

# Endogenous Muscle Lectin Inhibits Myoblast Adhesion to Laminin

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**Abstract.** L-14, a dimeric lactose-binding lectin with subunits of 14 kD, is expressed in a wide range of vertebrate tissues. Several functions have been postulated for this lectin, but definitive evidence for a specific biological role has been elusive. In muscle, L-14 is secreted during differentiation and accumulates with laminin in basement membrane surrounding each myofiber. Here we present evidence that laminin is a major

glycoprotein ligand for L-14 in differentiating mouse C2C12 muscle cells and that binding of secreted L-14 to polylectosamine oligosaccharides of substrate laminin induces loss of cell-substratum adhesion. These results suggest that one function of L-14 is to regulate myoblast detachment from laminin during differentiation and fusion into tubular myofibers.

As differentiating cells integrate into complex tissues, their changing interactions with neighboring cells and extracellular matrix are determined by the regulated expression of specific extracellular matrix components and receptors. Carbohydrate-binding proteins (lectins) are postulated to participate in this process by recognizing complementary glycoconjugate saccharides expressed on cells or in matrix at specific times or in specific locations (Barondes, 1984; Sharon and Lis, 1989). Evidence is provided here for a role of one such lectin in regulating matrix adhesion in developing muscle.

Most animal lectins have been classified into two major groups, C-type and S-lac, on the basis of biochemical properties and consensus amino acid sequences (Drickamer, 1988; Leffler et al., 1989). The C-type lectin family is primarily composed of integral membrane glycoproteins which require divalent cations for activity. This group includes the lectin cell adhesion molecules (LEC-CAM)<sup>1</sup> which mediate cell-cell recognition (Rosen, 1990; Springer, 1990) and other receptors which mediate intracellular glycoprotein transport. The S-lac family is composed of soluble proteins (not integrated into membranes) which specifically bind lactose derivatives. Biological functions of the S-lac lectins remain unclear, but they, too, have been postulated to participate in specific cell-cell or cell-matrix recognition by binding complementary glycoconjugates (Barondes, 1984, 1988). Here we provide evidence for a biological role of the most extensively studied S-lac lectin, L-14, in regulating detachment of developing myoblasts from a laminin matrix during formation of tubular, multinucleate myofibers.

L-14 is a soluble, homodimeric, lactose-binding lectin

with subunit relative molecular weight of 14 kD. During development, it is expressed at high levels in many vertebrate tissues, including cardiac, smooth, and skeletal muscle, where it is prominent in adult extracellular matrix (Barondes, 1984; Cerra et al., 1984; Catt et al., 1987; Wasano et al., 1990; Cooper and Barondes, 1990). We have recently described developmentally regulated secretion of L-14 from cultured muscle by a novel apocrine pathway (Cooper and Barondes, 1990). Undifferentiated myoblasts synthesize L-14, but rapidly degrade the protein and do not secrete it. With the onset of differentiation expression of L-14 activity peaks (Den et al., 1976; Nowak et al., 1976), its turnover ceases, and it is secreted at the time of myoblast fusion (Cooper and Barondes, 1990). Immunohistochemistry suggests that L-14 is secreted by an unusual mechanism involving concentration in vesicular evaginations of plasma membrane which pinch off and release L-14 into the extracellular space (Cooper and Barondes, 1990). Ultimately, secreted L-14 accumulates with laminin in nascent basement membrane surrounding each myofiber.

Several studies have raised the possibility that secreted L-14 might interact functionally with laminin. Indeed, carbohydrate-dependent recognition of purified laminin has been recently demonstrated for L-14 (Zhou and Cummings, 1990), as well as for related S-lac lectins of 29 (Woo et al., 1990) and 67 kD (Hinek et al., 1988; Mecham et al., 1989). Such binding was anticipated, because L-14 shows high affinity for polylectosamine oligosaccharides (Leffler and Barondes, 1986; Abbott et al., 1988; Merkle and Cummings, 1988), and laminin is one of the few glycoproteins known to be glycosylated in this manner, at least as normally purified from Englebreth-Holm-Swarm mouse tumors (Arumugham et al., 1986; Fujiwara et al., 1988; Knibbs et al., 1989).

L-14 recognition of laminin suggests that it might partici-

1. *Abbreviations used in this paper:* LEC-CAM, lectin cell adhesion molecule; TDG, thiodigalactoside.

pate in laminin's various biological activities (Kleinman and Weeks, 1989). In developing muscle laminin is believed to play a prominent role in promoting myoblast adhesion, migration, proliferation, and differentiation (Foster et al., 1987; Ocalan et al., 1988; Goodman et al., 1989a,b; von der Mark and Ocalan, 1989). Actively replicating myoblasts migrate from somite myotomes into peripheral mesenchyme, differentiate, and fuse to form postmitotic multinuclear myofibers (Mayne and Sanderson, 1985). As they differentiate, myoblasts initially increase their adhesion to substrate laminin, but with further differentiation they ultimately lose adhesion and responsiveness to laminin before fusing into myofibers (Goodman et al., 1989a). Mature myofibers then become individually ensheathed in a continuous, laminin-rich basement membrane (Mayne and Sanderson, 1985; Sanes et al., 1986; Kuhl et al., 1982, 1984). These complex developmental processes are believed to involve regulated expression of multiple extracellular matrix molecules and receptors (Podleski et al., 1979; Menko and Boettiger, 1987; von der Mark and Ocalan, 1989), including at least two distinct laminin receptors (Knudsen, 1990; Mercurio, 1990; Lesot et al., 1983).

The coincidence of L-14 secretion with the onset of laminin deposition in muscle basement membrane and a reduction in muscle cell adhesion to laminin suggests that L-14 might be involved in regulating these processes. Binding of certain plant lectins to laminin oligosaccharides inhibits substrate attachment for a variety of cell lines (Dean et al., 1990; Bouzon et al., 1990). Therefore, we have considered the possibility that L-14 might act like the plant lectins, but in a physiologically germane manner, to regulate muscle cell interactions with matrix laminin.

To assess potential biological relevance of L-14 interaction with laminin during muscle development we have studied a myogenic mouse cell line to determine: (a) the relative prominence of muscle cell laminin as an endogenous ligand for L-14; (b) the degree to which oligosaccharides recognized by L-14 colocalize with laminin; (c) the effect of competitive saccharide on deposition of laminin and L-14 into extracellular matrix; and (d) any effects of L-14 on muscle cell adhesion to substrate laminin. The results presented here provide strong support for the hypothesis that L-14, secreted during muscle differentiation, binds specifically to poly-lactosamine oligosaccharides on substrate laminin and thereby promotes substrate detachment of differentiating myoblasts.

## Materials and Methods

### Purification of Recombinant L-14

Recombinant L-14 was synthesized in *Escherichia coli* using a pET expression vector kindly provided by Dr. F. W. Studier (Department of Biology, Brookhaven National Laboratory, Upton, NY). A rat L-14 cDNA was modified using PCR oligonucleotide-directed mutagenesis to place an NcoI site at the initial methionine, and this restriction site was used to ligate the full coding sequence into the NcoI site of pET3d (Studier et al., 1990). *E. coli* BL21(DE3) transformed with this plasmid expressed L-14 at high levels. Recombinant L-14 was purified from sonicated cell extracts by standard lactosyl-Sepharose affinity chromatography (Leffler et al., 1989). To stabilize L-14 against oxidative inactivation, recombinant L-14 was treated with iodoacetamide (Whitney et al., 1986), and the alkylated lectin was repurified by lactosyl-Sepharose affinity chromatography, followed by water elution (Teichberg et al., 1988) and filter sterilization. Protein concentration was determined by a dye-binding assay (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA).

