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

Douglas N. W. Cooper and Samuel H. Barondes
Department of Psychiatry and Langley Porter Psychiatric Institute, University of California, San Francisco, San Francisco, California 94143-0984

Abstract. A soluble lactose-binding lectin with subunit Mr 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 Mr of ~14,500, here referred to as L-14, 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 (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 NH2 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 Mr's 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-Mr rat lung lectin.
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 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.

**Immunochemistry**

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 PBS. 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 then blocked.

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 0.1% glycerol PBS (pH 8.5). 10 μM p-phenylenediamine (Eastman Kodak Co., Rochester, NY). Fluorescence was viewed with a Leitz Dialux epifluorescence microscope using a Leitz 10× 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 (M2) was purchased from Ventrex Laboratories, Inc. (Pertland, ME). Biotinylated second antibodies were purchased from Acrurate Chemical and Scientific Corp. (Westbury, NY), and fluorescent streptavidin was purchased from InfereGen Co. (Bencia, 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 oligorucleotide primers and used to screen a lambda gtl 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 x 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 Sac I, and subcloned into Bluescript (Stratagene Corp., La Jolla, CA). The cDNA was sequenced as described above. Primer-directed amplification using Taq polymerase (Stratagene Corp.) extension of synthetic lambda gtl oligo-nucleotide primers with dideoxyribonucleic acid 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
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 Mr 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 35S-labeled L-14 with anti-L-14 antiserum. Samples were derived from C2 myoblast cultures (lanes 1-3) or myotube cultures (lanes 4-6). (Migration positions are indicated for protein molecular mass standards of 43.0, 25.7, 18.4, and 14.3 kD.) Immunoprecipitates of 35S-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 [35S]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.

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 [35S]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.

eperimental 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 [35S]methionine, L-14 remained associated with the cells, and <3% was detected in the culture medium (Fig. 4 A). 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.
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 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.](image)

![Figure 4. Comparison of L-14 metabolism in C2 myoblasts and myotubes. Myoblasts or myotubes were pulse-labeled with [35S]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 35S-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 35S-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.](image)
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 framshifts 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
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 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 outpocketed 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 parathyminosin, 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
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 globoseries. For example, L-14 is known to have a high affinity for

References

1. Abbott, W. M., and T. Feizi. 1989. Evidence that the 14 kDa soluble β-galactoside-binding lectin in man is encoded by a single gene. Biochem. J. 259:291–294.
2. Abbott, W. M., A. Mellor, Y. Edwards, and T. Feizi. 1989. Soluble bovine galactose-binding lectin: cDNA cloning reveals the complete amino acid sequence and an antigenic relationship with the major encephalitogenic domain of myelin basic protein. Biochem. J. 259:283–290.
3. Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes. 1986. Nucleotide sequence and an antigenic relationship with the major encephalitogenic domain of myelin basic protein. Biochem. J. 259:283–290.
4. Aromugham, R. G., T. C.-Y. Hsieh, M. L. Tanzer, and R. A. Laine. 1987. Structures of the asparagine-linked sugar chains of laminin. Biochem. Biophys. Acta. 883:112-126.
5. Bakouche, O., D. C. Brown, and L. B. Lachman. 1987. Subcellular localization of human monococyte interleukin 1: evidence for an inactive precursor molecule and a possible mechanism for IL-1 release. J. Immunol. 130:4229–4255.
6. Barondes, S. H. 1984. Soluble lectins: a new class of extracellular proteins. Trends Biochem. Sci. 9:54–58.
7. Barondes, S. H., and P. L. Haywood-Reid. 1981. Externalization of an endogenous chicken muscle lectin with in vivo development. J. Cell Biol. 91:568–572.
8. Beyer, E. C., K. T. Tokuyasu, and S. H. Barondes. 1979. Localization of an endogenous lectin in chicken liver, intestine, and pancreas. J. Cell Biol. 82:465–469.
9. Catt, J. W., L. F. Harrison, and J. S. Carleton. 1987. Molecular cloning, characterization, and expression of a human 14-kDa lectin. J. Biol. Chem. 264:1310–1316.
10. Den, H., D. A. Malinzak, and A. Rosenberg. 1976. Lack of evidence for the involvement of a β-D-galactosyl-specific lectin in the fusion of chick myoblasts. Biochem. Biophys. Res. Commun. 70:621–627.
11. Fabian, N., C. Auger, and J.-C. Monier. 1987. Immunolocalization of thymosin alpha 1, thymopetin and thymulin in mouse thymic epithelial cells at different states of culture: a light and electron microscopic study. Immunol. 63:721–727.
12. Hale, J. E., and R. E. Wuthier. 1987. The mechanism of matrix vesicle formation. J. Biol. Chem. 262:1916–1925.
13. Hirasahashi, J., and K. Kasai. 1988. Complete amino acid sequence of a β-galactoside-binding lectin from human placenta. J. Biochem. (Tokyo). 104:1–4.
14. Hirschi, J., H. Kawasaki, K. Suzuki, and K. Kasai. 1987. Complete amino acid sequence of 14 kDa β-galactoside-binding lectin of chick embryo. J. Biol. Chem. 101:775–787.
15. Hirschi, J., H. Kawasaki, K. Suzuki, and K. Kasai. 1987. Further characterization and structural studies on human placenta lectin, J. Biol. Chem. 263:10045–10047.
16. Haywood-Reid, T. P., and S. H. Barondes. 1985. Expression of a developmentally regulated antigen on the surface of skeletal and cardiac muscle cells. J. Cell Biol. 101:775–787.
17. Hjerrild, D. Gospodarowicz, and J. C. Fiddes. 1986. Nucleotide sequence and an antigenic relationship with the major encephalitogenic domain of myelin basic protein. Biochem. J. 259:283–290.
18. Hjerrild, D. Gospodarowicz, and J. C. Fiddes. 1986. Nucleotide sequence and an antigenic relationship with the major encephalitogenic domain of myelin basic protein. Biochem. J. 259:283–290.
mentally regulated lectin from embryonic chick pectoral muscle. *J. Biol. Chem.* 252:6026–6030.

