PURIFIED LECTIN FROM SKELETAL MUSCLE INHIBITS

MYOTUBE FORMATION IN VITRO

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ABSTRACT

A lactose-extractable lectin obtained from 14–16-d embryonic chick pectoral muscle and myotube muscle cultures by affinity chromatography inhibited myotube formation in culture. When applied to muscle cultures at 0.09 μg/ml, the purified lectin produced variable effects on the inhibition of myotube formation related to the time and length of application, suggesting that components of the culture medium and/or temperature produced inactivation. Hemagglutination assays showed that the lectin was inactivated by horse serum and by chick embryo extract but not by L-15 salt solution at 4°C. Incubation in L-15 solution at 37°C with or without 2 mM dithiothreitol resulted in inactivation in 2–3 h. To maximize the effect of the lectin on the inhibition of myotube formation, primary muscle cultures were grown in low $[Ca^{+2}]$ medium to inhibit fusion, and then $[Ca^{+2}]$ was increased to elicit fusion in the absence and presence of lectin with solution renewal every 2 h. Without lectin, myotube formation was normal, whereas, with lectin, it was inhibited by 93%. Continued incubation at 37°C without renewal of lectin resulted in myotube formation, suggesting reversibility by lectin inactivation.

It has been postulated that embryonic cells employ the appearance and/or disappearance of surface constituents during their development for specific tissue recognition (17, 24, 29). Evidence for the appearance of such factors has been obtained for retinal cells and, more recently, for retinal cell membranes that show specificity for type, developmental stage, and absence of recognition factors in older embryos (8, 9). Multivalent glycoproteins that promote species-specific sponge cell aggregation (10) and molecules that appear to be the receptors for these ligands on the cell surfaces (28) have been isolated in pure form. Simpson et al. (25) have characterized and purified a galactose-binding protein, discoidin, from the slime mold *Dictyostelium discoideum* in which the biosynthesis closely parallels the development of cell cohesiveness. Species-specificity is suggested because an-

other slime mold, *Polysphondylium pallidum*, also contains a developmentally regulated cell surface lectin that reacts with species-specific cell surface oligosaccharides during cellular cohesion (26).

A β-D-galactoside-specific lectin occurs in embryonic chick pectoral muscle and skeletal muscle cultures (4, 7, 19, 27). The highest concentration appears between 8 and 16 d of development (19), which is also the period of highest myoblast fusion, suggesting a possible correlation between the lectin and the recognition-fusion process. The hemagglutinating activity of this lectin is inhibited by lactose and thiodigalactoside. Primary cultures of chicken (6) and rat skeletal (4) muscle are unaffected by thiodigalactoside; however, the rat muscle cell lines L 6 (4, 7) and L 8 (4) are fusion inhibited.

The objective of this study was to determine the effect of direct application of the lectin to skeletal

muscle cultures on cell recognition-myoblast fusion. Application of crude extracts to monolayer primary cultures of chicken skeletal muscle showed a partial inhibition of myotube formation. Therefore, we purified the lectin by affinity chromatography, determined its unit molecular weight, and optimized the conditions for employing the lectin in culture to better test the effect on myotube formation. Our studies show that under conditions which minimized inactivation of the lectin, application in picomole concentrations inhibits myotube formation by >90%. While this study was in progress, Den and Malinzak (4) and Nowak et al. (20) reported the isolation of a similar lectin from chicken embryonic muscle. However, its effects on myotube formation have not been reported.

MATERIALS AND METHODS

Preparation of Purified Lectin

Extracts of chicken embryonic skeletal muscle and muscle cultures were prepared according to Nowak et al. (19). Pectoral muscles from 14–16-d embryos (20 g wet wt) were homogenized in an Omnimixer (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) in 0.15 M NaCl, 2 mM EDTA, and 2 mM dithiothreitol (DES)[1] with 0.3 M lactose, pH 8.0, at top speed for 1 min at 0°C and centrifuged for 1 h at 100,000 g. The supernate was dialyzed two times against 1 liter of DES without lactose for 12 h at 4°C, then two times against phosphate-buffered saline (0.15 M NaCl and 5 mM NaPO₄, pH 8.0 [PBS]) plus 2 mM dithiothreitol (DTT). Samples were frozen at −20°C until used. Primary myotube cultures (5-d) were rinsed three times with 4°C PBS (7 ml), scraped from the plates with a rubber policeman in DES plus 0.3 M lactose, extracted overnight at 4°C, and centrifuged at 100,000 g. The supernate was dialyzed as described for muscle extracts against DES and PBS plus DTT.