## Cell Culture and Metabolic Labeling

C2C12 mouse myoblasts (Yaffe and Saxel, 1977; Blau et al., 1983) were cultured and induced to differentiate as previously described (Cooper and Barondes, 1990). L-14 and laminin secretion kinetics were studied using pulse-chase metabolic labeling following methods previously described (Cooper and Barondes, 1990). For overnight metabolic labeling, differentiating myotubes in 75-cm<sup>2</sup> flasks were cultured for 24 h in 10 ml complete differentiation medium supplemented with 1 mCi of <sup>35</sup>[S]methionine and <sup>35</sup>[S]cysteine (Translabel; ICN Radiochemicals, Irvine, CA). To distinguish secreted extracellular proteins from cellular proteins, the conditioned culture medium was gently collected and the cell layer was scraped from the flask with 10 ml of ice cold TBS (0.05 M Tris-HCl, pH 7.2, 0.15 M NaCl) including 1.0% Triton X-100, 50 µg/ml PMSF, and 5 mM EDTA. The collected medium was adjusted to 50 µg/ml PMSF, and centrifuged at 4°C for 30 min at 25,000 g. The supernatant was collected and the pellet saved. Medium and cell samples were immediately frozen in liquid nitrogen. After all time points had been collected, samples were thawed, the cell lysates were centrifuged for 1 h at 100,000 g at 4°C, and the supernatant was collected. This pellet and the conditioned medium pellet were re-extracted with 1 ml TBS including 4 M guanidine chloride and 10 mM EDTA. Pellet extracts, diluted 10-fold with TBS to reduce the guanidine-HCl concentration, yielded negligible amounts of labeled protein recognized by either antibody to laminin or an L-14 affinity column.

### L-14 Affinity Chromatography

To create an L-14 affinity column, recombinant L-14 was conjugated to cyanogen bromide activated Sepharose using standard techniques (Powell and Whitney, 1984). This matrix was used to purify cognate glycoconjugates from myotube cultures metabolically labeled with <sup>35</sup>[S]methionine and <sup>35</sup>[S]cysteine. Conditioned culture medium or cell extracts were passed over the L-14 column, which was then washed with several column volumes of TBS, 0.1% Triton X-100, followed by several column volumes with 0.1 M cellobiose (to control for non-specific sugar elution), and finally with 0.1 M lactose to elute glycoconjugates specifically bound by L-14. Labeled proteins in each fraction were detected by SDS-PAGE fluorography.

### Immunoprecipitation

Immunoprecipitation followed standard techniques using affinity-purified, cross-adsorbed antibody to L-14, as previously described (Cooper and Barondes, 1990), or affinity-purified antibody to mouse laminin (EY Labs, Inc., San Mateo, CA). Precipitates were analyzed by SDS-PAGE fluorography using a 20% gel for L-14 and a 5% gel for laminin.

### Histochemistry

Immunohistochemical techniques have been previously detailed (Cooper and Barondes, 1990). To localize laminin, glycoconjugate ligands for L-14, and endogenous L-14, C2C12 myoblasts were cultured on glass coverslips and differentiated for up to four days. To visualize both intracellular and extracellular localization, cultures were first fixed with 2% paraformaldehyde, 0.1% glutaraldehyde in PBS for 30 min at 4°C, blocked with 1 mM borohydride for 30 min, followed by 1% BSA (Sigma Chemical Co., St. Louis, MO; RIA grade) for 1 h, permeabilized with 0.2% Triton X-100, and then incubated with fluorescein-labeled L-14, antibody to laminin or L-14, and finally fluorescently labeled second antibody. L-14 (200 µg/ml in PBS, 1 mM mercaptoethanol) was labeled with fluorescein by overnight incubation at 4°C with 10 mM iodoacetamido-fluorescein (Molecular Probes, Inc., Eugene, OR). Labeled L-14 was repurified twice by lactosyl-Sepharose affinity chromatography using water elution (Teichberg et al., 1988).

### Assay of Cell Adhesion and Spreading on Laminin

C2C12 myoblasts, beginning to differentiate due to high population density (80-100% confluence), were harvested by brief trypsinization in the presence of EDTA. Trypsin was inactivated with bovine serum and the cells were washed in serum-free DME medium. Tissue culture wells of 96-well plates (Gibco Laboratories, Grand Island, NY) were pre-coated for 2 h with EHS laminin (>95% pure from Gibco BRL, Gaithersburg, MD) in PBS, and remaining protein binding sites were blocked for 2 h with 1% BSA (Sigma Chemical Co., RIA grade, heat inactivated for 20 min at 80°C). Most experiments were conducted with serum-free medium to obviate potential competitive inhibition by serum glycoproteins. 2 × 10<sup>4</sup> cells were added to each well in DME alone or with added L-14 or thiodigalactoside (TDG).

After incubation for various times, wells were either fixed immediately by addition of 4% paraformaldehyde, 1% glutaraldehyde, or fixed after non-adherent cells had been removed by gently washing twice with PBS. Adherent cells were stained overnight with 1% toluidine blue in 2% formaldehyde, PBS. For quantitation of inhibition of adhesion or spreading, stained cells were counted in a 0.156-mm<sup>2</sup> microscopic field, and counts were averaged for duplicate wells.

To test the role of laminin oligosaccharides in myoblast adhesion, poly-lactosamines were digested with endo- $\beta$ -galactosidase (*Escherichia freundii*), kindly supplied by Mishiko Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). Tissue culture wells, which had been adsorbed with laminin (40  $\mu$ g/ml) and blocked with BSA, were incubated overnight at 37° with 0.1 U/ml of glycosidase in 0.1 M sodium acetate (pH 5.0) with 0.05% BSA. Wells were then washed twice in DME with 1% BSA. Cell attachment and spreading was assessed, as above, two hours after adding trypsinized, washed myoblasts with or without 100  $\mu$ g/ml L-14 in DME with 1% BSA.

To assess affinity of glycosidase-digested laminin for L-14, the alkylated lectin was radiiodinated using <sup>125</sup>I-Bolton-Hunter reagent (ICN Radiochemicals) according to standard procedures (Leffler and Barondes, 1986) and repurified by lactosyl-Sepharose affinity chromatography. Immulon II microtiter wells (Removawell strips; Dynatech Laboratories, Inc., Chantilly, VA) were coated by incubation for two hours with 40  $\mu$ g/ml laminin, washed and blocked with 1% BSA in TBS, and digested with endo- $\beta$ -galactosidase as described above. Wells were then incubated overnight at 4° with 0.5  $\mu$ g/ml (0.6  $\mu$ Ci/ml) radiolabeled L-14 in 1% BSA, 0.2% Triton X-100, TBS, and quickly washed twice with the same buffer. Bound L-14 was then quantified by counting gamma emission. Non-specific binding, determined by inclusion of 10 mM TDG, was quite low and was subtracted to determine specific binding.

