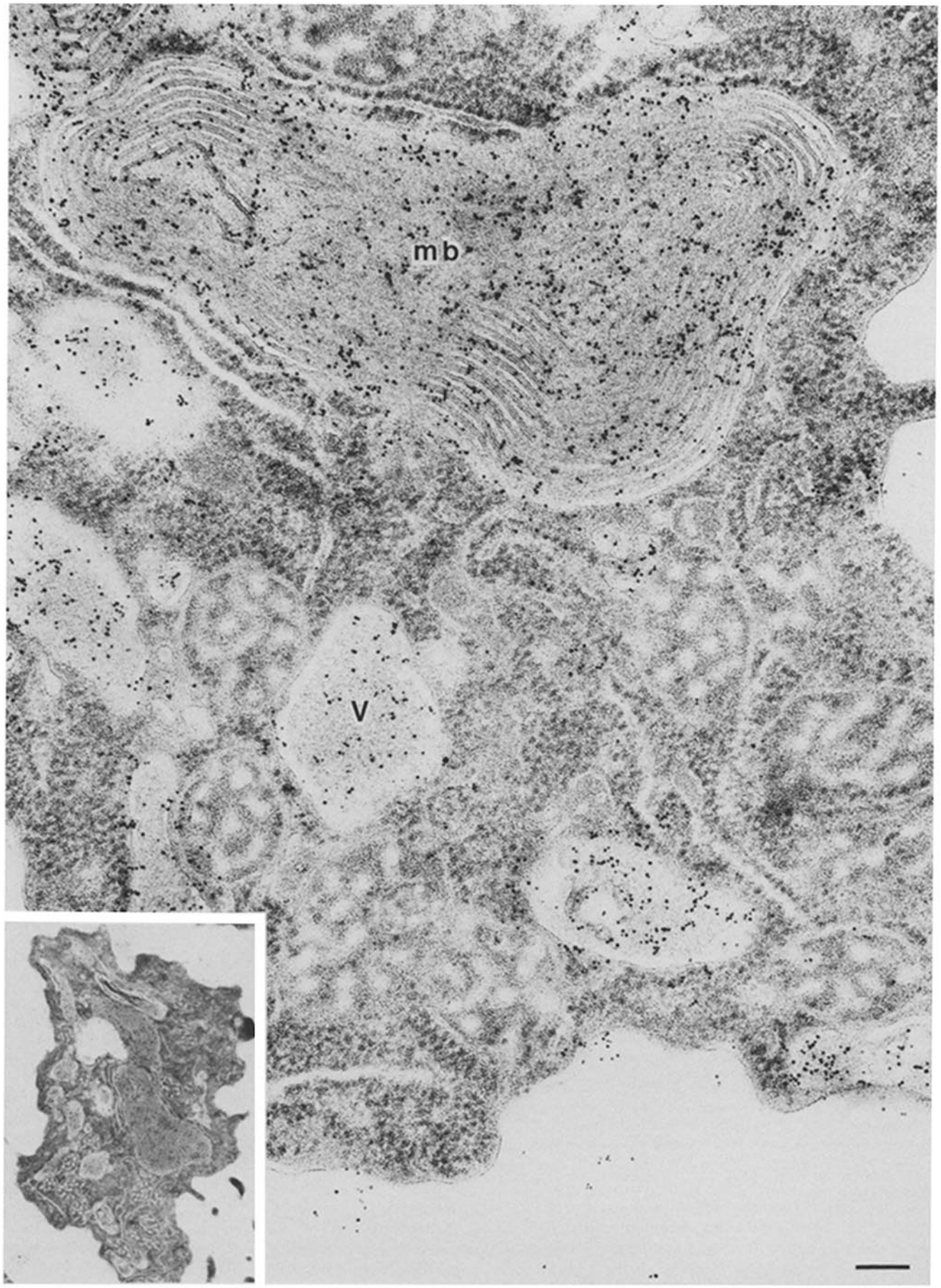

FIGURE 1 Immunohistochemical localization of discoidin I in differentiating *D. discoideum* cells. Cells were differentiated, fixed, embedded in Epon, then stained with affinity-purified rabbit anti-discoidin I and goat anti-rabbit IgG complexed with 4-nm colloidal gold as described in Materials and Methods. The inset is a lower power view to assist in orientation. (*mb*) Multilamellar body; (*V*) vesicle. Bar, 0.1 μm. Inset, × 16,000; main figure, × 105,000.

