Evidence for Export of a Muscle Lectin from Cytosol to Extracellular Matrix and for a Novel Secretory Mechanism

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Abstract. A soluble lactose-binding lectin with subunit M_r of 14,500 is believed to function by interacting with extracellular glycoconjugates, because it has been detected extracellularly by immunohistochemistry. This localization has been questioned, however, since the lectin lacks a secretion signal sequence, which challenges the contention that it is secreted. We have demonstrated externalization of this lectin from C2 mouse muscle cells by both immunoprecipitation of metabolically labeled protein and immunohistochemical localization. We further show that externalization of the lectin is a developmentally regulated process that accompanies myoblast differentiation and that the lectin codistributes with laminin in myotube extracellular matrix. Immunohistochemical localization during intermediate stages of externalization suggests that the lectin becomes concentrated in evaginations of plasma membrane, which pinch off to form labile lectin-rich extracellular vesicles. This suggests a possible mechanism for lectin export from the cytosol to the extracellular matrix.

A soluble lactose-binding lectin with subunit M_r of ~14,500, here referred to as L-14,[1] is abundantly expressed in a wide range of vertebrate tissues (6). Immunohistochemical studies (7–11, 17, 41) have repeatedly shown that L-14 is prominently localized on the cell surface or in extracellular matrix, where it is presumed to function by interaction with complementary glycoconjugates. However, secretion and extracellular function of this protein have been questioned (13, 43, 53), because its biochemical characteristics are more typical of cytoplasmic proteins.

L-14 is clearly not a typical secreted protein, since cDNA sequences that include the entire coding length for chicken (42), mouse (47, 53), rat (13), cow (2) and human (1, 15) L-14 have not revealed a recognizable secretion signal. Furthermore, within cells L-14 message is found on free cytoplasmic ribosomes (53) and L-14 protein is found in the cytosol (11), rather than in the membrane-bound compartments expected for secreted proteins. L-14 also has other features more characteristic of cytoplasmic proteins, such as an acetylated NH_2 terminus, absence of glycosylation, and a requirement for reducing conditions for carbohydrate-binding activity (13, 24–26, 33, 52).

These anomalous properties of L-14 are not unique. Certain other cytoplasmic proteins lack a signal sequence, yet are externalized and function extracellularly. These include interleukin 1 (37), fibroblast growth factor (3, 29), thymosin (21), parathymosin (14), and, possibly, anchorin CII (22, 44). Immunocytochemistry indicates that these proteins are localized in the cytosol without being enclosed in intracellular vesicles before their externalization (5, 19, 28, 44, 50). Thus, it has been proposed that they are externalized by some alternate secretory pathway.

These issues led us to investigate L-14 externalization with a combination of metabolic and histochemical techniques. We chose to study a myogenic cell line, because previous studies showed that L-14 is highly expressed in muscle tissue (16, 40) and is externalized from embryonic chick muscle in vivo (7). We here describe developmentally regulated externalization of L-14 in cultured muscle by both immunohistochemical and metabolic labeling techniques. A mechanism for externalization of this cytosolic protein is suggested from immunohistochemical studies which show that, with differentiation, cytosolic L-14 is concentrated in cellular ectoplasm beneath regions of the plasma membrane, which then appear to be evaginated to form labile extracellular vesicles.

Materials and Methods

Lectin and Antibody Purification

Soluble rat lung lactose-binding lectins with subunit M_rs of 14,500, 18,000, and 29,000 were purified by affinity chromatography or lactosyl-Sepharose and separated by ion exchange chromatography as previously described (12). To affinity purify rabbit antibody to L-14, 5 ml of a previously raised antiserum (12) was circulated over a 2-ml column of Reactigel CDI (Pierce Chemical Co., Rockford, IL) conjugated to 1 mg of purified rat lung L-14. Specifically bound antibody was eluted with low pH and neutralized. To remove cross-reactive antibodies, affinity-purified anti-L-14 was passed over a similar column conjugated with the purified 29,000-M_r rat lung lec-

1. *Abbreviations used in this paper*: APCA-anti-14, affinity-purified cross-adsorbed antibody to L-14; L-14, soluble lactose-binding lectin with subunit M_r of ~14,500; LDH, lactate dehydrogenase.

tin. Antibody specificity was assessed by immunoblotting of cell extracts or purified lectins denatured and resolved by SDS-PAGE on 17.5% gels and transferred onto nitrocellulose. Antigen-bound rabbit antibody was visualized by incubation with biotinylated goat antibody to rabbit immunoglobulin and avidin-conjugated peroxidase (Vectastain ABC; Vector Laboratories, Inc., Burlingame, CA) followed by reaction with 4-chloronaphthol.

Cell Culture and Differentiation

C2 mouse myoblasts (strain C12) were obtained from Dr. Zach Hall (Department of Physiology, University of California, San Francisco) and cultured as previously described (43). Replicating myoblasts were maintained at <60% confluence in DME with 20% FBS and 0.5% chick embryo extract (growth medium). To induce differentiation to form multinucleate myotubes, myoblast cultures at 80% confluence were transferred to medium with only 5% horse serum (fusion medium) for 2–3 d to allow for extensive fusion. Medium was changed every 24 h. For experiments distinguishing events dependent on cell fusion from other aspects of differentiation, (1.8 μM) EGTA was included in the fusion medium to reduce the calcium concentration. This blocked myoblast fusion as assessed by light microscopy, but allowed induction of differentiation as assessed by creatine phosphokinase activity.

Creatine phosphokinase activity was assayed by a quantitative colorimetric technique available as a commercial kit (CPK-520; Sigma Diagnostics, St. Louis, MO). The volumes were modified for use with a microtiter tray and microELISA reader (Dynatech Laboratories, Inc., Alexandria, VA). Samples were determined in ranges found to be linear with regard to time of reaction and volume of sample. Units are expressed as the absorbance at 500 nm for 200 μl of sample reaction mixture with 20 μl of sample after 1 h of incubation. Sample protein concentration was determined by the method of Lowry et al. (36).

Metabolic Labeling

To metabolically label nascent proteins, myoblast or myotube cultures in 60-cm dishes were starved for methionine for 20 min at 37°C in methionine-free growth or fusion medium, pulse-labeled for 20 min at 37°C in the same medium with 200 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL), and chased in 4 ml of growth or fusion medium supplemented with 1 mM cold methionine.

To distinguish soluble extracellular from cellular L-14, the culture medium was gently collected and the cell layer was washed twice with 37°C PBS (pH 7.3) and scraped from the dish with 2 ml of ice-cold TBS (0.05 M Tris-HCl, pH 7.2, 0.15 M NaCl) including 0.5% Triton X-100, 50 μg/ml PMSF, 5 mM EDTA, 1 mM mercaptoethanol, and 100 mM lactose. The dish was washed with another 2 ml, which was pooled with the first. The collected medium was adjusted to 50 μg/ml PMSF, 1 mM mercaptoethanol, and centrifuged at 4°C for 30 min at 25,000 *g*. The supernatant was collected and the pellet saved. Extraction of this pellet with 1% SDS yielded negligible amounts of labeled L-14 or labeled protein. Medium and cell samples were immediately frozen in liquid nitrogen. After all time points had been collected, samples were thawed and the cell lysates were centrifuged for 1 h at 100,000 *g* at 4°C. The supernatant was collected and the pellet was reextracted with 1% SDS or 4 M guanidine chloride.

