Externalization of an Endogenous Chicken Muscle Lectin with In Vivo Development

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ABSTRACT Chicken-lactose-lectin-I (CLL-I), an endogenous lectin that is developmentally regulated in embryonic muscle, was localized by immunohistochemical techniques in tissue samples taken at various stages of in vivo development and in primary muscle cultures. Lectin, which was diffusely distributed in myoblasts, became localized in myotubes in a distribution similar to that of the sarcoplasmic reticulum and T tubules. Later in development, lectin was predominantly extracellular. This sequence suggests that externalization may have occurred by migration in the T tubules, which are continuous with the extracellular space, although alternative explanations are possible. Only traces of lectin were found in the adult. These studies did not reveal the function of CLL-I in muscle development. However, we infer that it acts by organizing complementary glycoconjugates in the intracellular tubular network, on the muscle surface, and/or in extracellular materials.

Extracts of immature chicken muscle contain a dimeric protein that binds lactose and other β-galactosides (1–3), can be assayed as a hemagglutinin, and is referred to as a lectin. An identical protein is also found in other developing and adult chicken tissues (4–6). It is designated chicken-lactose-lectin-I (CLL-I) to distinguish it from another chicken lactose binding lectin (5) designated chicken-lactose lectin-II (CLL-II).

CLL-I levels in developing chicken pectoral muscle undergo striking changes with development (2, 6). Levels are very low in extracts of 8-d-old embryonic chicken pectoral muscle, rise to a maximum at ~12 d of embryonic development and then become barely detectable in the adult, as measured both by a hemagglutination assay and a radioimmunoassay (2, 6). These results suggest that, in muscle, CLL-I plays a specifically developmental role.

Because the lectin is expressed in both primary chick muscle cultures (3) and cultures of established rat cell lines (2, 7, 8), attempts have been made to determine its function in these culture systems. Some lectin is detectable on the surface of cultured myoblasts (8, 9), suggesting a role in cell surface or cellular interactions, but most is intracellular (9). Since thiodigalactoside, a potent inhibitor of lectin activity, can inhibit fusion of myoblasts from an established rat cell line (7), it was suggested that the lectin facilitates myoblast fusion. However, similar results were not found using primary chick muscle cultures (3). Other evidence for (10) and against (11) this hypothesis has been presented.

As a step in the elucidation of the function of this endogenous lectin in muscle development, we have studied the localization of CLL-I in vivo at various stages of chicken pectoral muscle development by immunohistochemical techniques. Our results show that the majority of the lectin in immature muscle cells is intracellular, in a distribution like that of the intracellular tubular system. We also show that with muscle maturation CLL-I becomes localized extracellularly. The localization studies do not support the inference that the lectin's primary role is in muscle cell fusion. Instead they suggest that the lectin plays a role in the organization of glycoconjugates of the cell surface membrane of myotubes, the intracellular membranes that are continuous with it, and/or of extracellular materials. These organizational functions are apparently especially important during muscle differentiation.

MATERIALS AND METHODS

Lectin and Antibody

CLL-I from embryonic muscle or adult liver was purified by affinity chromatography followed by isoelectric focussing, as described previously (5, 9). Antiserum raised in rabbits has been characterized previously (5, 6). It is highly potent and specific for CLL-I without crossreaction with CLL-II (6). We used a γ-globulin fraction prepared by ammonium sulfate fractionation and reconstituted to the initial serum volume. We employed dilutions of 1:100 to 1:500 for primary staining.

Tissue Preparation for Immunohistochemistry

Pectoral muscle from White Leghorn chickens was immersed in 3% paraformaldehyde, 0.2% glutaraldehyde in 75 mM phosphate buffer, 75 mM NaCl (phosphate-buffered saline [PBS]), pH 7.2, at 4°C and cut into ~2-mm^3 pieces. After 1 h, the pieces were placed in 0.3% paraformaldehyde, 30% sucrose in PBS at 4°C and stored overnight. Muscle pieces were then mounted on microtome chucks using O.C.T. compound (Miles Laboratories Inc., Research Products Div., Elkhart, Ind.) and 1.0 to 2.0 μm frozen sections were cut at −35°C in an H/I

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Bright Cryostat (Hacker Instruments, Inc., Fairfield, N. J.) fitted for sectioning with glass knives with an LKB "Ralph" knife adapter (LKB Instruments, Inc., Bromma, Sweden).