Affinity chromatography was performed as described by Baues and Gray (1) using aminoethyl Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, Calif.) coupled with lactose. Coupling was accomplished by a direct covalent attachment of reducing carbohydrates to an aminoethyl polyacrylamide gel via a reductive amination with cyanoborohydride at pH 9.1. The thawed extract was added to the column in PBS plus DTT at 4°C, and the column was washed with four times the sample volume of PBS plus DTT to remove unabsorbed protein. The lectin was eluted by passing 50 ml of PBS plus 0.3 M lactose through the column and collecting the eluate in 1-ml volumes. The first few volumes contained the lectin, as determined by absorbance at 280 nm and hemagglutination. Lactose was subsequently removed by dialysis against two changes of PBS plus DTT. Ap-

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[1] Abbreviations used in this paper: DTT, dithiothreitol; DES, 0.15 M NaCl, 2 mM EDTA, and 2 mM dithiothreitol; PBS, phosphate-buffered saline (0.15 M NaCl and 5 mM NaPO₄); BSA, bovine serum albumin; SDS-PAGE, SDS polyacrylamide gel electrophoresis; HS, horse serum; CEE, chick embryo extract; MEM, minimal essential medium.

proximately 1 mg of lectin was obtained per 20 g wet wt of dissected muscle.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the lectin's purity by the method of Kedinger et al. (14) using denaturing (1.25% SDS) and reducing (1.25% β-mercaptoethanol) conditions with a polyacrylamide gradient of 5–17.5%. Electrophoresis was run for 8 h at 100 V using 3 μg of purified lectin. Molecular weight was determined by passing lectin over a Sephadex G-75 superfine chromatography column using cytochrome c (12,400 daltons), ovalbumin (45,000 daltons), and bovine serum albumin (BSA) (67,000 daltons) as standards.

Assay for Lectin Activity

The hemagglutination assay was modified from that of Lis and Sharon (16). Human blood (type B positive) erythrocytes were prepared fresh for each assay by washing three times with cold PBS, trypsinizing for 1 h at 37°C with a 0.05% trypsin solution containing Eagle's minimal essential medium (MEM) with Spinner's salts, washing three more times with cold PBS, and making a stock 1% (vol/vol) suspension in PBS. The assay was performed by serial twofold dilutions of the lectin with PBS. The dilutions were mixed with an equal volume of the erythrocyte suspension in a microtiter plate with V-shaped wells (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The cells were allowed to settle for 1–2 h, and the titer was determined from the highest dilution showing aggregation. Specific activity was expressed as the titer⁻¹/mg of protein. Protein determinations were done by the method of Bradford (2) using graded concentrations of BSA as standards.

Skeletal Muscle Cultures

Primary breast muscle cultures from 12-d White Leghorn chicken embryos obtained from the Case Western Reserve University farm were prepared and grown as described by Sandra et al. (22) in 2.5 ml of L-15 medium supplemented with 15% horse serum (HS), 5% chick embryo extract (CEE), and 1% antibiotic-antimycotic (Grand Island Biological Co., Grand Island, N. Y.). To obtain cultures in which myoblast fusion was suppressed, [Ca⁺²] was decreased by dialyzing L-15 medium and HS against Eagle's MEM with Spinner's salts (23) (CEE was not dialyzed to reduce [Ca⁺²]). Cultures were plated for the first 24 h in fully supplemented medium, and then the medium was changed to the low [Ca⁺²] medium. At the time of testing, lectin was added with supplemented medium to promote myoblast fusion by the increased [Ca⁺²]. Identical cultures with no lectin were maintained, and the medium from these cultures was used as conditioned medium for medium changes.

Effect of Lectin on Myotube Formation

Lectin was added directly as a concentrated solution in a small volume (0.25 ml) of PBS to standard cultures or to cultures that previously had been grown in low [Ca⁺²] medium to yield a final desired concentration in the medium. Myotube formation was determined by light microscopy after the cultures had been fixed with cold 5% TCA and stained with 0.25% azure B in 0.05 M phosphate buffer at pH 6.5. Myotubes were defined as cells containing three or more nuclei in which the cytoplasmic staining in the adjacent cytoplasm was homogeneous in intensity and distribution. The total number of nuclei was determined in both mononucleated and multinucleated cells, and the number of nuclei in myotubes was expressed as a percentage of the total nuclei in the field. Because the total cell number was obtained,

the detrimental effects of the experimental conditions on cell viability and proliferation could be assessed. Twelve random fields per plate were counted to represent the culture dish. This area of 1.9 mm² per plate was determined in independent studies to accurately represent values obtained from assessing larger areas of the plate. 400–1,000 nuclei were counted per experimental point in duplicate or triplicate samples. Each experiment was done two or three times.