Incorporation of [^{35}S]methionine into total protein in cell extracts was assayed by drying between 1 and 5 μl of sample on cellulose filter paper, which was then incubated in ice cold 5% TCA for 10 min, washed in 5% TCA, boiled in 5% TCA for 10 min, washed in 5% TCA, washed in ethanol, washed in acetone, dried, and counted by liquid scintillation.

Immunoprecipitation and Quantitation

To 200 μl of each sample was added 800 μl of immunoprecipitation buffer (0.05 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.4% BSA, 1% Triton-X-100, 1% sodium deoxycholate, 5 mM EDTA, 50 μg/ml PMSF, 0.1 M lactose) and 20 μl of protein A-Sepharose (Sigma Chemical Company, St. Louis, MO). This was gently agitated at 4°C for 1 h and then centrifuged to remove the protein A-Sepharose. To the supernatant was added 4 μl of specific antiserum and this mixture was shaken overnight at 4°C. After a 1-h incubation with 20 μl of protein A-Sepharose, the mixture was centrifuged to pellet protein A-bound antigen–antibody complexes. The pellet was washed once with 1 ml of immunoprecipitation buffer, once with 1 ml TBS, 0.1% Triton X-100, 1 mM EDTA, and once with 1 ml 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. The pellet was then resuspended in 50 μl of SDS gel sample buffer and incubated at 100°C for 10 min. After centrifugation the supernatant was analyzed by SDS-PAGE using a 17.5% gel. After electrophoresis the gel was fixed and impregnated with 22% 2,5-diphenyloxazole in glacial acetic acid, which was precipitated by washing in cold water. Radioactive protein bands were visualized by exposure of the dried gel to prefogged Kodak XAR film at −80°C. After development, exposed areas of the film were roughly quantitated by densitometry (model 1312 Gel Scanner; ISCO, Inc., Lincoln, NE). Controls using nonimmune antiserum and varying amounts of sample established that the conditions used were sufficient to specifically precipitate >90% of the L-14 and that densitometry was performed in a linear range. Because the culture medium was changed every 24 h, amounts appearing in earlier medium samples were summed to calculate the accumulated amount of L-14 appearing in the medium at longer times.

Immunohistochemistry

Cells were cultured as above on glass coverslips for immunohistochemistry. At various stages of differentiation cultures were washed with 37°C PBS and fixed for 30 min at 4°C with PBS containing paraformaldehyde and glutaraldehyde. Varying concentrations of paraformaldehyde and glutaraldehyde were initially tested and 2% paraformaldehyde with 0.1% glutaraldehyde was chosen for further use because these concentrations did not noticeably diminish antibody access to L-14 antigen, gave adequate preservation of cellular morphology, and produced little tissue autofluorescence. However, it was found that this fixation permeabilized cell membranes in an uneven manner. Therefore, to immunostain only extracellular antigens, it was necessary to incubate the cultures at 4°C with antibody diluted in culture medium or 1% BSA in PBS for 30 min before washing with PBS and fixation. Fixation was stopped by incubation for 1 h with 1 mM sodium borohydride in PBS, and nonspecific binding sites were blocked by incubation for 1 h with 1% BSA in TBS. Coverslips immunostained as above but fixed with −20°C acetone revealed distributions very similar to the aldehyde fixed coverslips. Control coverslips stained with non-immune rabbit serum were blank.

For immunohistochemical staining, coverslips were incubated with primary antibody diluted in TBS, 1% BSA, 0.1% Triton X-100 for 1 h. Coverslips were washed thoroughly in the above buffer, incubated for 1 h with 5 μg/ml biotinylated anti-immunoglobulin, washed again, incubated for 1 h with 10 μg/ml fluorescein or rhodamine conjugated streptavidin, washed thoroughly, and mounted on glass slides with 90% glycerol PBS (pH 8.5), 10 mM *p*-phenylenediamine (Eastman Kodak Co., Rochester, NY). Fluorescence was viewed with a Leitz Dialux epifluorescence microscope using a Leitz 100× or 40× oil immersion lens. Photographs were taken with a Wild MPS 45 camera and Kodak T-Max 400 film. Frozen sections of mouse diaphragm, kindly provided by Dr. Zach Hall and Dr. Herman Gordon (Department of Physiology, University of California, San Francisco), were immunostained and viewed in the same manner.

Rabbit antiserum to laminin was purchased from Telios Pharmaceuticals, Inc. (San Diego, CA); rat monoclonal antibody to laminin was purchased from ICN Immunobiologicals (Lisle, IL); and rabbit antiserum to lactate dehydrogenase (M_4) was purchased from Ventrex Laboratories, Inc. (Portland, ME). Biotinylated second antibodies were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY), and fluorescent streptavidin was purchased from InFerGene Co. (Benicia, CA).

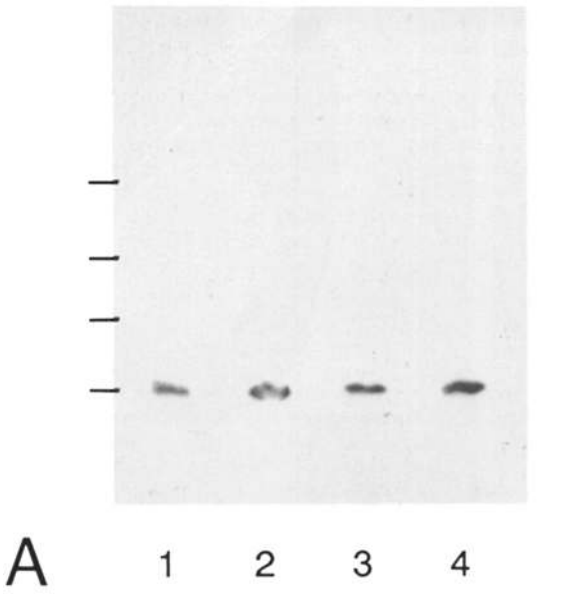
Molecular Cloning and Sequencing

A rat lung L-14 cDNA (previously isolated in this laboratory, unpublished) was radioactively labeled by polymerase extension of random oligonucleotide primers and used to screen a lambda gt11 library prepared from mouse skeletal muscle (Clontech Laboratories, Inc., Palo Alto, CA) by plaque hybridization. 10 positive plaques were identified from a total of 2×10^5. One of these was purified by successive rounds of enrichment. The cDNA insert was removed by digestion with restriction endonucleases, Kpn I and Sst I, and subcloned into Bluescript (Stratagene Corp., La Jolla, CA). The cDNA was sequenced as denatured double-stranded plasmid using Taq polymerase (Stratagene Corp.) extension of synthetic lambda gt11 oligonucleotide primers with dideoxynucleotide chain termination by standard techniques.