Immunoperoxidase Staining

Tissue sections were stained using the peroxidase-anti-peroxidase (PAP) procedure of Sternberger et al. (12) and 4-Cl-1-napthol-hydrogen peroxide reaction mixture as described by Li et al. (13). Slides were washed before staining for 1 h in two changes of PBS containing 1/20 vol normal goat serum (PBS+GS). Dilutions of reagents were made in PBS+GS and all incubations were done at room temperature.

For primary binding, slides were incubated for 30 min in immune or control γ-globulin followed by a 10-min wash in PBS+GS. Next, slides were incubated in 1 U/ml goat antibody to rabbit γ-globulin (Calbiochem-Behring Corp., La Jolla, Calif.) for 30 min followed by a 10-min wash in PBS+GS. Slides were then transferred to a 1:40 dilution of rabbit PAP (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) for 30 min followed by two 10-min washes in 0.05 M Tris-HCl, pH 7.6, 0.15 M NaCl (TBS).

The peroxidase reaction mixture was prepared immediately before use by adding 8 mg of 4-Cl-1-napthol (Polysciences, Inc., Warrington, Pa.) dissolved in 250 μl of 95% ethanol to 50 ml of TBS plus 0.2% hydrogen peroxide. Slides were incubated for 4 min in this reaction mixture then washed for 10 min in TBS. Slides were then osmicated for 10 min in 1% aqueous OsO_4 washed with TBS then overlaid with 90% glycerol and a glass cover slip for microscopy.

Immunofluorescence Staining

Immunofluorescence staining was done as described previously. Slides were washed before staining in PBS+GS, then incubated for 20 min in up to 20 μg/ml control or immune γ-globulin. After two 15-min washes in PBS+GS, sections were reacted for 20 min at room temperature with a 1:100 dilution of rhodamine-conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories Inc.). After two 15-min washes in PBS, cover slips were mounted in 90% glycerol.

Preparation and Staining of Primary Muscle Cultures

Pectoral muscle cultures were prepared as described previously (9) from 12-d-old embryos, except that cells were plated on rat tail collagen-coated glass slides. Cultures were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum for 24 or 48 h, then washed free of medium with Hank's balanced salt solution. The slides were removed from culture plates and the cells were fixed in 3% paraformaldehyde, 0.2% glutaraldehyde, 0.025% saponin (Sigma Chemical Co., St. Louis, Mo.) in PBS for 45 min at room temperature before reaction with antibodies. Cultures were treated with the same procedures used for tissue sections, except that 0.025% saponin was added to all incubation buffers used before development of the peroxidase reaction product.

Microscopy

Sections stained by the immunofluorescence technique were examined with a Leitz Dialux epifluorescence microscope using a Leitz × 40 (NA 1.3) oil immersion lenses. Immunoperoxidase stained sections were examined with Zeiss × 25 (NA 0.8) and × 40 (NA 0.9) Plan-neofluar oil immersion lenses. Micrographs were taken using a Wild MPS 45 camera. Kodak Tri-X film was used for fluorescence studies and Pan-X film for peroxidase studies. Exposures and subsequent printing for experimental and control slides were done under identical conditions.

RESULTS

CLL-I was found predominantly intracellularly in myoblasts or myotubes from pectoral muscle of 10-d-old chick embryos by the immunoperoxidase technique (Fig. 1 *a*). Lectin was widely distributed throughout the cytoplasm but was absent from the nuclei. Some lectin may also be associated with the cell surface, as observed with cultured myoblasts (8, 9). Using low antibody concentrations, antibody binding was not uniformly distributed even at this early stage. Rather, some relatively dark clumps of staining and other relatively clear areas were found (Fig. 1 *a*). With higher concentrations of antibody this localization was obscured. The immunoperoxidase staining was specific for CLL-I since adsorption of the antibody with 0.1 mg/ml of boiled highly purified CLL-I eliminated the staining (Fig. 1 *b*). Staining the sections with up to ten times as much normal rabbit γ-globulin followed by addition of the normal sequence of reagents also produced no significant labeling.