RESULTS

Lectin was purified by homogenization and centrifugation to obtain a postmicrosomal supernate that yielded purified lectin by a single passage through the aminoethyl Bio-gel P-150 coupled with lactose (Fig. 1). Before it was applied to the column, the supernate had a specific activity of ~100. Passage through the affinity column and subsequent elution with PBS showed by hemagglutination that the active component was retained by the column. After the column had been rinsed with PBS (four times the sample buffer), elution with DES plus 0.3 mM lactose released a bound protein that appeared in the first few volumes after the void volume. This material had a specific activity of ~110,000, yielding >1,000-fold purification.

Characterization of the purified protein included a determination of its purity by SDS-PAGE and of its molecular weight by Sephadex chromatography. Analysis of the lactose-eluted

fractions with the highest specific activity obtained from the affinity column produced a single, discrete, Coomassie Blue-stained band positioned between the markers cytochrome c and myoglobin. It had an estimated subunit molecular weight of 15,000. SDS-PAGE of the crude extract produced many stained bands, but, importantly, one of ~15,000 daltons that comigrated with the purified lectin was present. After Sephadex G-75, a 15,000-dalton subunit and a larger dimer fraction at 30,000 daltons were shown by hemagglutination activity.

Crude embryonic muscle extract in PBS, with a specific activity of 100, was applied to muscle cultures in conditioned medium as single and as multiple daily treatments (Fig. 2). Application of the extraction buffer and changes of the medium had no noticeable effect on myotube formation. A single treatment with crude extract (2.6 mg protein/ml; titer⁻¹ of 256) at the onset of culture partially inhibited myotube formation on days 4 and 5 of culture, but the extent of myotube formation on day 6 was equivalent to that of untreated cultures. Sequential daily treatments with crude extract partially inhibited myotube formation during the entire culture period. Cell numbers at all points tested were equal in untreated and treated cultures. A similar experiment was performed with a crude extract of day-5 cultured
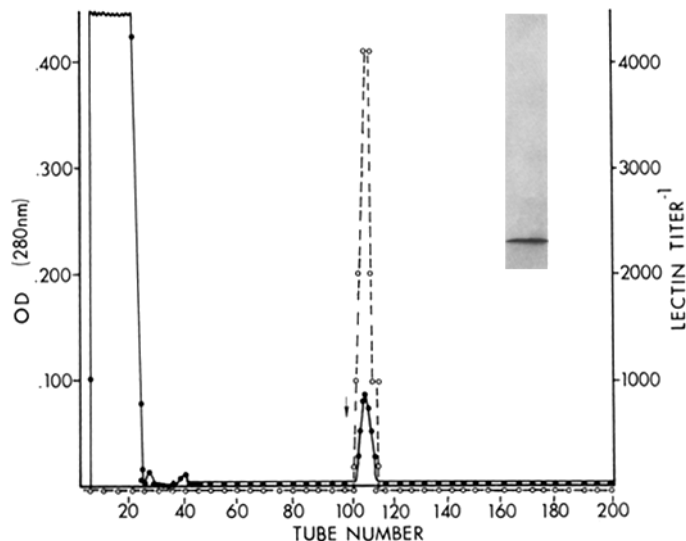

FIGURE 1 Affinity chromatography of embryonic muscle lectin on aminoethyl Bio-Gel P-150 coupled with lactose by reductive amination. The sample was applied at $t = 0$ and washed with PBS. At the point indicated by the arrow (tube 100), PBS plus 0.3 M lactose was added. Absorbance at 280 nm is indicated by (●), and the titer of lectin activity determined by hemagglutination assay is indicated by (○). (*Insert*) SDS-PAGE analysis of purified lectin (3 µg) on a 5–17.5% gradient gel stained with Coomassie Blue.
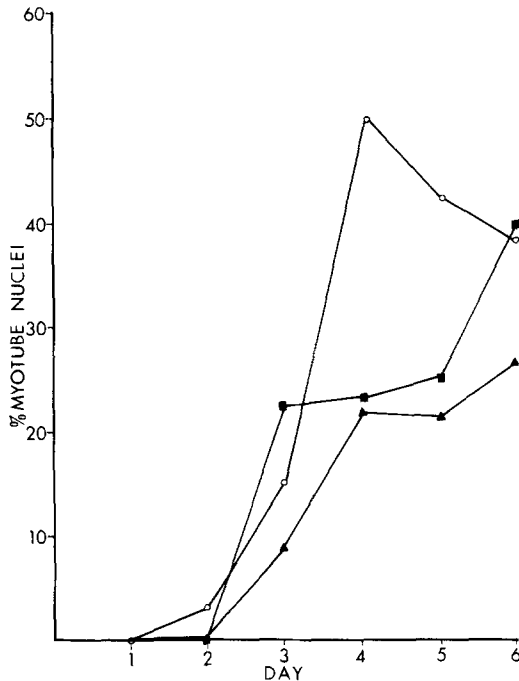
FIGURE 2   The effect of the 100,000-g supernate from 14-16-d embryonic muscle dialyzed against DES plus DTT without lactose followed by PBS plus DTT on myotube formation at various times in culture. The crude extract with a hemagglutination titer^{-1} of 16 was added to the supplemented L-15 medium. ○, Control untreated cultures. ■, Culture receiving a single addition on day 1 of incubation. ▲, Culture receiving daily additions of lectin with fresh conditioned medium appropriate for the time in culture.

muscle (Fig. 3). Inhibition of myotube formation was minimal after a single addition of crude extract and was most pronounced after multiple daily additions (the effect was assessed on days 3 and 4). No effect was found on days 5 and 6.