Results

Antibody Specificity

Rabbit antiserum raised against L-14 was affinity purified using a column conjugated with purified L-14. The small



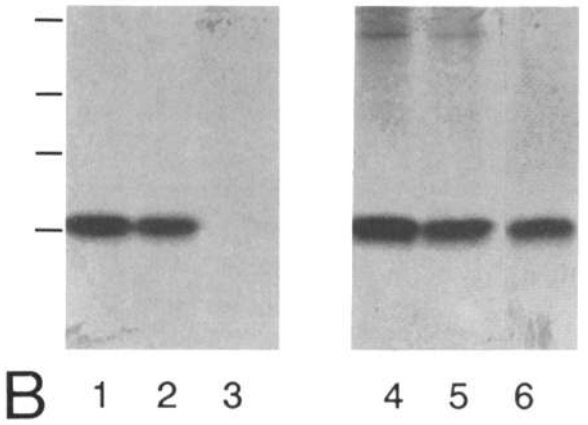


Figure 1. Demonstration of antibody specificity by immunoblotting and immunoprecipitation. (*A*) Immunoblots after SDS-PAGE immunostained with APCA-anti-14. Sample in lane *1* included equal amounts of L-14 and two other rat lectins with subunit M_r 18,000 and 29,000 (12). Samples in the other lanes included extracts in 2% SDS, 5% mercaptoethanol of rat thigh muscle (lane *2*); mouse C2 myoblasts (lane *3*); and mouse C2 myotubes (lane *4*). (Migration positions are indicated for protein molecular mass standards of 43.0, 25.7, 18.4, and 14.3 kD. (*B*) Immunoprecipitates of ^{35}S-labeled L-14 with anti-L-14 antiserum. Samples were derived from C2 myoblast cultures (lanes *1–3*) or myotube cultures (lanes *4–6*) that had been pulse-labeled with [^{35}S]methionine. Cell layers were extracted as described in Materials and Methods after chase incubations of 0 (lanes *1* and *4*) or 24 h (lanes *2* and *5*). The soluble proteins from this detergent extract or from the conditioned medium after 24 h (lanes *3* and *6*) were immunoprecipitated, resolved by SDS-PAGE, and detected by fluorography. The illustrated fluorograph was overexposed to emphasize specificity.

amount of antibody that cross-reacted with a related lectin of subunit M_r 29,000 (12) was removed by passage through a column conjugated with that purified protein. The resultant affinity-purified, cross-adsorbed antibody to L-14 (APCA-anti-14) is highly specific, revealing only a 14.5-kD band upon immunoblotting of a mixture of rat lung lectins or of extracts of rat thigh muscle, mouse C2 myoblasts, or mouse C2 myotubes (Fig. 1 *A*). Rabbit antiserum to lactate dehydrogenase (LDH) also bound to a single band of $\sim M_r$ 35,000.

Immunohistochemical Localization of L-14 in Cultures of C2 Myoblasts or Myotubes

To study the distribution of L-14 in muscle cells, C2 mouse myoblasts were cultured on glass coverslips and induced to differentiate to form large multinucleate myotubes. To visualize only extracellular antigen, cultures of myoblasts or myotubes were incubated with APCA-anti-14 at 4°C before fixation. To visualize both intracellular and extracellular antigen, cultures were initially fixed, permeabilized with Triton X-100, and then incubated with APCA-anti-14. For comparison, parallel cultures were stained as above with rabbit antibody to laminin, a secreted basement membrane protein, or rabbit antibody to LDH, a nonsecreted, soluble, cytosolic enzyme.

Immunofluorescent visualization of L-14 or LDH in myoblasts revealed a diffuse, homogeneous, cytosolic distribution (Fig. 2, *A* and *C*), which contrasts with laminin localization in intracellular vesicles (Fig. 2 *B*). Incubation with these antibodies before permeabilization prevented immunostaining, which indicates that, as has been shown for laminin (43), there is no detectable extracellular L-14 in myoblast cultures. In contrast, for well developed myotubes the immunostaining patterns for L-14 and laminin (Fig. 2, *D* and *E*) are very similar and are independent of permeabilization, whereas immunostaining of LDH still appears diffusely cytosolic (Fig. 2 *F*) and requires permeabilization. Thus, L-14 must be primarily extracellular in developed myotube cultures, as has been shown for laminin (43). In double label experiments with well differentiated C2 cultures using a monoclonal antibody to laminin and APCA-anti-14, we found extensive overlap of the two antigens (data not shown), which suggests that L-14, like laminin, is deposited in the basement membrane surrounding differentiated muscle cells.

Immunohistochemical Localization of L-14 and Laminin in Sections of Adult Muscle

Because of the possibility that externalization of L-14 and its colocalization with laminin might be artifacts of tissue culture, we determined the localization of L-14 and laminin in adult muscle tissue. Frozen sections of unfixed mouse diaphragm were immunostained for either laminin or L-14. The staining patterns for the two antigens were nearly indistinguishable (Fig. 3) and, thus, confirm that L-14 is primarily localized in extracellular matrix surrounding individual myofibers in differentiated muscle. Some faint intracellular staining was also evident for L-14, but not for laminin.

Metabolism of L-14 in C2 Myoblasts and Myotubes

To evaluate the cellular processing of L-14, we metabolically pulse-labeled C2 cells with [^{35}S]methionine under several