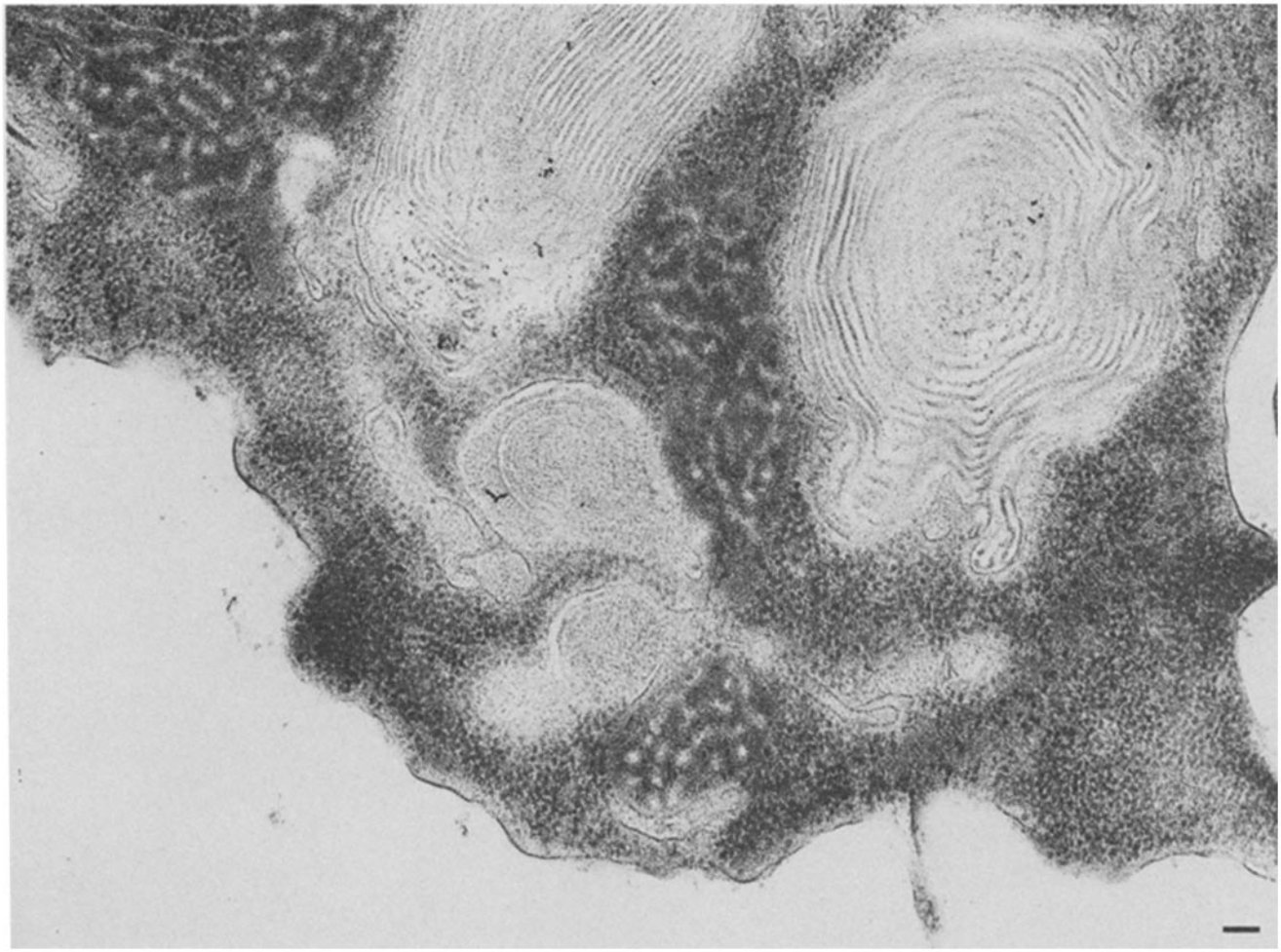

FIGURE 2 Absence of immunohistochemical staining with control antibody. All conditions were as in Fig. 1 except that the primary antibody was affinity-purified anti-discoidin II that had been cross absorbed with discoidin I. Bar, 0.1 μm. × 54,000.

coidin I concentrated in externalized multilamellar bodies has active carbohydrate binding sites.

Given these findings, we determined the distribution of the glycoconjugate ligand for discoidin I by staining sections of aggregating *D. discoideum* with highly purified lectin conjugated to colloidal gold. This stained intracellular vesicles and multilameller bodies (Fig. 6), and no significant ligand was detected elsewhere in the cells. In some instances, the label was concentrated at the periphery of vesicles (Fig. 6*b*), but the vast majority was found distributed throughout large multilamellar bodies (Fig. 6*a*). Binding of the discoidin I–gold complex to ligand was specific since it was completely blocked by *N*-acetylgalactosamine, with which the active site reacts (Fig. 6*c*). In contrast, there was extensive binding to ligand in the presence of *N*-acetylglucosamine (Fig. 6, *a* and *b*), with which the lectin does not interact. Binding in the presence of this inactive sugar was indistinguishable from that in other experiments (not shown) in which all sugar was omitted.