### Genetic Engineering and Cell Transfection

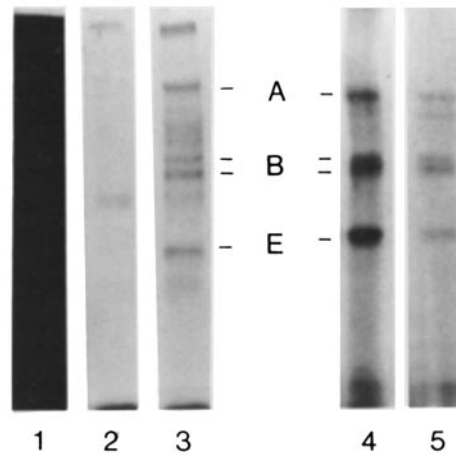
The secretion signal leader peptide from a human  $\alpha$ -1-antitrypsin cDNA (Rogers et al., 1983) was cleaved using TaqI and BamHI and ligated at the XmnI site of the 5' untranslated region of a rat L-14 cDNA to yield an in-frame fusion (leader-L-14) coding for the  $\alpha$ -1-antitrypsin 24 amino acid leader peptide, plus two amino acids beyond the signal peptidase cleavage site, 5 amino acids (LFA5I) not normally coded in either protein, followed by the complete L-14 coding sequence. The derived sequence was confirmed by double strand plasmid sequencing using standard dideoxynucleotide chain termination techniques.

To express the leader fused L-14 or the native L-14 cDNA, they were ligated into the pHBApR1 vector behind a human  $\beta$ -actin promoter, which drives high level expression in both myoblasts and myotubes (Gunning et al., 1987). Stably transfected clones were selected for neomycin resistance (Gunning et al., 1987) following co-transfection with a 20:1 ratio of the leader-L-14 or native L-14 plasmid expression vector and pRSVneo (Gorman, 1985) using standard calcium phosphate precipitation methods. Cultures were then selected in Geneticin (Gibco BRL), cloned by limiting dilution, and cultured in the absence of Geneticin for two weeks to eliminate possible drug effects. To identify transiently transfected cells, C2C12 myoblasts were co-transfected as above with a 20:1 ratio of the leader-L-14 or native L-14 plasmid expression vector in combination with the pHBApR1 vector including an *E. coli*  $\beta$ -galactosidase sequence (Picard and Yamamoto, 1987) kindly provided by Drs. F. Picard and K. Yamamoto (Department of Biophysics and Biochemistry, University of California, San Francisco). These cultures were histochemically stained for  $\beta$ -galactosidase activity (Goring et al., 1987) 48 h after transfection.

## Results

### Preparation of Recombinant L-14

Although L-14 is a relatively abundant protein, access to the large amounts of protein required for these studies was facilitated by preparation of recombinant L-14. Cloned rat L-14 was expressed in *E. coli* and purified as described in Materials and Methods. Silver staining of the purified lectin resolved by SDS-PAGE revealed only a single band. Comparison of recombinant L-14 with L-14 purified from rat muscle (Cooper and Barondes, 1990) revealed no differences detectable by HPLC peptide mapping and mass spectrometry, other than absence of NH<sub>2</sub>-terminal acylation, in accord



**Figure 1.** Identification of L-14 binding glycoproteins secreted by differentiated C2C12 cultures. An L-14-conjugated column was used to purify cognate glycoconjugates from conditioned culture medium from C2C12 myotubes metabolically labeled for 24 h with <sup>35</sup>[S]methionine and <sup>35</sup>[S]cysteine after one day of differentiation. Aliquots of fractions which flowed through the L-14 column (lane 1), eluted with 0.1 M cellobiose (to control for non-specific sugar elution) (lane 2), or subsequently eluted with 0.1 M lactose (lane 3) were resolved by 5% SDS-PAGE and fluorography. Aliquots of starting material (lane 4) and lactose-eluted fractions (lane 5) were also immunoprecipitated with affinity-purified anti-laminin. In each case aliquot volumes were adjusted to represent equivalent portions of the initial volume of conditioned medium. Positions are indicated for bands presumed to represent laminin A, B1 and B2 subunits (marked A and B) and the tightly associated protein entactin (marked E).

with previous reports (Hirabayashi et al., 1989; Couraud et al., 1989). This recombinant L-14 was used for all studies described below.

For use in cell culture, recombinant L-14 was alkylated with iodoacetamide (Whitney et al., 1986) to stabilize the lectin against oxidative inactivation of its carbohydrate-binding activity. Otherwise, L-14 is reported to be inactivated within 30 min in the oxidizing environment of tissue culture medium (MacBride and Przybylski, 1986; Whitney et al., 1986). The possibility is discussed below that extracellular oxidation might function biologically to limit the duration of activity of secreted L-14. The stabilized lectin remained dimeric even when diluted in tissue culture medium, as assessed by molecular sieve HPLC.

### L-14 Affinity Purification of Complementary Glycoproteins from C2C12 Myotubes

To determine the range of muscle glycoproteins recognized by L-14 and the relative prominence of laminin, recombinant L-14 conjugated to Sepharose (Powell and Whitney, 1984) was used to affinity purify ligands from C2C12 cultures metabolically labeled for 24 h with <sup>35</sup>[S]methionine and <sup>35</sup>[S]cysteine. For these experiments cultures were differentiated for only one day, because such immature myotubes synthesize and secrete both L-14 and laminin into the culture medium, but do not deposit them as nascent basement membrane until later in differentiation (Olwin and Hall, 1985; Cooper and Barondes, 1990). Thus, laminin purification at this stage should not be hampered by insolubility which is found at later stages.

Several radiolabeled proteins in myotube conditioned medium were bound to an L-14 affinity column and specifically eluted with lactose (Fig. 1). Following resolution by SDS-PAGE and fluorography, the most prominent bands had molecular weights suggestive of laminin A ( $\approx 400$  kD), B1 ( $\approx 220$  kD), and B2 ( $\approx 200$  kD) chains and the tightly associated protein entactin ( $\approx 150$  kD) (Kleinman and Weeks, 1989). In contrast to the medium, extracts of the myotube cell layers included no specifically bound ligands. It should be noted that other low affinity (Merkle and Cummings, 1988; Zhou and Cummings, 1990) or scarce glycoprotein ligands might be missed and that glycolipid ligands would not be detected by these methods.

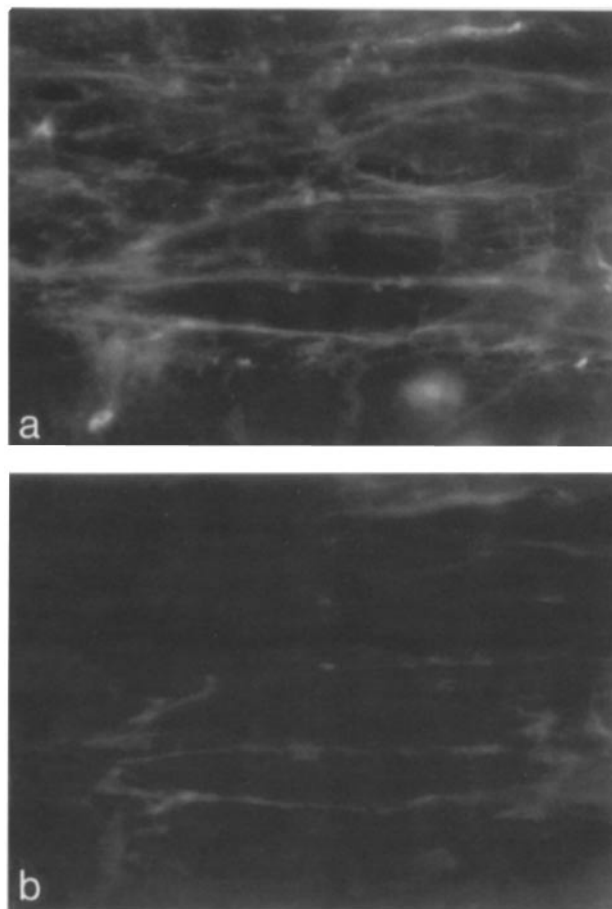
### **Laminin is a Major Glycoprotein Ligand for L-14 in Myotube Cultures**