Purified lectin obtained from the affinity column was tested for its effect on myotube formation to show a relationship between the amount of inhibition and the concentration of the lectin applied. In early experiments, the purified lectin was added to cultures in supplemented conditioned medium. Under these conditions, in contrast to the crude extract, purified lectin had no effect on myotube formation. Consequently, conditions were modified. Purified lectin was diluted with PBS instead of conditioned medium before it was added to cultures containing supplemented medium. Muscle cultures were initially grown with decreased [Ca^{+2}]. To test its effect on the inhibition of fusion calcium was added without lectin or with

increasing concentrations of lectin to promote myoblast fusion. Control cultures containing the normal [Ca^{+2}] showed maximal myotube formation on day 3 of culture. At this time, calcium deprivation resulted in essentially no myotube formation. Fig. 4 shows that, in the absence of lectin, elevation of [Ca^{+2}] resulted in myotube formation in the ensuing 8 h. Lectin with a specific activity of 110,000 added at a concentration of 0.0225 μg/ml (titer^{-1} of 2.5) had no effect on myotube formation. Higher concentrations of lectin, i.e., 0.045 μg/ml (titer^{-1} of 5.0) to 0.090 μg/ml (titer^{-1} of 10), inhibited myotube formation 50 to 90%, 6 h after lectin addition. 8 h after lectin was added, its effect diminished, and the amount of myotube formation approached that of untreated cultures. Addition of 0.3 M lactose with the lectin cancelled its effect.

That lectin did not effect myotube formation when it was diluted in supplemented medium suggested that inactivation was occurring that could account for the temporary inhibition of myotube formation observed in the experiments
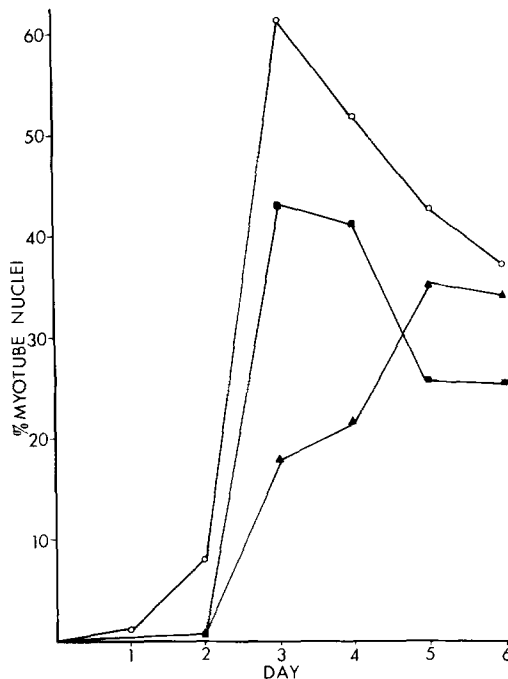

FIGURE 3   The effect of the dialyzed 100,000-g supernate obtained from day-5 myotube cultures on myotube formation. The extract, 30 μg protein/ml with a hemagglutination titer^{-1} of 16, was added to cultures grown in supplemented L-15 either as a single addition on day 1 of culture (■) or as multiple daily additions (▲) with a change of conditioned supplemented medium. ○, Untreated control cultures.
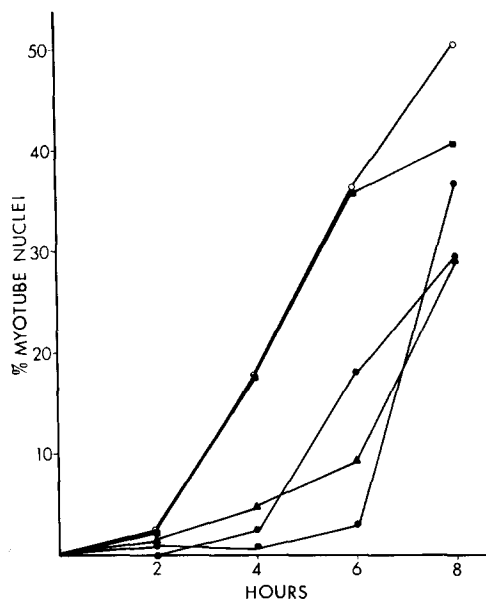
FIGURE 4 The effect of the concentration of lectin purified by affinity chromatography on myotube formation. Muscle culture were grown in L-15 medium with decreased [Ca^{+2}] for 3 d. [Ca^{+2}] was increased by a medium change with supplemented L-15 without lectin (○) or with increasing amounts of lectin. The lectin concentrations used were (■) 0.0225 µg/ml (titer^{-1} of 2.5), (●) 0.045 µg/ml (titer^{-1} of 5.0), (▲) 0.067 µg/ml (titer^{-1} of 7.5), and (●) 0.09 µg/ml (titer^{-1} of 10). The percent myotube nuclei was determined at various times after lectin and calcium were added.