Like discoidin I, the ligand which binds it remained associated with multilamellar bodies upon release (Fig. 7). However, in cases when the extracellular multilamellar bodies had unfolded, the discoidin I and ligand within them tended to be distributed differently. Ligand appeared to be more closely associated with the membranes of the multilamellar bodies (Fig. 7*b*) than was endogenous lectin (Fig. 7*a*). This could be due to differences in the staining procedure in that ligand is visualized by direct staining with lectin conjugated to colloidal gold, whereas endogenous lectin is visualized by a two-step procedure.

DISCUSSION

These experiments show that as *D. discoideum* differentiates to an aggregating stage, the intracellular discoidin I that is synthesized becomes localized in vesicles and multilamellar bodies. It is unclear how discoidin I crosses intracellular membranes to reside in these structures, since it lacks an N-terminal signal peptide, but there are precedents for this (17). The multilamellar bodies appear to be the vehicle for release of the lectin around the aggregating cells.

Localization in multilamellar bodies was very unexpected and might be interpreted in several ways. One possibility is that formation of the multilamellar bodies, which may be critical for excretion of undigested bacterial products (16), depends on discoidin I. However, this is ruled out since vegetative *D. discoideum* that feed on bacteria also form multilamellar bodies (16), although such cells do not contain discoidin I (1). Another possibility is that the lectin really functions in the cytoplasm, where it is presumably synthesized as a soluble protein, and is ultimately excreted as a functionless waste product in the multilamellar bodies. This seems unlikely since virtually all the lectin is found in multilamellar