The distribution of lectin in muscle cells underwent changes with development. In maturing myotubes, lectin was frequently seen as dense intracellular bands running parallel to the long axis of the cells (Fig. 1 *c*). In some cases, lectin was not only concentrated parallel to the long axis but also in clusters that ran perpendicular to these (Fig. 1 *d*). The same type of distribution is seen in cross section in a sample of 19-d embryonic muscle, in which unstained zones devoid of lectin are surrounded by dense lectin concentrations (Fig. 1 *e*). Similar sections prepared from muscle taken from chickens 2 d after hatching also showed this type of distribution, although most of the lectin was concentrated extracellularly, but non-uniformly, around the enlarging myotubes (Fig. 1 *f*). In longitudinal sections of adult tissue, staining was very faint (Fig. 1 *g*), barely exceeding the staining seen when nonimmune-γ-globulin was used (Fig. 1 *h*).

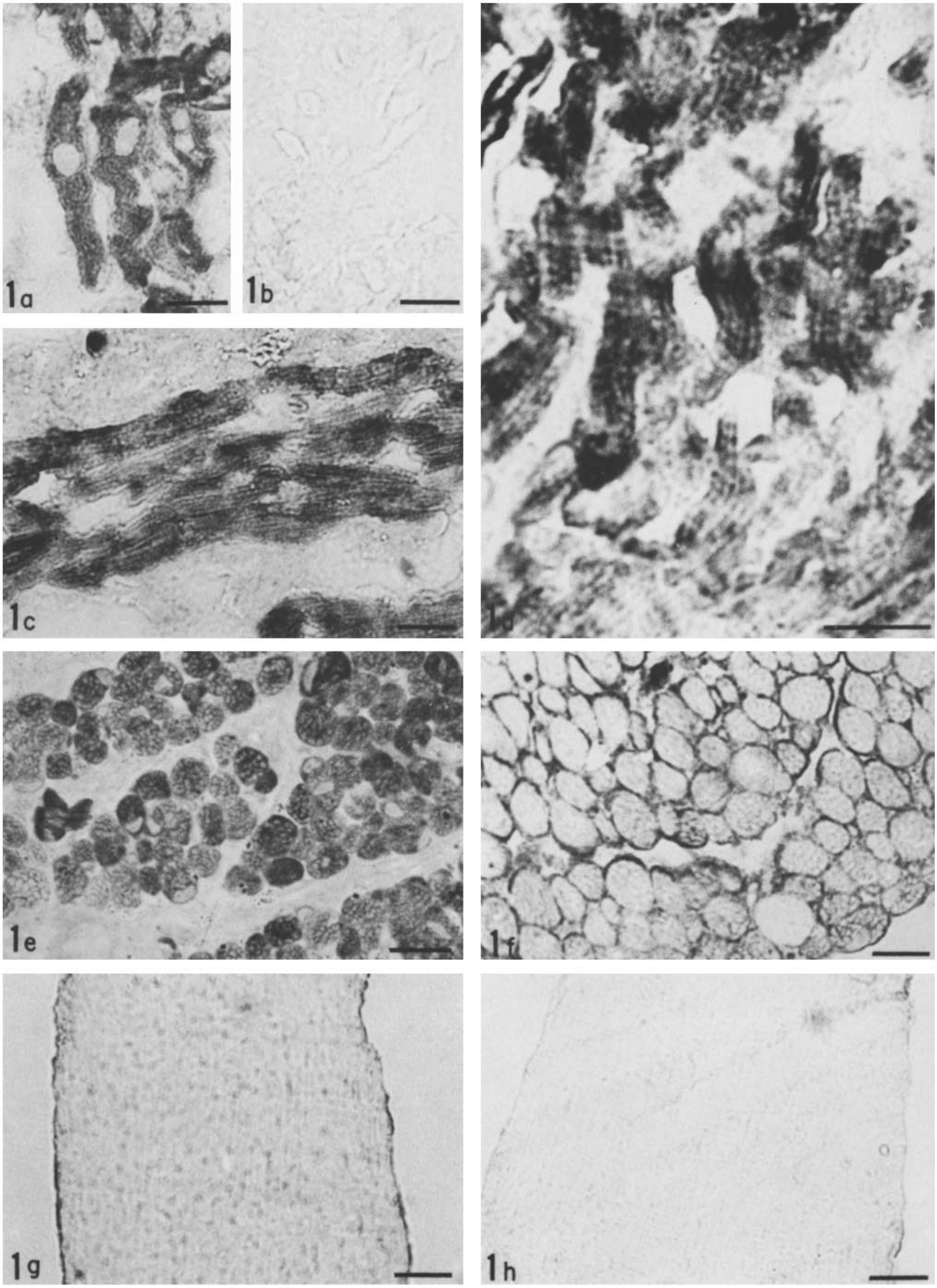
Similar results were obtained with the immunofluorescence technique. A cross section of 15-d-old pectoral muscle showed intense intracellular staining of cytoplasm, but not nuclei (Fig. 2 *a*). The intracellular staining with this technique appears more uniform than with the peroxidase technique, emphasizing that some antigen is fairly widely distributed at this stage. However, using lower antibody concentrations, staining patterns resembled those with the peroxidase technique. In tissue obtained 2 d after hatching, lectin was concentrated extracellularly, both around myotubes and in intercellular material (Fig. 2 *b*). With nonimmune-γ-globulin there was no significant staining (Fig. 2 *c*).