described above. We tested for temperature lability and possible inactivation by components in the supplemented medium by hemagglutination assays. Table I demonstrates that the lectin in PBS at 37°C showed heat lability by 30 min when the test solution (titer^{-1} of 10) was diluted 16-fold. Lower dilutions showed heat lability by 3 h, indicating nearly complete inactivation. Tests repeated in the presence of 2 mM dithiothreitol showed no retardation of inactivation. The same test applied to purified lectin with a titer^{-1} of 10 in complete culture medium showed complete inactivation of hemagglutination at 0.5 h. Therefore, the major components of the complete medium were tested separately. The L-15 solution without additives gave results identical to tests with PBS. A solution of L-15 containing either 15% HS or 5% CEE produced inactivation of the lectin (titer^{-1} of 10) within the first 30 min. Co-addition of lectin plus 0.3 M lactose resulted in no activity. We tested lectin modification during inactivation by first exposing lectin in PBS to 37°C for various lengths

of time from 0.5 to 2.0 h as before and then subjecting the samples to 17.5% SDS-PAGE (18). No difference in banding pattern or migration was detected.

On the basis of these results, we applied lectin purified by affinity chromatography in PBS with L-15 solution (1.5 mM Ca^{+2}) to calcium-deprived cultures with and without HS and CEE to test for maximal effects of the lectin on myotube formation (Fig. 5). We minimized potential heat inactivation in the absence of HS and CEE by replenishing with fresh lectin. Fusion occurred normally and on schedule in lectin-free control cultures with HS and CEE after [Ca^{+2}] was increased. Fig. 6A and B depicts myotubes formed under these conditions. Deletion of HS and CEE produced slightly reduced myotube formation (Fig. 5). Fig. 6 C and D shows that the cells have become more elongate and that the amount of fusion is diminished but still quite evident. Although the myotubes are narrow, they still meet our criteria for the definition of myotubes. Cultures treated singly with lectin in the absence of HS and CEE showed partial inhibition of myotube formation up to 12 h after [Ca^{+2}] was elevated, but myotube formation approximated control cultures by 14 h. Cultures in which the lectin was replenished every 2 h in the presence of 1.5 mM Ca^{+2} showed myotube formation below 5% up to 12 h, with a slight increase thereafter. Fig. 6E shows no myotubes

TABLE I

Inactivation of Lectin at 37°C as Determined by Hemagglutination

Time	Dilution			
	1:2	1:4	1:8	1:16
h				
0.5	+	+	+	−
1.0	+	+	+	−
1.5	+	+	+	−
2.0	+	−	−	−
2.5	+/−	−	−	−
3.0	−	−	−	−

Lectin (titer^{-1} of 10) purified by affinity chromatography was tested for heat inactivation in PBS. At the times indicated, the lectin was diluted in PBS as shown and tested for hemagglutinating activity as described in Materials and Methods. Addition of 2 mM dithiothreitol or substitution of L-15 solution for PBS did not change the results. Addition of HS and/or CEE gave no hemagglutination at the initial time point.
+, presence of hemagglutination.
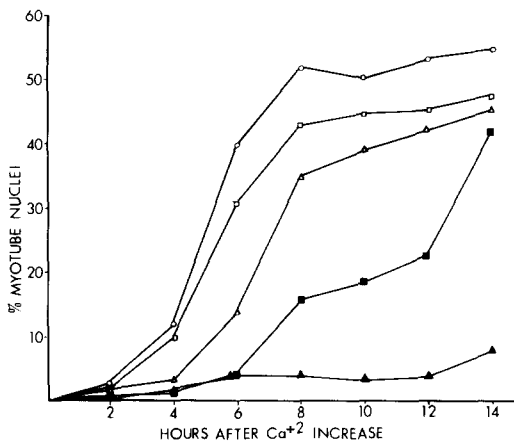−, absence of hemagglutination.