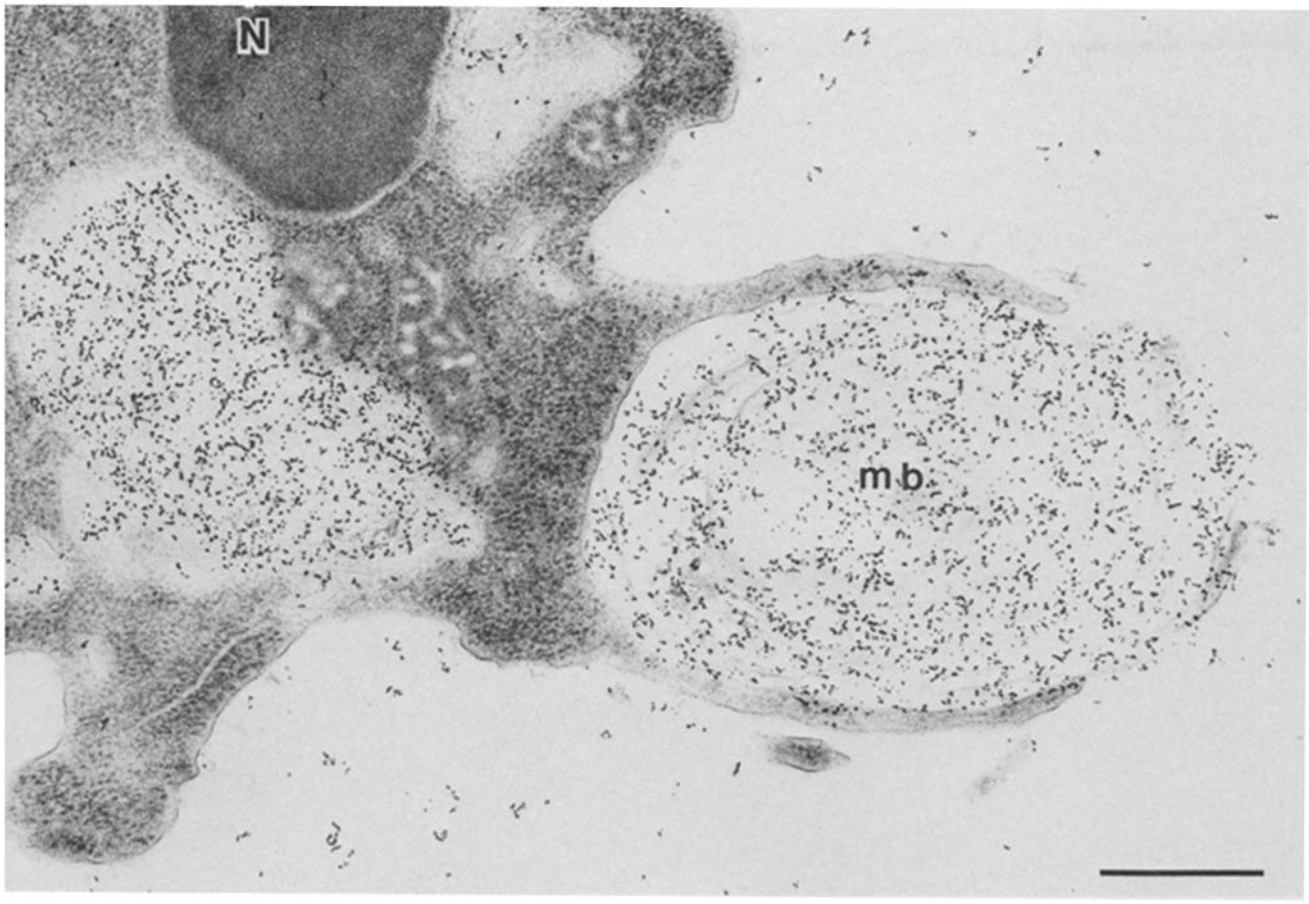

FIGURE 3 Immunohistochemical staining of a differentiating *D. discoideum* cell apparently in the process of releasing a multilamellar body (*mb*). The extracellular accumulations of colloidal gold represent specific anti-discoidin I staining and were not observed with control antibody as in Fig. 2. (*N*) Nucleus. Colloidal gold, 4 nm. Bar, 0.5 μm. × 45,000.

bodies, even at a developmental stage when it is being actively synthesized, and very little if any is detectable in the cytoplasm. Furthermore, the lectin is not degraded in the multilamellar bodies even when they are externalized. Therefore, the most likely explanation is that the multilamellar bodies, which originated as excretory structures, have been adapted to transport functional discoidin I from the cell to the surrounding extracellular materials. This interpretation is supported by evidence that the released discoidin I plays a role in cell–substratum adhesion (3) and is consistent with studies with vertebrate lectins, which also suggest extracellular functions (18). Since the evidence for a specific extracellular role for discoidin I is fairly convincing (3), externalization of the lectin by multilamellar bodies must be biologically significant.