Affinity purified antibody to the laminin complex was used to confirm the identity of the material purified from conditioned medium. Aliquots of unfractionated medium and lactose-eluted fractions were immunoprecipitated and resolved by SDS-PAGE and fluorography (Fig. 1). The major bands, as well as several of the minor bands, purified from the conditioned medium were specifically immunoprecipitated with this antibody. This confirms that laminin is at least one of the most abundant muscle glycoproteins recognized by L-14 and suggests that many of the minor bands eluted from the L-14 column might represent laminin degradation products. Bands with  $\approx 300$  and  $100$  kD appeared in the L-14 column eluate, but were not precipitated by anti-laminin. The intensity of these unidentified bands varied greatly in three separate experiments. Possible contribution of laminin variants (Kleinman and Weeks, 1989) has not yet been assessed.

### **Immunohistochemical Colocalization of L-14 Binding Sites with Laminin in Myotube Cultures**

The prominence of laminin as an L-14 ligand in mature myotubes was confirmed by histochemical comparison of the distributions of laminin and glycoconjugates recognized by L-14. For these experiments we studied C2C12 cultures differentiated for one to four days. Whereas laminin is secreted at high levels throughout myoblast differentiation, it is not efficiently deposited into nascent basement membranes until three to four days postfusion. Laminin can then be easily detected on the surface of mature myotubes by immunohistochemistry (Olwin and Hall, 1985; Cooper and Barondes, 1990). To localize binding sites for L-14, purified L-14 was conjugated with iodoacetamido-fluorescein, re-purified by lactose affinity chromatography, and used for fluorescence histochemistry.

Double-label staining with fluorescent L-14 and specific antibody to laminin revealed extensive co-distribution of laminin and glycoconjugate binding sites for L-14 on the surface of mature myotubes three to four days postfusion (Fig. 2). No intracellular binding of labeled L-14 was detected at any developmental stage, and no extracellular binding was detectable in cultures less than three days postfusion. Binding of the labeled L-14 was completely blocked by incubation in the presence of  $100$  mM TDG, the most potent of the readily available, simple saccharide ligands for this lectin. Thus, the major glycoconjugate binding sites for L-14 primarily coincide with laminin in basement membrane deposited

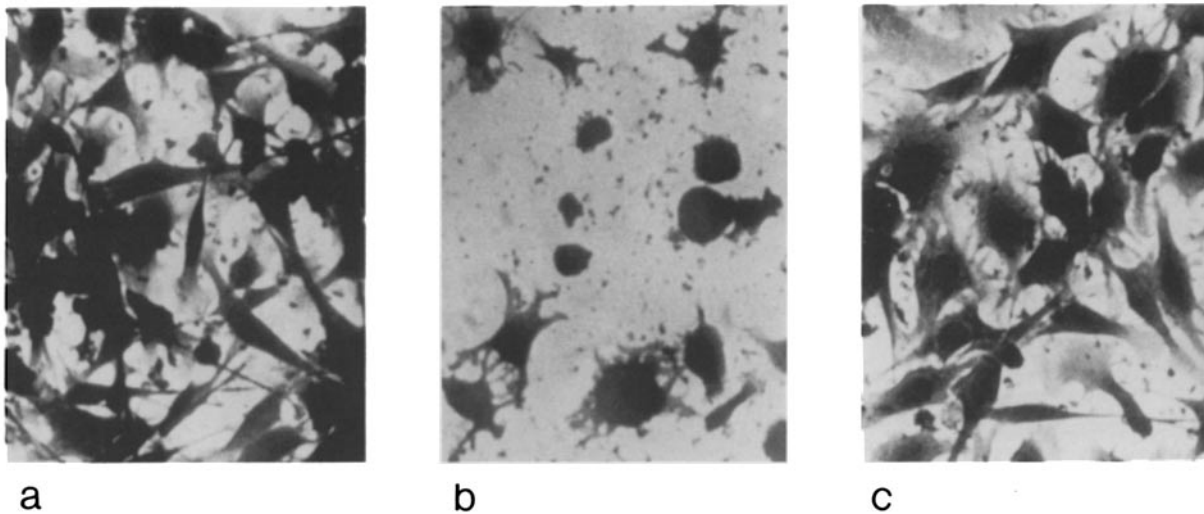


**Figure 2.** Comparison of the localization of laminin and glycoconjugates recognized by L-14. Differentiated C2C12 cultures were fixed, detergent permeabilized, and co-stained with rabbit anti-laminin, followed by rhodamine-conjugated goat anti-rabbit antibody (a) and fluorescein-L-14 (b).

on mature myotubes. Based on the affinity purification results described above, it seems likely that such co-localization reflects specific binding of the labeled L-14 to laminin oligosaccharides.

Endogenous L-14 also co-localizes with laminin in muscle basement membrane (Cooper and Barondes, 1990). This suggests that deposition of secreted L-14 in basement membrane is a result of specific recognition of laminin oligosaccharide chains. To test this we compared the intensity of immunohistochemical staining for endogenous L-14 in myotube matrix in cultures differentiated with or without  $40$  mM TDG in the medium. TDG inhibited matrix deposition of L-14 for two to three days postfusion. With further development to four days postfusion, L-14 accumulated with laminin in myotube matrix even in the presence of  $40$  mM TDG. These results suggest that L-14 matrix localization arises, at least initially, from binding to laminin oligosaccharides. Later in differentiation L-14 deposition either is not totally dependent on carbohydrate binding or the affinity of this potentially multivalent interaction becomes too great for effective competition by concentrations of TDG compatible with cell culture.

In these same experiments, no evidence was found for L-14 regulation of laminin deposition. Although faint laminin



**Figure 3.** L-14 inhibition of differentiating myoblast adhesion to laminin. Differentiating C2C12 myoblasts were incubated on tissue culture plastic precoated with 40  $\mu\text{g/ml}$  of EHS laminin and blocked with BSA. Cells were added to each well in DME alone (a) or with 100  $\mu\text{g/ml}$  L-14 without (b) or with (c) 10 mM TDG. After 2-h incubation, the wells were gently washed to remove non-adherent cells and then stained with toluidine blue and photographed.