FIGURE 5 The effect of purified lectin addition and increased [Ca^{+2}] on myotube formation under various lectin-inactivating conditions. Cultures were grown for 3 d in low [Ca^{+2}] L-15 medium, rinsed with the test medium (shown at $t = 0$ on this graph), and then incubated in medium with 1.5 mM Ca^{+2} for various periods of time. The following conditions were tested: ○, Supplemented L-15 medium containing HS and CEE but no lectin with no medium changes. □, L-15 solution without HS, CEE, or lectin. Medium was not changed. △, L-15 solution without HS, CEE, or lectin. Medium was changed every 2 h. ■, L-15 solution without HS or CEE but with 0.09 μg/ml (titer^{-1} of 10) lectin. Medium was not changed. ▲, As in ■ above but with a change every 2 h to replenish lectin that may have been inactivated by incubation at 37°C.

present. Fig. 6 F shows the typical relationship of cells passing over one another or contacting one another but evidently not with continuous cytoplasm.

DISCUSSION

A lectin extracted from 14–16-d pectoral embryonic chicken muscle with DES and lactose (19) was purified using Bio-Gel P-150 coupled with lactose by reductive amination (1). This procedure usually produced about 1 mg of purified lectin from 20 g wet wt of embryonic muscle. Affinity chromatography was used similarly by Den and Malinzak (4) with an asialofetuin Sepharose column and by Nowak et al. (20) with a Sepharose 4 B coupled to α-aminophenyl-β-lactoside. Lectins isolated by these methods showed a subunit molecular weight of 15,000 on SDS-PAGE and a dimeric molecular weight of 30,000 by Sephadex G-100 gel filtration (4, 20). Furthermore, Nowak et al. (20) showed that the lectin had an isoelectric

point of 4.0. All of the purified proteins, including the ones described in this report, had hemagglutinating activity that was inhibited by lactose (4, 20). By these criteria, the proteins have similar identities and probably are similar to those reported by Teichberg et al. (27) and by Gartner and Podleski (7).

Isolation of these lectins from muscle tissue or muscle cultures of salt-sugar extraction probably involves both cytoplasmic and cell surface material. Lectin activity was reported in the particulate and soluble fractions from L 6 skeletal muscle culture homogenates (7). Immunochemistry showed that the lectin existed in cultured skeletal muscle myoblasts and myotubes both as constituents of the cell surface and diffusely spread throughout the cytoplasm (20). Although such information does not exist for embryonic muscle tissue, it is presumed that a similar distribution exists.

The presence of high concentrations of the endogenous lectin during the myoblast fusion phase in both muscle tissue and cultures suggests that it is a developmentally regulated protein, perhaps involved in the recognition and fusion process (7, 19). Den and co-workers (4, 6) concluded that there is insufficient evidence to warrant such conclusions. They treated chicken muscle cultures with various galactose sugars but were unable to inhibit myotube formation, i.e., the end product of cell recognition, adhesion, and fusion. In contrast, inhibition of myotube formation was obtained for rat muscle cell lines (4, 7). This species and/or cell line difference in response has not been resolved. Our unpublished observations show that continuous treatment of chicken muscle cultures with lactose results in retardation of myotube formation by 48 h, but the inhibition is overcome, with the final extent of fusion equivalent to untreated cultures. Cultures were treated continuously from 24 h after plating with four sugars added directly to the culture medium at 0.15 M. At 72 h, α-methyl-D-mannoside, α-methyl-D-glucoside, and α-L(−)fucose did not significantly affect fusion, as determined by visual inspection. Lactose-treated cultures contained little myotube formation; cell division was not impaired, as evidenced by the confluent cell layer. At 96 h, all experimental and control cultures were comparable with regard to the extent of myotube formation, suggesting that lactose had transiently inhibited fusion.