The advantage of externalization by this mechanism is not clear. One possibility is that these externalized structures act as a repository for the lectin, gradually releasing it to guide the migration of other cells. This would be consistent with the finding that lectin plays a role in ordered cell migration of *D. discoideum* (3). These structures might also function to bring discoidin I together with a complementary glycoconjugate ligand. The latter may have been made by the cell or derived from bacterial polysaccharides that are known to interact with discoidin I (10). Whatever the origin of the ligand, its co-localization with discoidin I in multilamellar bodies suggests that their interaction is functionally significant rather than a trivial coincidence. It remains to be determined if this interaction is important merely to release discoidin I in a bound form or if it persists upon disintegration of the multilamellar body.

This specific process of externalization is not, however, indispensable for further development of this organism since a mutant, AX3, which can grow on soluble nutrients without bacteria, does not make multilamellar bodies (19), but still externalizes the lectin into the matrix around aggregates (7). The use of multilamellar bodies to bring discoidin I to the extracellular environment may, therefore, have proved expedient for these organisms which normally eat bacteria, but can apparently be accomplished by another mechanism. The alternative mechanism used by the axenically grown mutant may, however, be disadvantageous. It may, for example, result in a subtle abnormality like that recently detected (3) with a discoidin I–deficient mutant which appeared normal to more casual inspection (20).

Externalization of biologically significant extracellular materials via multilamellar bodies has precedents. In the lung, a surfactant is externalized from type II cells in multilamellar bodies and spreads out to form a film over the alveolar surface (21). In skin, multilamellar bodies are extruded into the intercellular spaces where the released materials play a role in the construction of a barrier that limits water penetration (22, 23). It may also be relevant that the extracellular materials on which a terrestrial slug migrates are released in membrane-bound vesicles and apparently contain a lectin (24).