Lectin in myoblasts and small myotubes studied in culture was predominantly intracellular (Fig. 3) as observed previously (9). A small fraction of the reaction product followed the outlines of the cells, consistent with the previous finding that some lectin is detectable on the cell surface (8, 9). In our experiments some cell surface lectin was also found when cells were not permeabilized with saponin. However, the external localization that becomes apparent late in in vivo development (Figs. 1 *f* and 2 *b*), is clearly not prominent in young cultured cells. The non-uniform distribution of the lectin in myoblasts and myotubes in vivo is also seen in these cultured preparations, which show both linear clustering of lectin as well as small clumps.

DISCUSSION

Previous studies have shown that CLL-I, which is scarce in early development of pectoral muscle, becomes prominent in 12-d-old embryos, and then declines to very low levels in adult muscle (2, 6). Our studies indicate that the cellular localization of this lectin also undergoes marked changes.

In myoblasts, lectin is predominantly intracellular. Although present throughout the cytoplasm, it does not appear to be uniformly distributed even at this early stage. Striking segregation within the cells becomes more apparent with myotube formation and the synthesis of contractile proteins. As this occurs, lectin is concentrated primarily in a linear pattern parallel to the long axis of the myotubes, but also in lines perpendicular to this. This distribution is reminiscent of the intracellular tubular network of sarcoplasmic reticulum and T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tubules. Using the light microscope, it is not possible to tell whether the lectin is actually associated with this tubular network and whether it is on its cytoplasmic side or within the lumen of the tubules. Since CLL-I becomes localized extracellularly later in muscle development, the results raise the possibility that it becomes externalized by migration through the T tubules that are continuous with the extracellular space (14, 15). Whether or not lectin in the sarcoplasmic reticulum could migrate directly into the T tubules when these tubular systems associate (14, 15) remains to be determined.