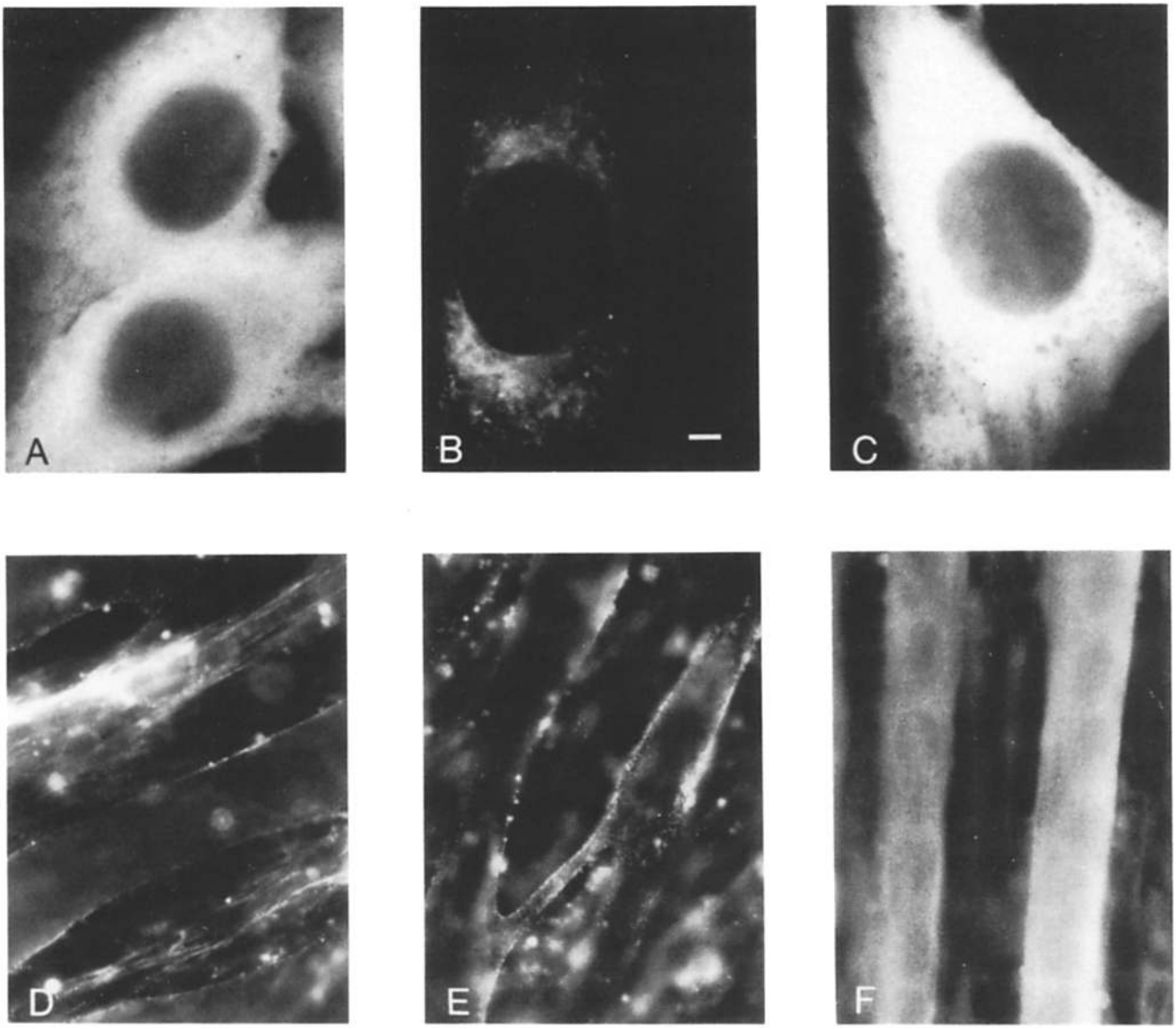


Figure 2. Immunofluorescent localization of L-14 and laminin in C2 myoblasts and myotubes. Myoblast (*A*, *B*, and *C*) or myotube (*D*, *E*, and *F*) cultures were fixed and permeabilized prior to incubation with APCA-anti-14 (*A* and *D*), rabbit antilaminin (*B* and *E*), or rabbit anti-LDH (*C* and *F*). Primary antibody binding sites were visualized with biotin-conjugated anti-rabbit antibody and fluorescein-conjugated streptavidin. Bar, (*A*, *B*, and *C*) 1 μm; (*D*, *E*, and *F*) 4 μm.

experimental conditions. Labeled L-14 was immunoprecipitated from culture media or detergent cell extracts, resolved by SDS-PAGE and fluorography, and roughly quantitated by densitometric scanning of fluorograms. The L-14 in detergent cell extracts appears to include lectin from extracellular matrix, since no further L-14 was released by subsequent extraction with 1% SDS or 4 M guanidine chloride. No evidence was found for posttranslational processing of L-14 in either myoblast or myotube cultures, as assessed by SDS-PAGE. Immunoprecipitates were highly specific and L-14 migrated at the same position in all fractions at times ranging from 0 to 72 h of chase after a 20-min pulse-label (Fig. 1 *B*).

Both the metabolic stability of L-14 and its externalization were highly dependent on the state of differentiation of the cells. This was established in three separate experiments, one of which is summarized in Fig. 4. Differentiation was monitored by measurement of creatine phosphokinase activity (Fig. 4 *C*). When actively replicating myoblasts were labeled with [^{35}S]methionine, L-14 remained associated with the cells, and <3% was detected in the culture medium (Fig. 4 *A*). In such cultures the recovery of labeled L-14 from cell lysates declined by 70% over 48 h, presumably due to metabolic turnover. In contrast, when differentiated myotube cultures were labeled, there was no significant degradation of labeled L-14, and after 24 h of chase, 65% of the initially labeled L-14 was found in the medium and 33% in the cell extract (Fig. 4 *A*). The striking difference between myoblast and myotube processing of L-14 is distinct from their processing of other labeled cellular proteins (Fig. 4 *B*). Both types of culture externalized only a small fraction of total labeled protein within 24 h; and metabolic degradation of total labeled protein was similar in both cultures over this period.

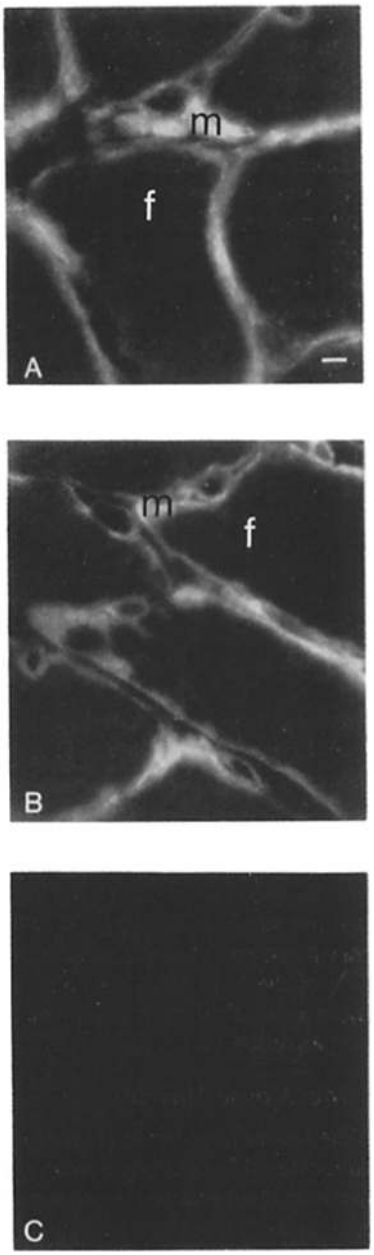


Figure 3. Immunofluorescent localization of L-14 and laminin in adult skeletal muscle. Frozen sections of mouse diaphragm were immunostained with APCA-anti-L-14 (*A*), rabbit antilaminin serum (*B*), or non-immune rabbit serum (*C*). Primary antibody binding sites were visualized with biotin-conjugated anti-rabbit antibody and fluorescein-conjugated streptavidin. (*m* = extracellular matrix, endomysium; *f* = myofiber cytoplasm). Bar, 2.5 μm.