In this study, we show that myotube formation is reproducibly inhibited by added 15,000-dalton
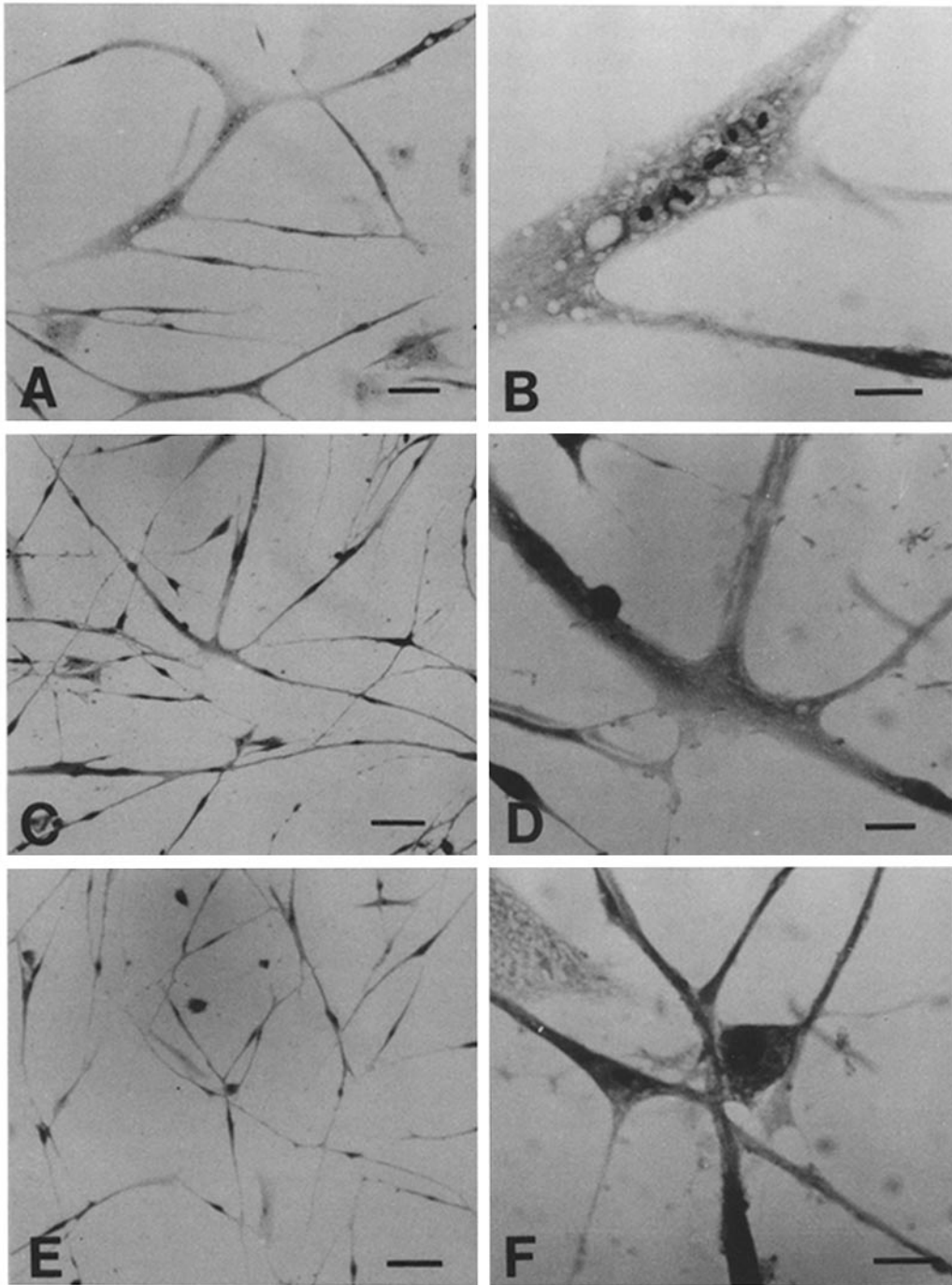
FIGURE 6 Representative areas of Ca^{+2}-deprived cultures 10 h after release from low $[Ca^{+2}]$ block. (A and B) Views of cultures receiving L-15 with HS and CEE but no lectin. Myoblasts are fused into myotubes with continuous cytoplasm. Cytoplasm also appears to be continuous for cells joined at the right. (A) × 200. (B) × 920. (C and D) Views of culture receiving only L-15 and no HS, CEE, or lectin. Myotubes are again visible, although very elongate, with nuclei far apart. Cells joining myotube from the top appear to also have cytoplasm continuous with the myotube. (C) × 200. (D) × 620. (E and F) Views of cultures receiving L-15 and lectin but no HS or CEE. Few multinucleate myotubes are visible. Cells seem to contact each other but appear to keep their cytoplasmic integrity. (E) × 200. (F) × 920. (A, C, and E) Bar, 50 μm. (B, D, and F) Bar, 10 μm.

muscle lectin. The various degrees of inhibition obtained were the result of the lectin's heat lability at 37°C and inactivation by components of HS and CEE. Under conditions which minimized inactivation by the elimination of HS and CEE and by the replenishment of potentially inactivated, purified lectin, myotube formation was reproducibly inhibited by >90% in short-term experiments. Inhibition was cancelled by co-addition of 0.3 M lactose. The specific components in HS and CEE responsible for the inactivation are unknown. Furthermore, it is unknown whether inactivation is by degradation or competitive interactions of the unknown components in HS and CEE with cell surface sites. Lectin was more unstable when added to the supplemented medium in the purified form than as a component of a crude extract, which suggests that secondary components may be associated with the lectin that serve as protective agents. It is assumed that added lectin reacts with the cell surface, resulting in incomplete incorporation and thereby leaving the lectin accessible for rapid inactivation.