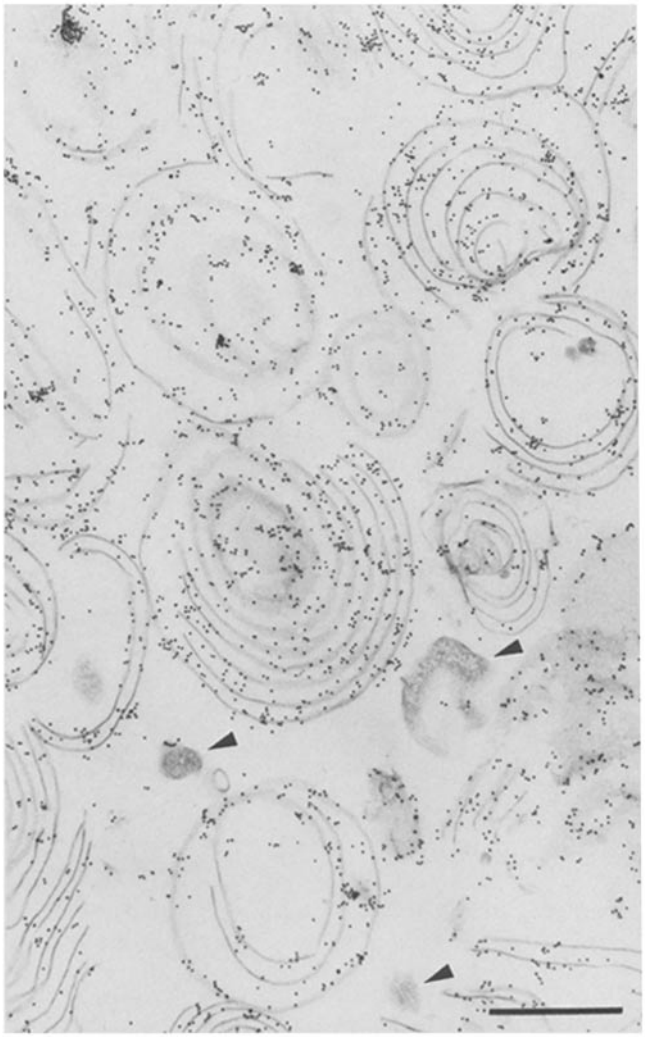

FIGURE 4 Immunohistochemical staining of multilamellar bodies released by *D. discoideum* differentiating in liquid suspension. The multilamellar bodies were purified by differential centrifugation as described in Materials and Methods. The fraction shows slight contamination with small membrane-bound particles containing cytoplasm (arrowheads) which may be pinched off filopodia. Colloidal gold, 9 nm. Bar, 0.5 μm. × 37,000.

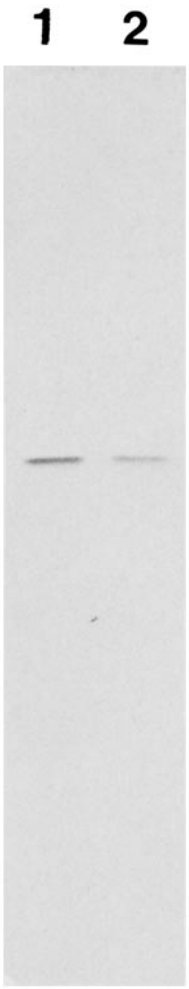

FIGURE 5 Immunoblots of multilamellar bodies and pure discoidin I. An aliquot of the preparation shown in Fig. 4 was solubilized, electrophoresed, and immunoblotted (lane *1*), along with pure discoidin I (lane *2*), as described in Materials and Methods.

TABLE I

Extraction of Discoidin I from Multilamellar Bodies Is Dependent on the Hapten Sugar, N-acetylgalactosamine

Addition to medium	Discoidin I		
	Soluble	Particulate	% Soluble
	μg		
None	0.1	1.9	5
0.2 M Glucose	0.1	1.9	5
0.2 M *N*-acetylgalactosamine	1.4	0.6	70

Extracellular multilamellar bodies purified as described in Materials and Methods were briefly sonicated in buffer with and without 0.2 M glucose or 0.2 M *N*-acetylgalactosamine. The soluble and particulate fractions were then treated with detergent to assure solubilization of discoidin I, and the content of the lectin in each fraction was determined by immunoassay (7).