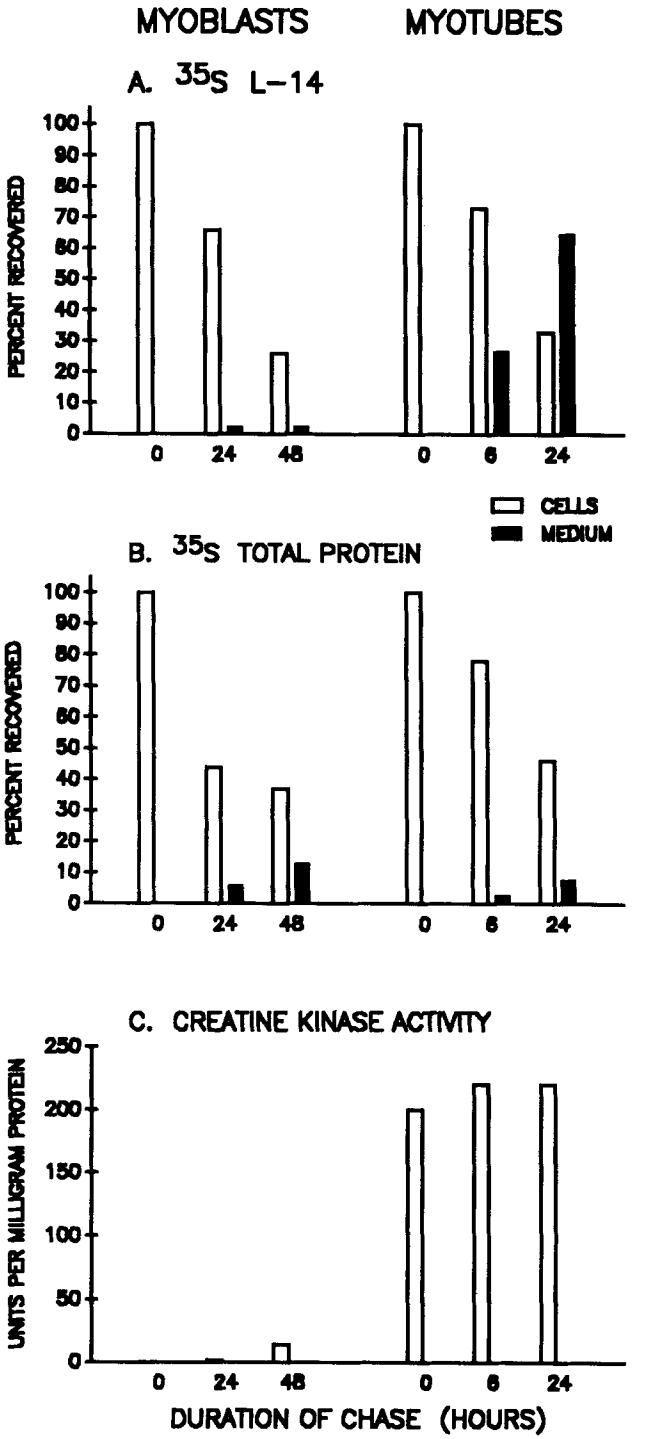


Figure 4. Comparison of L-14 metabolism in C2 myoblasts and myotubes. Myoblasts or myotubes were pulse-labeled with [^{35}S]methionine. Myoblasts were chased for 0, 24, and 48 h; myotubes for 0, 6, and 24 h. (A) Detergent-soluble extracts of the cell layers (*white bars*) and conditioned media (*black bars*) were immunoprecipitated, electrophoresed, and fluorographed as in Fig. 1 *B*, and labeled L-14 was estimated by densitometry. The total recovered ^{35}S-labeled L-14 in the cell extract and medium at time 0 was defined as 100%, and recovery at later times is reported relative to the initial value. (*B*) TCA-precipitable ^{35}S-labeled protein from the cell extract and medium is expressed in the same way. (*C*) Creatine phosphokinase activity is reported for cell extracts; no activity was detectable in the culture medium.

To determine whether the cytosolic L-14 already made by myoblasts would be externalized in response to experimentally induced differentiation, parallel cultures of replicating myoblasts were pulse-labeled and then cultured in either growth-promoting medium to maintain undifferentiated replication or in differentiation-inducing medium (Fig. 5). For these experiments, we used cultures at a higher cell density (50% confluence) than the myoblast cultures shown in Fig. 4 (25% confluence), because induction of differentiation is very slow at the lower cell density. Differentiation occurs in

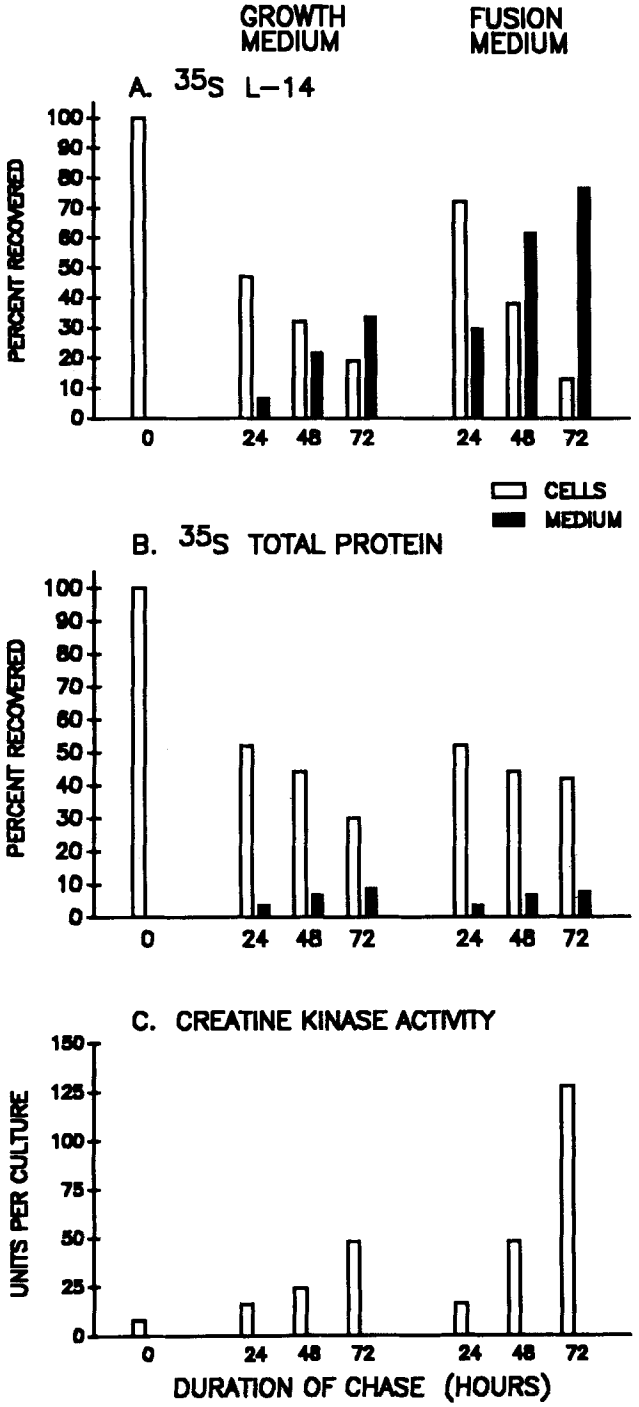


Figure 5. Comparison of L-14 metabolism in C2 myoblasts maintained in growth medium or induced to differentiate. Myoblast cultures were pulse-labeled with [^{35}S]methionine and chased for 0, 24, 48, and 72 h in either medium with 20% FBS and 0.5% chick embryo extract to maintain replication of undifferentiated cells or in medium with only 5% horse serum to induce differentiation and fusion. Detergent soluble extracts of the cell layers (*white bars*) and conditioned media (*black bars*) were analyzed for ^{35}S-labeled L-14 (*A*), ^{35}S-labeled protein (*B*), and creatine phosphokinase activity (*C*), as described in Fig. 4.

both media, as indicated by creatine kinase levels, but it is accelerated by differentiation inducing medium.