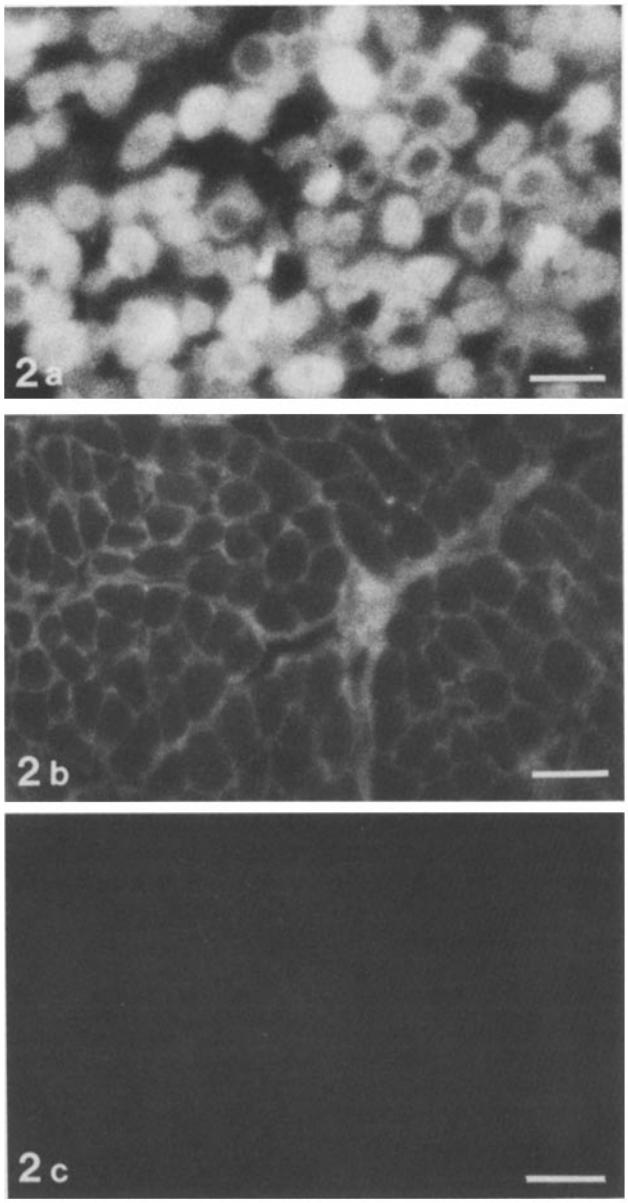

FIGURE 2 Immunohistochemical localization of CLL-I in pectoral muscle by the immunofluorescence technique. Sections of pectoral muscle from a 15-d-old embryo (*a*) and a chick 2 d after hatching (*b* and *c*) were reacted either with γ-globulin prepared from antiserum raised against CLL-I (*a* and *b*) or with an identical concentration of nonimmune γ-globulin (*c*). Bound antibody was visualized with rhodamine labeled goat anti-rabbit IgG. Bars, 5 μm. × 2,300.