Plant lectins have been shown in similar studies to interfere with myotube formation. Den et al. (5) tested the effects of concanavalin A (con A), wheat germ agglutinin, soybean agglutinin, abrin, and Lens culinaris lectin on cultures that had been calcium deprived. They found myotube formation to be inhibited 0–60%. On the basis of these observations, they suggested that the effects were the result of either binding to specific glycoproteins essential for fusion or nonspecific steric hinderance of cell contact necessary for fusion. Sandra et al. (21, 22) studied more closely the effect of con A and showed that this lectin would inhibit myotube formation under standard culture conditions, provided the cells were mildly pretrypsinized before lectin addition. This trypsin-lectin treatment resulted in long lasting effects. It produced 60% inhibition of myotube formation without crosslinking and 80% inhibition with con A and glycogen. Cell surface replicas show that normal myotube formation coincided with the dispersed state of con A binding sites on myoblast cell surfaces. Inhibition of myotube formation correlated closely with a clustered state of the con A surface receptors. On the basis of these data, they suggested that con A binding affected the membrane fluidity by cross-linking, and that it is unclear whether cell surface glycoproteins are involved in fusion, are cross-linked, or are adventitiously trapped. The effect was due more to physical interference of the

surface than to actual involvement of fusion sites. Unfused myoblasts continued to make contact at both their end processes and their lateral surfaces.

The lactose-binding lectin isolated from embryonic muscle is more effective than plant lectins in inhibiting myotube formation. The 15,000-dalton muscle lectin yielded >90% inhibition at picomole concentrations, suggesting that it has higher specificity and a greater potential for physiological effects than the plant lectins that yielded effects at millimole concentrations. Such differences may result from varied experimental conditions. In current studies, cultures are handled in the same way as that described for Fig. 5 and are treated with 15,000-dalton muscle lectin (0.09 μg/ml), con A, or peanut lectin (0.09–125 μg/ml), either as single additions without a medium change or as multiple additions with medium changes. Initial results show a decrease in myotube formation with the muscle lectin comparable to that depicted in Fig. 5. In contrast, the plant lectins did not decrease myotube formation.

We propose that the endogenous lactose-binding lectin has a natural role in muscle development. Our studies show that it does not affect cell proliferation and cell motility (unpublished time-lapse studies), and that in this way it is comparable to con A (22). In untreated skeletal muscle cultures, myoblasts align before fusion in such a way that contacts are made at the ends and lateral surfaces of the cells. In cultures in which [Ca^{+2}] is decreased to inhibit myotube formation, our studies and those of others (11) show that cell contact occurs by an elongate microspike. These initial sites of contact may result in gap junction formation, as has been shown in closely contacted myoblasts (12) and in fusing myoblast membrane preparations (3). These junctions could establish a "communication" between the cells, promoting the modification of the cell surfaces for fusion perhaps, as suggested by Kalderon and Gilula (13), via the incorporation of intracellular vesicles within the cell membrane to produce phospholipid-enriched domains free of intramembrane particles. However, regions of the cell membrane containing gap junctions would not be the sites of membrane dissolution and eventual cytoplasmic continuity between adjacent cells. Such continuity can only be achieved if the adjacent myoblasts are closely apposed. We propose that the endogenous lactose-binding lectin is involved in the close apposition of myoblasts.

We interpret the results of our studies in the

following way. Once the initial cell contact and communication is accomplished between myoblasts, a prolonged cell-cell contact is necessary to allow fusion of the respective membranes. Continued cell-cell apposition, promoted by increasing the $[Ca^{+2}]$, involves the lactose-binding lectin. When present in sufficient quantity in the cell membrane, it binds with specific lactose-containing receptors on adjacent cells and thus acts to hold the cells together. However, if exogenous lactose-binding lectin is present in active form, the adhesive sites are experimentally covered, as indicated by the minimal amount of lateral cell surface apposition observed in our studies. Inactivation of the added lectin by heat or components of HS and CEE leads to continued myotube formation. However, it appears that this occurs by the cytoplasm's becoming confluent at the initial sites of cell contact near the microspikes inasmuch as the myotubes formed are initially long and thin. This suggests that myoblast fusion can occur in discrete regions and that the added lectin is capable of temporarily inhibiting membrane apposition at these sites.

One would assume that the proposed role for the endogenous lactose-binding lectin in developing skeletal muscle requires specificity. That similar lectins are present in a variety of tissues (15) speaks against such specificity. However, we suggest that too little information currently exists to conclude that they are identical. It is not unreasonable to surmise that portions of these lectins are similar and thus provide for similar means of isolation and common antigenic determinants. The variable regions of the molecules would provide for specificity of function and, thus, for variations in distribution within tissues.

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