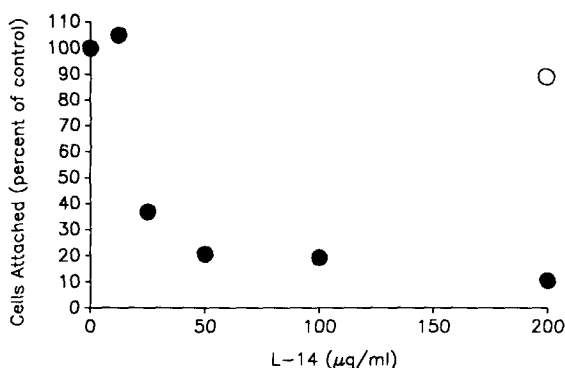
staining appeared on the plastic substrate at all developmental times, progressive accumulation on the surface of differentiating myotubes began before any detectable accumulation of L-14, and neither time of onset, extent, nor gross organization of laminin deposition was noticeably affected by differentiation in 40 mM TDG.

#### L-14 Inhibits Myoblast Adhesion to a Laminin Substrate

The affinity of L-14 for laminin and the prominent role of laminin as a substrate for myoblast adhesion suggest that L-14 might modulate substrate adhesion of differentiating myoblasts. To assess this hypothesis, stabilized recombinant L-14 was added to myoblasts cultured on a laminin-coated substrate. Myoblasts just beginning to differentiate because

of high density culture were used for these experiments, because myoblast attachment to limiting concentrations of laminin has been reported to peak during the first day of differentiation (Goodman et al., 1989a), which we confirmed for C2C12 cells. Adhesion was assayed two hours after plating, before cells would be expected to deposit their own substrate matrix. On plates coated for 2 h with concentrations of laminin ranging down to 40  $\mu\text{g/ml}$ , myoblasts attached and began spreading within 20 min and were well spread by one hour. Because adhesion and spreading decreased markedly with laminin coating concentrations below 40  $\mu\text{g/ml}$ , wells were coated using 40  $\mu\text{g/ml}$  for all subsequent experiments.

In the presence of added L-14, myoblast spreading and adhesion was strikingly inhibited. Gentle washing removed most cells incubated in the presence of 100  $\mu\text{g/ml}$  L-14, and

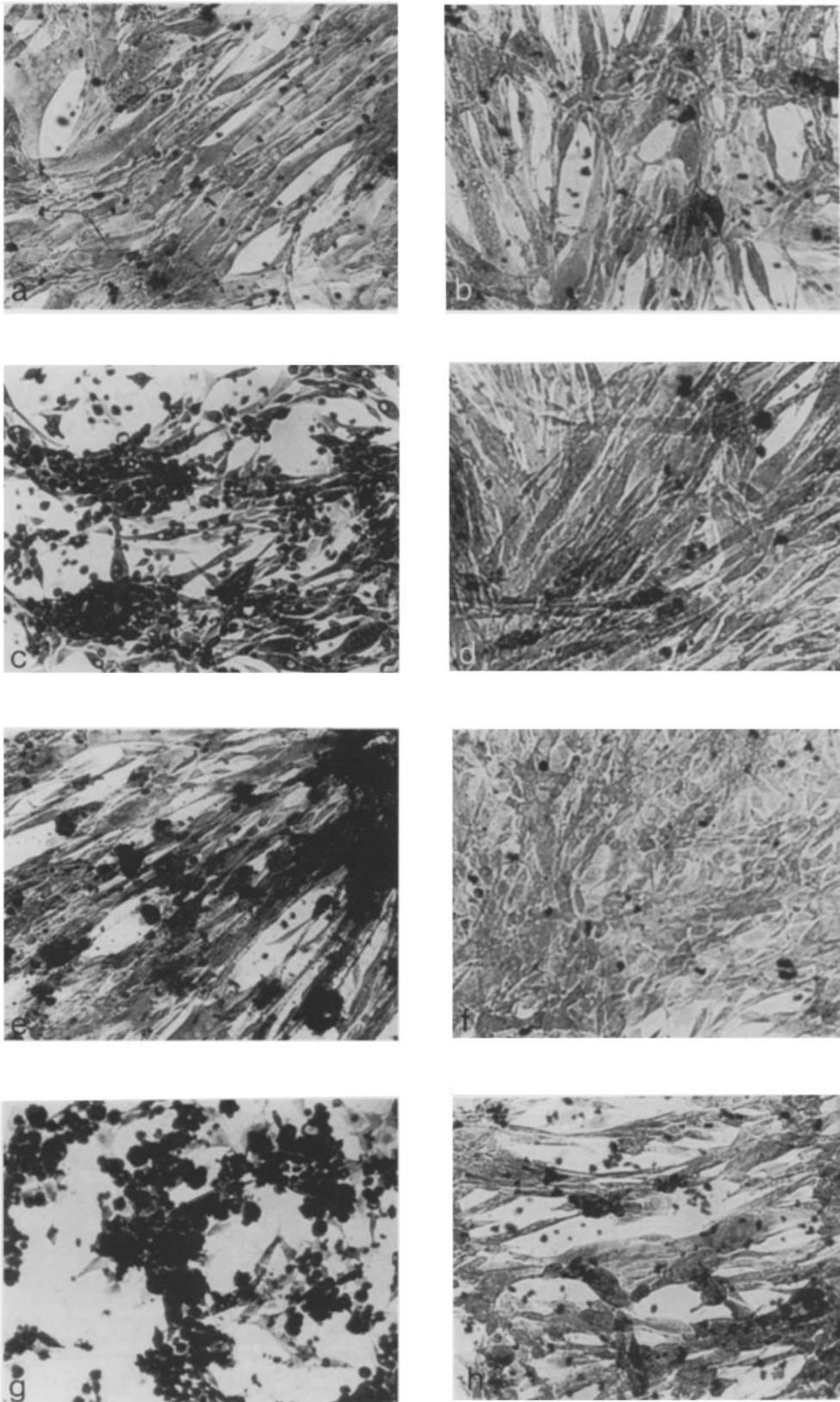


**Figure 4.** Quantitation of L-14 inhibition of C2C12 adhesion to laminin. C2C12 cells were assayed for adhesion to laminin in the presence of varying concentrations of L-14 without (●) or with 10 mM TDG (○). After 2-h incubation at 37°C, culture wells were washed to remove nonadherent cells, stained, and the number of remaining cells per microscopic field were counted. In the absence of L-14, counts averaged 690 cells per 100 $\times$  field. Counts of adherent cells in the presence of L-14 are expressed as a percentage compared to counts obtained in the absence of added L-14.

**Table I.** Percent of Normal or Transfected Myoblasts Which Spread on Untreated or Endo- $\beta$ -Galactosidase Digested Laminin and Effects of Added L-14 and TDG

Cell line	Untreated laminin		EndoB-Treated Laminin
	no addition	TDG	no addition
C2C12	50	50	60
C2C12 + L-14	5	60	30
Leader-L-14 transfected	5	40	70