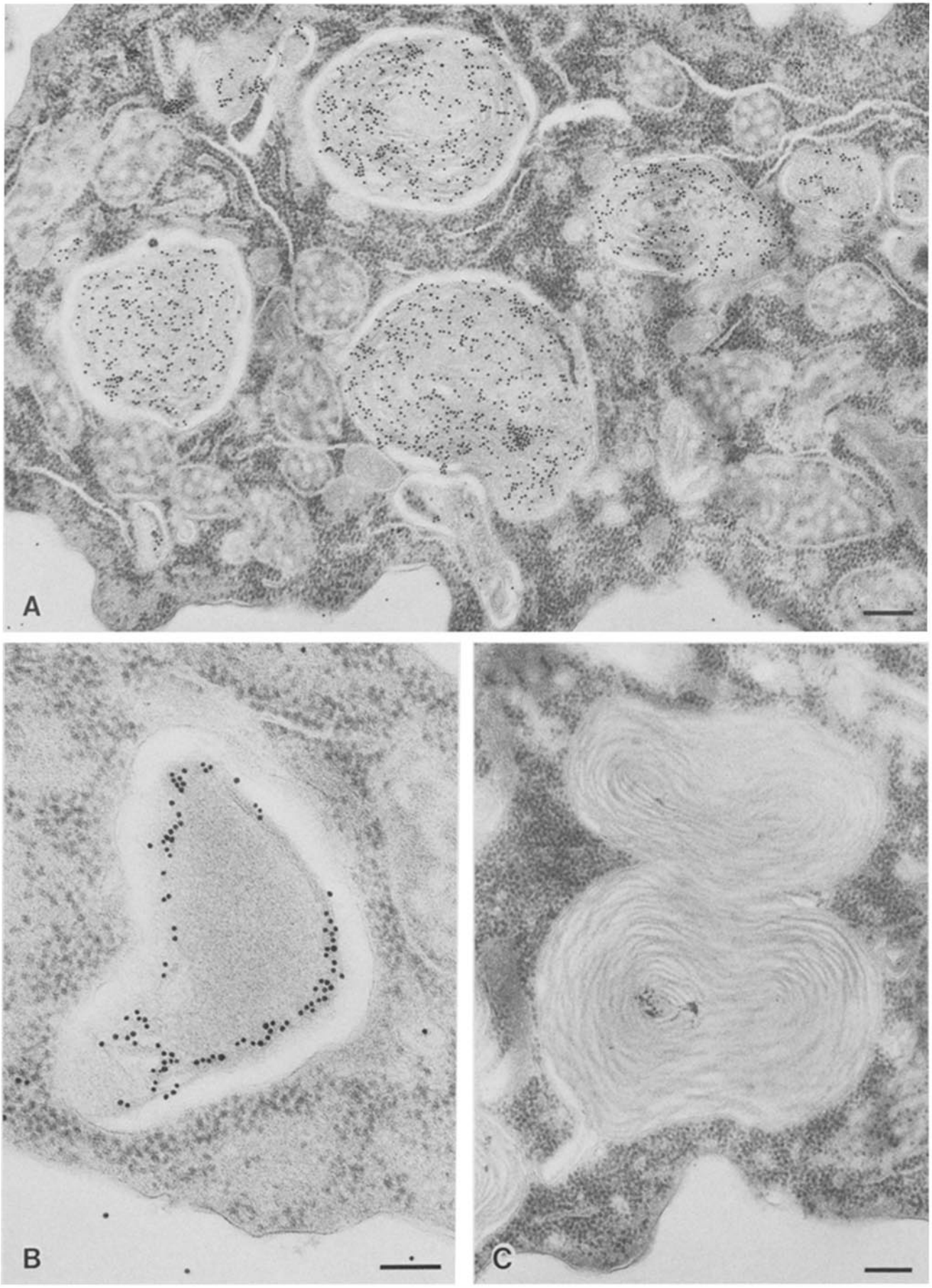

FIGURE 6 Discoidin I–colloidal gold binding to sections of differentiating *D. discoideum*. Binding was done as described in Materials and Methods in the presence of *N*-acetylglucosamine (*A* and *B*) which does not interact with discoidin I or *N*-acetylgalactosamine (*C*), the monosaccharide that binds best to the lectin. Most of the discoidin I ligand was located in multilamellar bodies (*A*), but vesicles like that in (*B*) were also frequently seen. Bar: (*A*) 0.5 μm; (*B*) 0.1 μm; (*C*) 0.5 μm. (*A*) × 45,000; (*B*) × 114,000; (*C*) × 44,000.