When these cultures were maintained in growth-promoting medium, only 7% of the labeled L-14 was externalized in the first 24 h of chase, and the total recovery of L-14 declined by half. During the succeeding 48 h, however, these cultures progressively differentiated due to increasing cell density, turnover of L-14 ceased, and about two-thirds of the remaining L-14 was externalized. When induced to differentiate more rapidly with fusion inducing medium, turnover of L-14 ceased immediately and appearance in the medium commenced within the first 24 h. Two-thirds of the lectin was already extracellular by 48 h of chase. By 72 h, 80% was extracellular. Thus, externalization of L-14 synthesized in undifferentiated myoblasts occurs with differentiation, and the rates of L-14 turnover or externalization are dependent on the rate of differentiation.

The developmental timing of L-14 externalization suggested that it might be related not only to cellular differentiation, but specifically to the process of myoblast fusion. To investigate this possibility, we determined whether blocking fusion by depleting the medium of calcium would influence the disposition of L-14. We found that myoblast fusion could be effectively blocked without inhibiting externalization of L-14 as assessed by either immunohistochemistry or immunoprecipitation from metabolically pulse-labeled cultures (data not shown).

In all of the above experiments the cells appeared healthy. Furthermore, little of the total ^{35}S-labeled protein and none of the creatine kinase activity was detected in the culture medium. As a further check that cell lysis did not account for L-14 externalization, LDH was immunoprecipitated from pulse-labeled myotube cultures and roughly quantified as for L-14. Less than 1% of the initially labeled LDH could be recovered from the medium after 24 h, whereas 75% could be recovered from the cell extract. This contrasts sharply with the recovery of 65% of the L-14 from the medium after 24 h. These results argue against cell lysis as a primary cause of the extensive externalization of L-14.

Sequence of L-14 cDNA from Mouse Muscle

Although L-14 cDNAs isolated from lung tissue (13), fibroblasts (2, 53), leukemic leukocytes (15), and hepatoma cells (1, 20) have no obvious secretion signal sequences, it seemed possible that muscle L-14 might be synthesized from a variant cDNA. Therefore, we sequenced an L-14 cDNA clone isolated from a lambda gt11 library prepared from adult mouse muscle mRNA. The nucleotide sequence of this cDNA (Fig. 6) extends from 52 bases upstream of the translation initiation site to an internal Eco RI site. A partial mouse L-14 cDNA, lacking coding region for the 20 most NH_2-terminal amino acids, but including the rest of the coding region, has previously been isolated from 3T3 fibroblasts (53). By comparison with that sequence, the muscle cDNA is identical in the 261-base region of overlap and, thus, encodes an amino acid sequence that includes all but the carboxy-terminal 28 amino acids. The muscle L-14 cDNA sequence is also identical to the sequence reported for L-14 cDNA isolated from a mouse fibrosarcoma (47), except for a few single base insertions. These are evidently due to minor sequencing errors for the fibrosarcoma cDNA, since they result in translation frameshifts in an otherwise conserved nucleotide sequence. Translating the complete mouse L-14 from these cDNA clones reveals 96% identity with the amino acid sequence determined for rat L-14 (13). As with

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MML14     GCTGACTGCTGGTGGAGCAGGTCTCAGGAATCTCTTCGCTTCAAGCTTCAATC

          M   A   C   G   L   V   A   S   N   L   N   L   K   P
MML14     ATG GCC TGT GGT CTG GTC GCC AGC AAC CTG AAT CTC AAA CCT

          G   E   C   L   K   V   R   G   E   V   A   S   D   A
MML14     GGG GAA TGT CTC AAA GTT CGG GGA GAG GTG GCC TCG GAC GCC
M3T3L                         CGG GGA GAG GTG GCC TCG GAC GCC

          K   S   F   V   L   N   L   G   K   D   S   N   N   L
MML14     AAG AGC TTT GTG CTG AAC CTG GGA AAA GAC AGC AAC AAC CTG
M3T3L     AAG AGC TTT GTG CTG AAC CTG GGA AAA GAC AGC AAC AAC CTG

          C   L   H   F   N   P   R   F   N   A   H   G   D   A
MML14     TGC CTA CAC TTC AAT CCT CGC TTC AAT GCC CAT GGA GAC GCC
M3T3L     TGC CTA CAC TTC AAT CCT CGC TTC AAT GCC CAT GGA GAC GCC

          N   T   I   V   C   N   T   K   E   D   G   T   W   G
MML14     AAC ACC ATT GTG TGT AAC ACC AAG GAA GAT GGG ACC TGG GGA
M3T3L     AAC ACC ATT GTG TGT AAC ACC AAG GAA GAT GGG ACC TGG GGA

          T   E   H   R   E   P   A   F   P   F   Q   P   G   S
MML14     ACC GAA CAC CGG GAA CCT GCC TTC CCC TTC CAG CCC GGG AGC
M3T3L     ACC GAA CAC CGG GAA CCT GCC TTC CCC TTC CAG CCC GGG AGC

          I   T   E   V   C   I   T   F   D   Q   A   D   L   T
MML14     ATC ACA GAG GTG TGC ATC ACC TTT GAC CAG GCT GAC CTG ACC
M3T3L     ATC ACA GAG GTG TGC ATC ACC TTT GAC CAG GCT GAC CTG ACC

          I   K   L   P   D   G   H   E   F   K   F   P   N   R
MML14     ATC AAG CTG CCA GAC GGA CAT GAA TTC
M3T3L     ATC AAG CTG CCA GAC GGA CAT GAA TTC AAG TTC CCC AAC CGC

          L   N   M   E   A   I   N   Y   M   A   A   D   G   D
M3T3L     CTC AAC ATG GAG GCC ATC AAC TAC ATG GCG GCG GAT GGA GAC

          F   K   I   K   C   V   A   F   E
M3T3L     TTC AAG ATT AAG TGC GTG GCC TTT GAG TGA

M3T3L     AGCCAGCCAGCCTGTAGCCTCAATAAAGGCAGCTGCT
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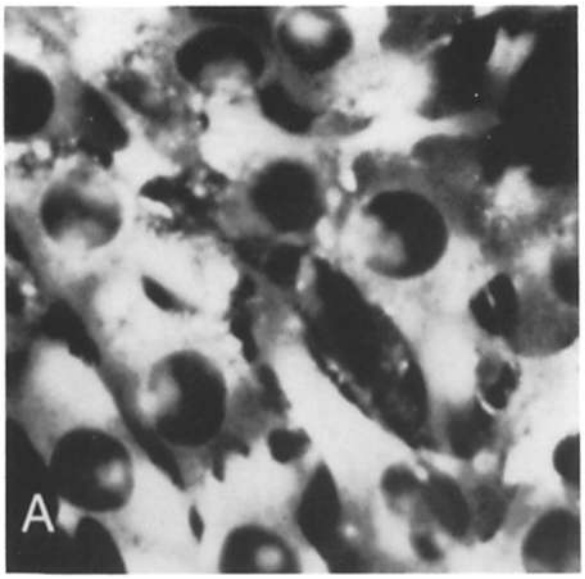
Figure 6. Nucleotide sequence and predicted amino acid sequence of cDNA for mouse L-14. (*MML14*) mouse muscle L-14 cDNA; (*M3T3L*) mouse 3T3 cell L-14 cDNA (53). These sequence data are available from EMBL/GenBank/DDBJ under accession number X51903.

other L-14 cDNAs, the mouse sequence does not code for a classical leader secretion signal and includes no peptide sequences of sufficient hydrophobicity to suggest an internal secretion signal.