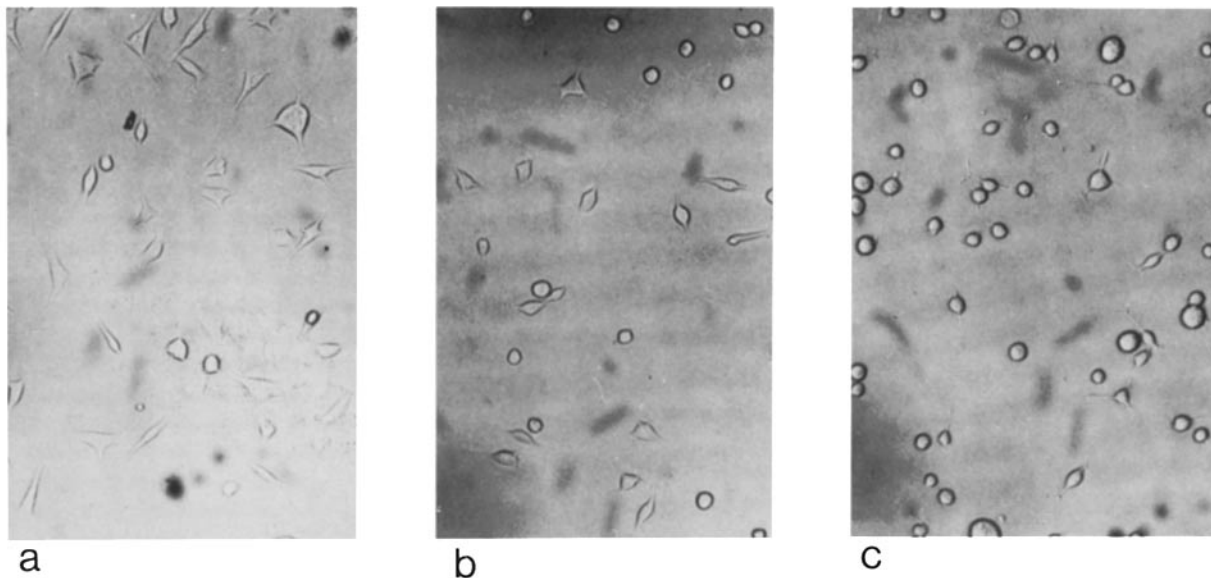
Effects on L-14 inhibition of myoblast adhesion were determined after glycosidase digestion of substrate-bound laminin. Tissue culture wells, which had been adsorbed with laminin and blocked with BSA were incubated overnight with 0.1 U/ml of endo- $\beta$ -galactosidase (EndoB). Wells were then washed free of enzyme with 1% BSA in DME, and  $2 \times 10^4$  trypsinized, washed myoblasts were added per well in 0.05% BSA, DME with or without 10 mM TDG. Myoblasts used were either standard C2C12 or a clonal line stably expressing transfected leader-L-14 at 200% of endogenous L-14 levels. Standard C2C12 myoblasts were also incubated with or without 100  $\mu\text{g/ml}$  L-14. After two hours incubation, cells were fixed, stained, and examined by light microscopy. Cells showing any degree of spreading were counted in a 0.156 mm<sup>2</sup> microscopic field. Counts were then adjusted for total well area and expressed as a percentage of the total input cells. Percentages for duplicate wells varied by <5% and are reported as rounded averages.



**Figure 5.** Effects of L-14 and TDG on morphology of differentiated C2C12 and clones expressing transfected leader-L-14. Normal C2C12 without added L-14 (*a* and *b*) or in 100  $\mu\text{g/ml}$  L-14 (*c* and *d*) and clones stably expressing leader-L-14 at 35% (*e* and *f*) or 200% (*g* and *h*) of the level of endogenous L-14 were differentiated without added saccharide (*a*, *c*, *e*, and *g*) or in the presence of 40 mM TDG (*b*, *d*, *f*, and *h*) on tissue culture plastic. Three days after induction of differentiation, cultures were fixed with formaldehyde, lightly stained with toluidine blue and photographed.

even those cells remaining bound appeared rounded and poorly adherent (Fig. 3). Counting bound cells over a range of L-14 concentrations revealed 50% inhibition of adhesion at 25  $\mu\text{g/ml}$  (Fig. 4). In contrast, adhesion to fibronectin or to untreated tissue culture plastic was not significantly affected by L-14.

L-14 inhibition of myoblast adhesion is dependent on carbohydrate binding, because 10 mM TDG blocked inhibition almost completely (Figs. 3 *c* and 4; Table I). In contrast, TDG by itself had no noticeable effect on adhesion or spreading on laminin. Furthermore, L-14 which had not been alkylated to prevent oxidative inactivation had little or no



**Figure 6.** Reduced adhesion to substrate laminin of C2C12 clones selected for stable expression of transfected leader-L-14. C2C12 clones selected for stable expression of transfected leader-L-14 were tested for their ability to adhere to a laminin-coated substrate before differentiation. Control clones not expressing leader-L-14 or standard C2C12 cells (a) were compared to clones stably expressing leader-L-14 at 35 (b) or 200% (c) of the level of endogenous L-14.

effect on myoblast adhesion. We attribute this to loss of carbohydrate-binding activity, because non-alkylated L-14 failed to rebind to lactosyl-Sepharose after incubation in tissue culture medium.

#### ***L-14 Inhibits Myoblast Adhesion and Fusion on Endogenously Synthesized Substrate***

Although L-14 clearly inhibited adhesion to an artificial laminin substrate, we were interested in whether it could influence adhesion to more natural substrates elaborated by the myoblasts themselves. To evaluate this we allowed cells to condition tissue culture plastic for one day, and then induced the cells to differentiate with or without added saccharide or L-14. When barely confluent cultures were induced to differentiate, over the next day the myoblasts appeared to decrease their substrate adhesion. They became less spread and more spindle-shaped, and many cells rounded up. With continued differentiation, the myoblasts aligned and fused to form increasingly large myotubes.

In the presence of 100  $\mu\text{g/ml}$  L-14, myoblasts appeared to behave normally during the first day of differentiation, but with further differentiation loss of attachment became progressively more pronounced. The cells became very rounded and showed little fusion even after three days (Fig. 5 b). Inclusion of 10 mM TDG blocked the accentuated detachment induced by L-14 (Fig. 5 c). Myoblasts differentiated in the presence of both L-14 and TDG behaved indistinguishably from those differentiating in TDG alone (Fig. 5 d), which had no apparent effect.

#### ***Premature Secretion of L-14 Inhibits Both Adhesion and Fusion of Differentiating Myoblasts***

As an alternative approach to assessing L-14 function, we engineered myoblasts to constitutively secrete the lectin regardless of differentiation. To accomplish this, we took advantage of the fact that the specialized apocrine pathway described for secretion of endogenous L-14 is developmentally

regulated (Cooper and Barondes, 1990), whereas myoblasts constitutively secrete other proteins by the standard signal sequence mediated pathway. Therefore, addition of a standard leader secretion sequence to L-14 would be expected to result in constitutive secretion of L-14 by the classical secretion pathway (Walter and Lingappa, 1986).

To add a standard secretion signal to L-14, we engineered a fusion of the leader sequence from human  $\alpha$ -1 antitrypsin (Kurachi et al., 1981; Verbanac and Heath, 1986) in frame to coding sequence of rat L-14 cDNA. Translation of message transcribed from this plasmid should yield the  $\alpha$ -1 antitrypsin 24 amino acid leader sequence plus two amino acids (ED) beyond the signal peptidase cleavage site, then five amino acids (LFAST) not normally coded in either protein, then the complete L-14 sequence, including the initial methionine. Even after cleavage of the leader peptide, the extra  $\text{NH}_2$ -terminal amino acids (EDLFASTIM) yield a sufficient increase in protein size to allow the fusion product to be distinguished from native L-14 by SDS-PAGE.

To express this leader-L-14 or the native L-14 cDNA, they were ligated into the pHBAPr1 vector, which uses a human  $\beta$ -actin promoter to drive high level expression in both myoblasts and myotubes (Gunning et al., 1987). Stably transfected clones were selected for Geneticin resistance following cotransfection with pRSVneo (Gunning et al., 1987).