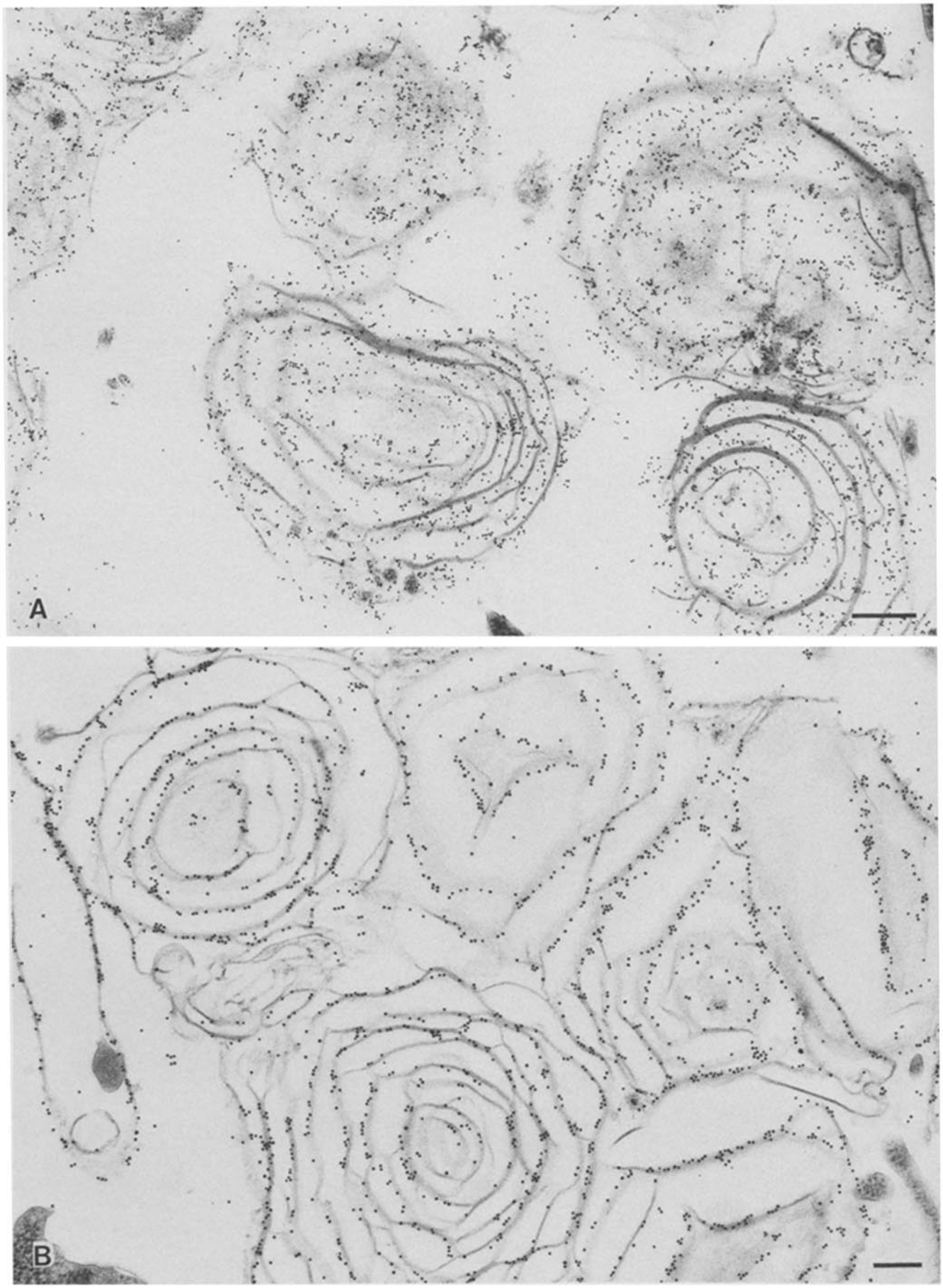

FIGURE 7 Staining of multilamellar bodies released from *D. discoideum* cells differentiating on an agar surface with (*A*) anti-discoidin I or (*B*) discoidin I-gold. Cells and released materials were fixed on agar, embedded in Epon, and an area rich in released multilamellar bodies was examined. Colloidal gold: (*A*) 4 nm; (*B*) 9 nm. Bar, 0.5 μm. (*A*) × 60,000, (*B*) × 44,000.

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