Immunohistochemical Localization of L-14 during Differentiation

To investigate the process of L-14 externalization, we immunohistochemically localized the lectin at intermediate stages of myoblast differentiation. This is difficult under standard culture conditions, since differentiation is highly asynchronous and formation of overlying myotube layers obscures observation of differentiating myoblasts. We found that observation of myoblast differentiation is facilitated under conditions of low cell density, so that differentiation is slowed and formation of obscuring myotube layers is avoided.

After induction of differentiation, the first detectable change is a concentration of cytosolic L-14 in cortical cytoplasm (ectoplasm) next to the plasma membrane. This shift is observed by varying the microscope focal plane through the cells and is difficult to illustrate with fluorescence photomicrographs. The change becomes more apparent, however, as L-14 becomes concentrated in progressively more restricted ectoplasmic domains (Fig. 7 *A*). These subplasmalemmal concentrations appear to be evaginated in protrusions of plasma membrane, which pinch off to form lectin-rich extracellular vesicles (Fig. 7 *B*).



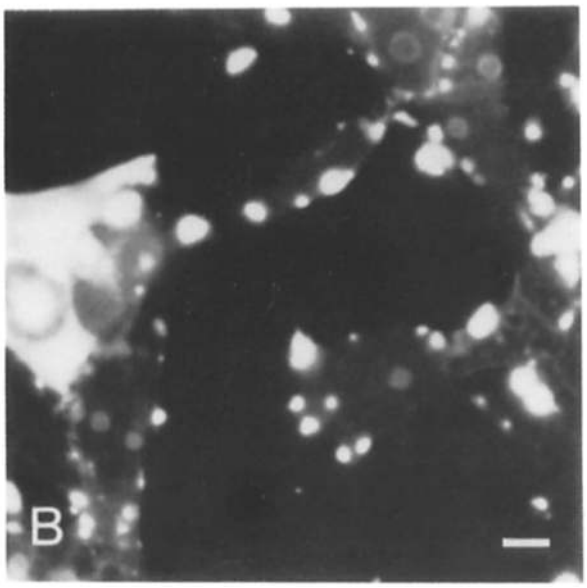


Figure 7. Immunofluorescent localization of L-14 during export from differentiating myoblasts. Myoblast cultures were induced to slowly differentiate at low cell density to localize L-14 at stages intermediate between its diffuse cytosolic distribution in myoblasts and its extracellular distribution in myotubes. Cells were fixed and permeabilized before incubation with APCA-anti-14. Primary antibody binding sites were visualized as in Fig. 2. After 24 h of differentiation (*A*), L-14 still is primarily intracellular, but shows a very patchy distribution. By varying the focal plane we found that these patches are concentrated in the outermost layer of cytoplasm (ectoplasm). After 48 h (*B*) L-14 appears highly concentrated in vesicles formed by evagination of plasma membrane. Bar, 2.5 μm.

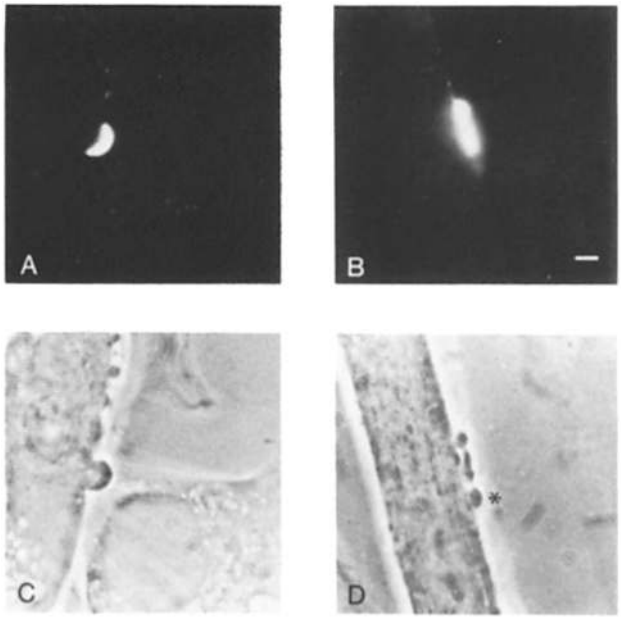


Figure 8. Immunofluorescent localization of L-14 during export from myotubes. Shortly after commencement of fusion, myotube cultures were fixed, permeabilized, and incubated with APCA-anti-14. Primary antibody binding sites were visualized as in Fig 2. Comparison of fluorescent (*A* and *B*) and phase-contrast (*C* and *D*) images reveals that L-14 is highly concentrated in evaginations of plasma membrane (*A* and *C*) and in extracellular vesicles (*B* and *D*). Not all extracellular vesicles are enriched in L-14 (*asterisk*). Bar, 1 μm.

The various distinct localizations of L-14 described above can also be seen in cultures forming multinucleate myotubes at higher cell densities. An ectoplasmic concentration of L-14 is evident in some young myotubes. In more mature myotubes, most of the cytosol appears empty of L-14 except for rare concentrated patches. Evagination of these in outpockets of plasma membrane may also be occasionally observed (Fig. 8, *A* and *C*), and lectin-rich vesicles are abundant 24 h after induction of fusion (Fig. 8, *B* and *D*). Detergent permeabilization is required to immunostain L-14 in ectoplasmic patches or membrane evaginations, but at least some extracellular vesicles are sufficiently permeable to allow penetration of anti-L-14 antibody without detergent treatment. The final step in L-14 externalization may involve disintegration of the extracellular vesicles, thus releasing lectin into the culture medium. Because we have been unable to concentrate L-14 from freshly collected conditioned medium by centrifugation, little of the L-14 in the medium remains in vesicles.

We have been concerned that the extracellular vesicles that stain for L-14 might represent artifactual membrane blebs induced by adverse conditions of culture or fixation. However, there is a notable specificity of vesicle staining in myotube cultures, such that L-14 appears highly concentrated in some, but not all, extracellular vesicles and little is visualized in myotube cytoplasm (Fig. 8). In contrast, immunostaining for LDH reveals that it remains cytosolic in myotubes (Fig. 2 *F*). Such selectivity seems unlikely to arise purely as an artifact. Also, while we have noted the occurrence of previously described glutaraldehyde-induced blebbing (49), when myotubes are fixed at 37°C, there is an increase in the number of extracellular vesicles, but not in the number of vesicles which stain for L-14. Furthermore, lectin-rich extracellular vesicles are also observed after alternative fixation with acetone at −20°C.