To identify clones expressing the leader-L-14 fusion, undifferentiated cultures were metabolically pulse labeled, and both leader-L-14 and endogenous, native L-14 were roughly quantified by densitometry of fluorographs following immunoprecipitation and SDS-PAGE. Representative clones expressing leader-L-14 at 0, 10, 35, or 200% of the level of the endogenous L-14 were selected for further study. Pulse-chase studies showed that the leader signal peptide was rapidly cleaved and successfully directed constitutive secretion of the fused L-14 regardless of differentiation, in contrast to the endogenous L-14, which was still only secreted after differentiation (data not shown). After metabolic cleavage of the leader peptide, the fused L-14 was active

as a lectin, as assessed by purification on lactosyl-Sepharose. Pulse-chase metabolic labeling revealed no significant changes in laminin secretion kinetics for the transfected cells, either as myoblasts or myotubes (data not shown).

These myoblasts expressing leader-L-14 showed defects in adhesion and fusion similar to those described above for addition of purified L-14 to normal myoblast cultures. As undifferentiated myoblasts, synthesizing their own substratum on tissue culture plastic, all clones expressing leader-L-14 appeared to adhere and proliferate as well as control clones. However, as they proceeded to differentiate, the leader-L-14 expressing clones lost adhesion, rounded up and fused poorly. The extent of these defects was greater for clones expressing higher levels of leader-L-14 (Fig. 5 *e* and *g*). When tested for adhesion to a laminin-coated substrate, the leader-L-14 expressing clones adhered and spread poorly either as undifferentiated or differentiating myoblasts. Again, the level of impairment of laminin adhesion appeared to be greater for clones expressing higher levels of leader-L-14 (Fig. 6, *b* and *c*).

We infer that the impaired substrate adhesion of myoblasts expressing leader-L-14 is specifically due to binding of constitutively secreted L-14 to cognate glycoconjugates, because 40 mM TDG markedly improved cell adhesion and spreading. Improvement was observed for attachment to a laminin-coated substrate (Table I) as well as to endogenously conditioned substrate (Figure 5, *f* and *h*).

We nevertheless considered the possibility that defective adhesion of the leader-L-14 expressing clones might have arisen from chance isolation of unusual phenotypes unrelated to leader-L-14 expression. We took this seriously because standard C2C12 cultures are markedly heterogeneous and subclones display a variety of distinct phenotypes, which diverge with continued culture to again include a range of phenotypes. Therefore, specificity of the leader-L-14 induced phenotype was checked in an uncloned, unselected population after transient transfection.

Because only a small fraction of the cells in transfection cultures actually take up and express added DNA, it was necessary to mark those cells which were successfully transfected with the expression vector. To accomplish this we took advantage of the fact that transfection "competent" cells are able to incorporate multiple DNA fragments and, thus, can be simultaneously co-transfected with different plasmids (Cullen, 1987). By co-transfecting myoblast cultures with an expression vector for  $\beta$ -galactosidase at a 1:20 ratio to the leader-L-14 vector, virtually all cells identified as expressing  $\beta$ -galactosidase should also be expressing leader-L-14. Cells expressing  $\beta$ -galactosidase are easily distinguished by blue staining after histochemical reaction with X-gal (Goring et al., 1987).

In differentiating cultures transiently co-transfected with the leader-L-14 expression vector and the  $\beta$ -galactosidase expression vector >90% of those myoblasts which stained blue (indicating expression of transfected DNA) appeared rounded and poorly adherent to their endogenous substratum. In contrast, in cultures similarly transfected with the expression vector for  $\beta$ -galactosidase alone or in combination with a vector expressing unmodified rat L-14, blue stained myoblasts remained well spread. Thus, inhibition of cell adhesion was specifically obtained in cells transfected with the form of L-14 that is constitutively secreted.

### ***L-14 Inhibition of Myoblast Adhesion Is Dependent on Binding to Laminin Polylectosamines***

The inhibitory effect of L-14 on myoblast adhesion could be because of its interaction with glycoconjugates on either the substrate laminin or the myoblast surface. One way to distinguish these possibilities is to preincubate the laminin substrate with L-14 and then wash out unbound lectin before adding cells. Only the small amount of lectin initially bound to the laminin substrate would remain available to potentially interact with the myoblast surface. We found that when laminin was preincubated with 100  $\mu$ g/ml L-14, cell spreading was still inhibited during the first hour after adding myoblasts. Because <1% of added radio-iodinated L-14 bound to the laminin substrate under the same experimental conditions, the L-14 concentration remaining after washing out unbound lectin was well below the soluble amount required to inhibit cell adhesion. Thus, L-14 inhibition of adhesion must have been a result of its binding to the laminin substrate rather than to the myoblast surface.

Another way to distinguish these possibilities is to specifically block L-14 binding to the laminin, as opposed to the cells, by predigesting the coated substrate with endo- $\beta$ -galactosidase, which specifically cleaves polylectosamine chains (Fukuda et al., 1978). This treatment reduced binding of radio-iodinated L-14 to laminin by ~50% (data not shown). Myoblasts still adhered and spread on the glycosidase digested laminin to the same extent as found for non-digested laminin, but inhibition by added L-14 was greatly reduced (Table I). Again this shows that L-14 inhibition of myoblast attachment to laminin must be caused by binding to oligosaccharides on the laminin substrate.

We also found that laminin predigestion with endo- $\beta$ -galactosidase greatly improved adhesion and spreading of cell lines stably expressing leader-L-14. For the line expressing leader-L-14 at 200% the level of endogenous L-14, only 5% of the cells spread on untreated laminin, but 70% spread on the glycosidase pre-treated laminin (Table I). These results strongly support our conclusion that defective adhesion of these transfected cells is due to binding of secreted L-14 to substrate laminin, as opposed to some other effect of high level expression of the leader-L-14 construct or inadvertent cloning of cells with impaired adhesion because of an unrelated mutation.

### ***Discussion***

Although L-14 is an abundant and widely expressed protein which has been studied in a variety of species and tissues, convincing evidence for its involvement in defined biological functions has been elusive. L-14, has long been postulated to function in mediating cell-cell or cell-matrix interactions, and several recent studies have presented evidence for mitogenic (Sanford and Harris-Hooker, 1990), cytostatic (Wells and Mallucci, 1991), and immunomodulatory (Offner et al., 1990) activities. Such effects on animal cell growth and differentiation are familiar and useful properties of plant lectins (Sharon, 1987), but it remains unclear whether these are physiologically relevant activities of endogenous L-14.

The evidence presented here indicates that one of L-14's physiological functions is to modulate muscle cell adhesion to laminin. We show that L-14 has particular affinity for



laminin and becomes colocalized with laminin coincident with major changes in muscle cell interaction with laminin. Furthermore, one of these changes, decreased cell adhesion to laminin, can be prematurely induced, prolonged, and exaggerated by increasing the extracellular concentration of L-14 by addition of exogenous L-14 or by engineering constitutive secretion of L-14. Thus, we propose that one biological function of L-14 is to regulate developmental changes in muscle cell adhesion to matrix laminin.