41. Oda, Y., Y. Ohyama, A. Obinata, H. Endo, and K. Kasai. 1989. Endogenous beta-galactoside binding lectin expression is suppressed in retinol-induced mucous metaplasia of chick embryonic epidermis. *Exp. Cell Res.* 182:33–43.

42. Ohyama, Y., J. Hirabayashi, Y. Oda, S. Ohno, H. Kawasaki, K. Suzuki, and K. Kasai. 1986. Nucleotide sequence of chick 14k beta-galactoside-binding lectin mRNA. *Biochem. Biophys. Res. Commun.* 134:51–56.

43. Olwin, B. B., and Z. W. Hall. 1985. Developmental regulation of laminin accumulation in the extracellular matrix of a mouse muscle cell line. *Dev. Biol.* 112:359–367.

44. Pfaffle, M., F. Ruggiero, H. Hofmann, M. P. Fernandez, O. Selmin, Y. Yamada, G. Garrone, and K. von der Mark. 1988. Biosynthesis, secretion and extracellular localization of anchorin CII, a collagen-binding protein of the calpain family. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2335–2342.

45. Pitts, M. J., and D. C. H. Yang. 1981. Mitogenicity and binding properties of beta-galactoside-binding lectin from chick-embryo kidney. *Biochem. J.* 195:435–439.

46. Powell, J. T. 1988. Evidence against lung galaptin being important to the synthesis or organization of the elastic fibril. *Biochem. J.* 252:447–452.

47. Raz, A., P. Carmi, and G. Pazerini. 1988. Expression of two different endogenous galactoside-binding lectins sharing sequence homology. *Cancer Res.* 48:645–649.

48. Rogelj, S., R. A. Weinberg, P. Fanning, and M. Klagsbrun. 1988. Basic fibroblast growth factor fused to a signal peptide transforms cells. *Nature (Lond.).* 331:173–175.

49. Scott, R. E. 1976. Plasma membrane vesiculation: a new technique for isolation of plasma membranes. *Science (Wash. DC).* 194:743–745.

50. Singer, I. I., S. Scott, G. L. Hall, G. Limjuco, J. Chin, and J. A. Schmidt. 1988. Interleukin 1 beta is localized in the cytoplasmic ground substance but is largely absent from the golgi apparatus and plasma membranes of stimulated human monocytes. *J. Exp. Med.* 167:389–407.

51. Whitney, P., S. Maxwell, U. Ryan, and D. Massaro. 1985. Synthesis and binding of lactose-specific lectin by isolated lung cells. *Am. J. Physiol.* 248:C258–C264.

52. Whitney, P. L., J. T. Powell, and G. L. Sanford. 1986. Oxidation and chemical modification of lung beta-galactoside specific lectin. *Biochem. J.* 238:683–689.

53. Wilson, T. J. G., M. J. Firth, J. T. Powell, and F. L. Harrison. 1989. The sequence of the mouse 14 kDa beta-galactoside binding lectin and evidence for its synthesis on free cytoplasmic ribosomes. *Biochem. J.* 261:847–852.