Discussion

The present results clearly establish that L-14, a cytosolic protein that is synthesized without a secretion signal sequence, accumulates extracellularly in cultured muscle cells. Externalization of the lectin is developmentally regulated and is accomplished by what appears to be a novel secretory mechanism.

Externalization of cytosolic L-14 is clearly demonstrated by both immunoprecipitation of metabolically labeled lectin and by immunohistochemical localization. Metabolic labeling studies show that the bulk of the L-14 made in myoblasts can be externalized with differentiation, whereas creatine kinase and lactate dehydrogenase remain intracellular. Therefore, extracellular L-14 cannot be accounted for by death and lysis of a small fraction of the cultured cell population or by transient membrane disruptions (38). Metabolic labeling studies also rule out the possibility that L-14 externalized by differentiated myotubes represents a different gene product than that made in undifferentiated myoblasts, because L-14 made in myoblasts was directly shown to be externalized with differentiation. Thus, the onset of L-14 externalization reflects a developmental change in muscle cell physiology rather than a change in the L-14 gene product. In light of these findings, the lack of L-14 externalization reported for cultured lung fibroblasts (46, 51) may reflect their physiological status. It is possible that these cells, too, might release L-14 under appropriate conditions.

We have used the term externalization instead of secretion to describe the export of cytosolic L-14, because secretion has come to imply passage out of the cell through a well characterized series of membrane-bound intracellular compartments. By examining intermediate stages of myoblast differentiation in tissue culture, we found evidence that the externalization of L-14 involves a very different mechanism, which is shown schematically in Fig. 9. As myoblasts differentiate, L-14 appears to be concentrated in cytoplasm directly beneath the plasma membrane, which has been referred to as ectoplasm. The ectoplasmic L-14 then becomes progressively concentrated into patches beneath localized regions of plasma membrane. These regions show no morphological specialization that we can detect with phase-contrast microscopy. The concentrated patches of ectoplasmic L-14 are then evaginated in outpockets of the plasma membrane to yield extracellular vesicles enriched in lectin. These vesicles do not appear to be connected to the cell. An mAb raised against a different rat muscle antigen has also been shown to stain evaginations of plasma membrane formed with differentiation (31).

The final step in externalization of L-14 is presumed to occur when evaginated vesicles are disrupted. Although we have not specifically visualized this final step, we observe that at least some of these vesicles are sufficiently permeable without detergent to allow penetration of antibody to L-14. Also, from the metabolic labeling studies, it is clear that the bulk of the L-14 synthesized in developing myotubes can be ultimately recovered as soluble protein in the culture medium. Once released, L-14 would be free to bind to cognate glycoconjugates on the cell surface or in extracellular matrix.

Aspects of the externalization suggested for L-14 are similar to processes previously described. In the process of mineralization of calcifying tissues, membrane-bound extracellular particles referred to as "matrix vesicles" have been detected that are specifically enriched in certain proteins originally found in the cytosol (23, 27). During erythrocyte maturation transferrin receptors are specifically shed by a somewhat different process involving internalization into multivesicular bodies which then bleb from the plasma membrane (30). The extracellular vesicles formed in these cases are believed to represent physiologically normal intermediate structures. On the other hand, a variety of culture conditions, such as temperature shift (34) or exposure to serum components (18), have been reported to induce membrane blebbing from some cells. This sort of artifact is difficult to rule out. It is notable, however, that L-14 appears highly enriched in extracellular vesicles, whereas LDH, another cytosolic protein, does not. Furthermore, not all extracellular vesicles are enriched in L-14. This implies at least some specific subcellular distribution for L-14 in relation to the plasma membrane and suggests physiological relevance.

Other proteins, such as fibroblast growth factor, interleukin I, thymosin, and parathymosin, are also externalized without classical secretion signal sequences. For these cytokines, utilization of an alternative secretion pathway may be important for prevention of excessive autocrine stimulation. For example, it has been shown that direction of fibroblast growth factor into the classical secretion pathway by

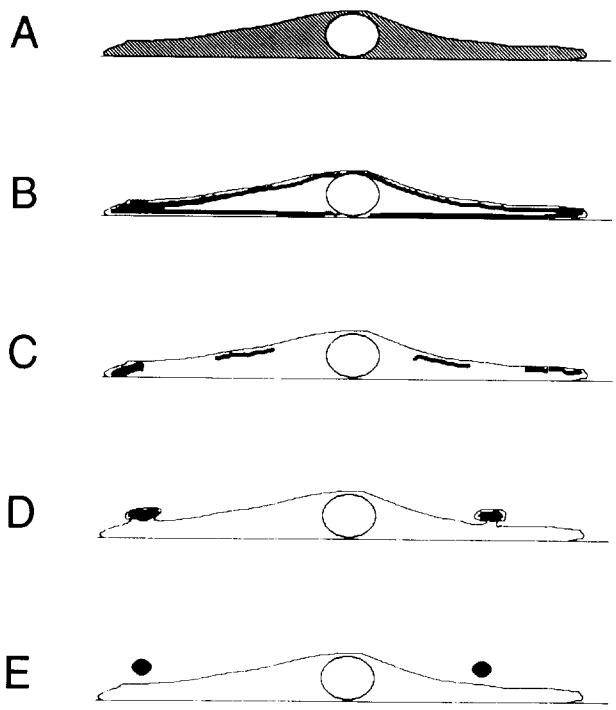


Figure 9. Schematic representation of pathway postulated for export of L-14. As myogenic cells progressively differentiate, the L-14 that is initially distributed diffusely throughout the cytosol (*A*) becomes concentrated in cytosol next to the plasma membrane (ectoplasm) (*B*) and then accumulates in restricted regions of ectoplasm (*C*). These L-14 concentrations are then included in protrusions of plasma membrane (*D*), which are evaginated to form extracellular vesicles highly enriched in lectin (*E*). Vesicle degeneration is presumed to release L-14 into the extracellular milieu where it may bind to the cell surface or extracellular matrix.

fusion to a functional signal sequence causes neoplastic transformation of cells expressing this construct (48). Because L-14 has been reported to be mitogenic for lymphocytes (35, 45) it, too, might function as a cytokine and, therefore, require segregation from potential receptors within the cell.

The unusual externalization of L-14 may also serve to prevent it from associating with complementary glycoconjugates that are being processed in normal secretory vesicles. For example, L-14 is known to have a high affinity for polylactosamines (32, 39) and it may be critical to limit interaction of L-14 with proteins containing these side chains, such as laminin (4), until after externalization. An alternative export pathway, such as that suggested here for L-14, may, therefore, be important for delivering critical materials to the extracellular environment while initially segregating them from other extracellular materials processed by the classical secretory pathway.

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