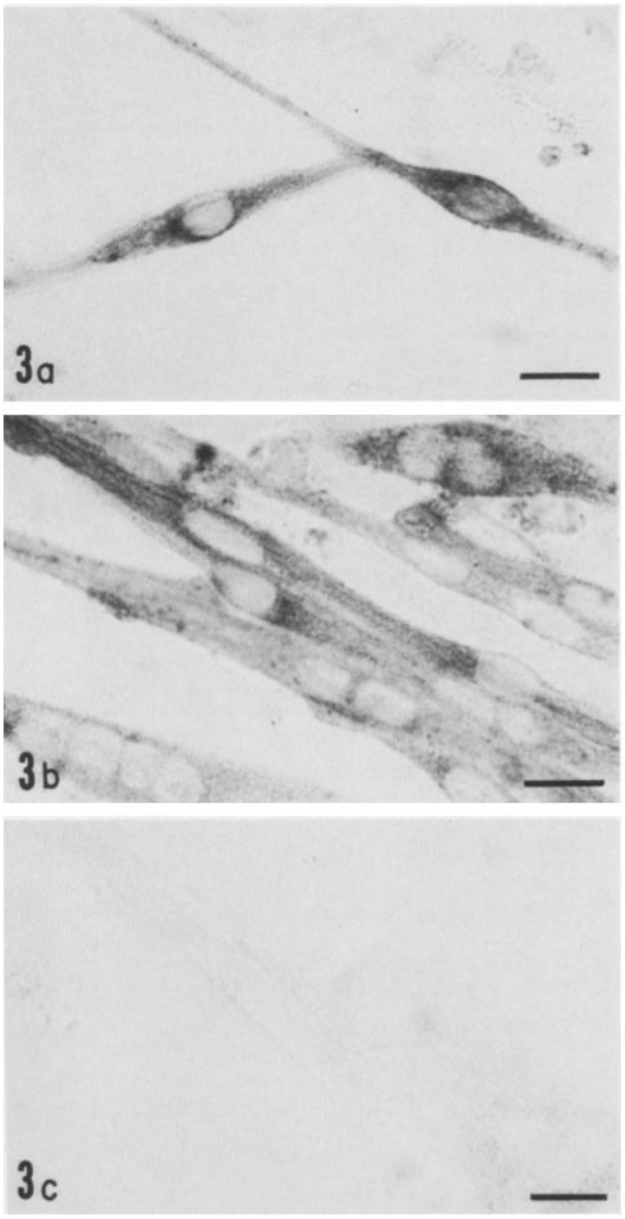

FIGURE 3 Localization of CLL-I in primary cultures of chick myoblasts. Cultures were studied 24 h (*a* and *c*) or 48 h (*b*) after plating as described in Materials and Methods. Cells were made permeable with saponin to permit intracellular localization. Specimens were treated with either γ-globulin prepared from antiserum raised against CLL-I (*a* and *b*) or identical concentrations of nonimmune γ-globulin (*c*), and antibody binding was visualized by the PAP reaction. Bars, 5 μm. × 2,300.

FIGURE 1 Immunohistochemical localization of CLL-I in embryonic and adult muscle by the PAP technique. Sections were prepared from pectoral muscle of: 10-d-old embryos (*a* and *b*); 17-d-old embryos (*c* and *d*); 19-d-old embryos (*e*); chicks 2 d after hatching (*f*); and adult chickens (*g* and *h*). All specimens (except *a*, *b*, and *h*) were treated with a 1:100 dilution of a γ-globulin fraction prepared from immune serum raised against CLL-I. Specimen *a* was treated with a 1:500 dilution of this antiserum because staining was too intense with the 1:100 dilution. *b* was treated with a 1:500 dilution that had been adsorbed by reaction with heat inactivated pure CLL-I. *h* was treated with normal rabbit γ-globulin at the same concentration as the immune γ-globulin that was generally used. Bars, 5 μm. All × 2,300, except *d*, × 4,100.

The changing localization of CLL-I with development is reminiscent of externalization of another endogenous lectin, CLL-II, in chicken intestine (16). This lectin is localized in secretory vesicles of the intestinal goblet cells, and is externalized as these vesicles fuse with the plasma membrane of the intestinal goblet cells (16). CLL-I, which is present at 0.5% of the concentration of CLL-II in these vesicles, may also be externalized in this way (16). Although the morphology and function of muscle cells differs strikingly from that of intestinal goblet cells, some basic mechanisms of lectin transport could well be similar. In both tissues, lectin is apparently passed through a membrane upon or after synthesis. In the intestine, further processing leads to its storage in a secretory vesicle which fuses with the plasma membrane to externalize the lectin. In muscle an analogous process of externalization might be effected by fusion of the T tubules, invaginations of the plasma membrane, with the sarcoplasmic reticulum of developing muscle.

Despite this new information on the localization of CLL-I, its function in developing muscle remains unknown. From the evidence presented here, CLL-I could play a role in organizing complementary glycoconjugates in the intracellular tubules, on the muscle surface, or in the extracellular materials. Its distribution and presumed function differs from fibronectin, which is very abundant in the extracellular matrix outside cultured myoblasts before fusion and then becomes confined to discrete patches associated with the plasma membrane (17, 18).

A better understanding of the function of CLL-I awaits identification of the endogenous muscle glycoconjugates that bind it and a more detailed investigation of its distribution with the electron microscope. Participation of CLL-I in myoblast fusion, while not excluded by these studies, is not suggested by the evidence presented here. However, it remains possible that CLL-I has multiple functions in muscle, just as it may play different roles in the many tissues in which it is found (6, 19). Its relationship to a heparin-inhibitable lectin from embryonic chicken muscle (20) that is externalized after myoblast fusion in culture (21) remains to be determined.

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