Evidence that L-14 binds specifically to laminin in muscle basement membrane derives from three findings: (a) laminin represents a major fraction of glycoprotein ligands purified from myotube extracts by affinity chromatography on an L-14 conjugated column; (b) glycoconjugate ligands for L-14 primarily co-localize histochemically with both endogenous L-14 and laminin in myotube basement membrane; and (c) L-14 deposition in nascent basement membrane is inhibited when muscle cultures are differentiated in the presence of competitive saccharide.

Evidence that L-14 and laminin interact functionally during myoblast differentiation also derives from several experiments: (a) in short-duration experiments designed to test adhesion to a laminin substrate, addition of stabilized L-14 inhibits myoblast adhesion. This effect is blocked by inclusion of competitive saccharide or by enzymatic cleavage of laminin polylactosamine chains; (b) in this type of assay myoblasts genetically engineered to constitutively secrete L-14 also show defective adhesion and spreading, which are greatly improved by inclusion of competitive saccharide or by removal of laminin polylactosamines; (c) in longer duration assays designed to test adhesion to endogenously synthesized substrate, when normal C2C12 myoblasts are induced to differentiate while barely confluent, they appear to transiently decrease their substrate attachment in the process of forming tubular, multinucleate myofibers. Addition of L-14 exacerbates such differentiation-induced loss of attachment and also inhibits fusion. Again, this effect of L-14 is blocked by inclusion of competitive saccharide; and (d) myoblasts genetically engineered to constitutively secrete L-14 also show exaggerated differentiation-induced loss of substrate adhesion and impaired fusion, and both defects are improved by inclusion of competitive saccharide.

These results suggest that as myoblasts differentiate their substrate adhesion becomes dependent on laminin and inhibitable by L-14. The exact mechanism by which L-14 inhibits myoblast attachment to laminin remains unclear. As suggested for the inhibitory effects of certain plant lectins on cell adhesion to laminin (Dean et al., 1990; Bouzon et al., 1990), we presume that L-14 sterically inhibits laminin recognition by a cell surface receptor, but the relevant receptors and binding sites on laminin remain to be determined. Since antibodies to the integrin class of laminin receptors (Mercurio, 1990) inhibit myoblast substrate adhesion (Greve and Gottlieb, 1982; Neff et al., 1982; Menko and Boettiger, 1987), it may be that L-14 blocks laminin interaction with integrin. For certain other cell types, spreading on laminin has been reported to require laminin oligosaccharides (Dean et al., 1990), and in some cases this has been attributed to recognition of laminin polylactosamines by a cell surface galactosyltransferase (Shur, 1989). However, we found that differentiating myoblasts adhere and spread normally on laminin which has been digested with endo- $\beta$ -galactosidase.

Also, whereas cell spreading mediated by galactosyltransferase is inhibited by  $\alpha$ -lactalbumin (Shur, 1989), we have found no effect on myoblast attachment to laminin by  $\alpha$ -lactalbumin even at 5 mg/ml (unpublished results).

On the basis of our results we propose the following model for L-14 function in differentiating muscle: (a) developmental regulation of L-14 metabolism (Cooper and Barondes, 1990) might allow a rapid, large buildup of intracellular L-14, which is secreted only when cells reach an appropriate stage of differentiation; (b) the specialized apocrine secretion of L-14 (Cooper and Barondes, 1990) might serve to regulate extracellular delivery of accumulated L-14 while segregating it from laminin processed by the classical secretory pathway; (c) secreted L-14 might bind locally to previously deposited laminin and interfere with laminin recognition by cell surface receptors; and (d) consequent changes in myoblast adhesion to laminin might be critical for regulating a variety of cell behaviors during muscle development, including proper myoblast migration, alignment, and fusion.

Evidence for a role of L-14 in muscle fusion was first obtained more than a decade ago (Gartner and Podleski, 1976) at a time when cell-matrix interactions were poorly understood and laminin itself had not yet been discovered. Inhibition of muscle fusion by L-14 was confirmed in succeeding studies (Knudsen and Horowitz, 1978; MacBride and Przybylski, 1980; MacBride and Przybylski, 1986), but a range of evidence has been presented which argues against direct participation of L-14 in the fusion process (Den et al., 1976; Den and Chin, 1981; Kaufman and Lawless, 1980). Instead, our results suggest that effects of L-14 on fusion might be indirectly a result of altered cell adhesion to laminin. Perhaps during normal development the regulated secretion of L-14 facilitates myoblast fusion into tubular myofibers by promoting myoblast detachment from surrounding matrix.

Oxidative inactivation of L-14 carbohydrate-binding activity might function to limit the duration and physical range of its activity. Regulation of L-14 activity by extracellular oxidation might, thus, be comparable to regulation of alpha-1-antitrypsin activity by its oxidation sensitivity (Travis and Salvesen, 1983). While it is clear that purified L-14 must be stored under reducing conditions to retain activity, it is not known how rapidly L-14 is oxidized after secretion in vivo. For the in vitro assays reported here, protection of purified L-14 against oxidation was required for inhibition of cell adhesion, but genetically engineered constitutive secretion of L-14 also inhibited cell adhesion and that L-14 was not protected from oxidation.

For the assays used here, inhibition of myoblast adhesion to laminin requires a relatively high concentration of L-14 (in the range of 25  $\mu$ g/ml), which raises the question of whether such concentrations are ever obtained physiologically. It would be very difficult to measure the relevant concentration of active L-14 secreted between the cell surface and substratum. However, based on carbohydrate affinity purification from whole muscle tissue, estimates of L-14 concentration per gram wet weight of skeletal muscle have been near 10  $\mu$ g (0.1% of soluble protein) in adults and up to 50  $\mu$ g (0.5% of soluble protein) in embryonic muscle (de Waard et al., 1976; Nowak et al., 1977; Den and Malinzak, 1977; Childs and Ten Feizi, 1979; Beyer and Barondes, 1982; Cerra et al., 1985). Thus, even when calculated over

the total volume of muscle tissue, L-14 concentrations are on the order of those found here to inhibit myoblast adhesion to laminin. Furthermore, when one considers the high degree of enrichment of L-14 in extracellular matrix indicated by immunohistochemistry (Barondes and Haywood-Reid, 1981; Catt et al., 1987; Cooper and Barondes, 1990; Wasano et al., 1990), extracellular concentrations of L-14 could greatly exceed levels required to inhibit cell adhesion.

The role proposed here for L-14 in modulating cell-matrix interactions is similar to roles which have been indicated for other soluble lectins. For instance, during differentiation of the slime mold, *Dictyostelium discoideum*, a galactose-binding lectin, discoidin I, is secreted into extracellular matrix (Cooper and Barondes, 1984) where it binds to a polysaccharide ligand (Cooper et al., 1986) and modulates changes in cell-substratum adhesion important for developmental cell migration (Springer et al., 1984). In vertebrates, a soluble 67-kD lactose-binding lectin has been identified as one subunit of a cell surface receptor complex (Hinek et al., 1988) which mediates chemotaxis on laminin or elastin (Mecham et al., 1989). That complex has been suggested to influence both substratum attachment and detachment.

Like laminin itself, matrix-bound L-14 might functionally influence other muscle cell activities in addition to cell adhesion. For instance, L-14 is reported to modulate proliferation of certain cell types (Sanford and Harris-Hooker, 1990; Wells and Mallucci, 1991). Thus, L-14 might also influence muscle cell growth. An analogy can be drawn with fibroblast growth factor, which is also cytoplasmic, atypically secreted, and bound in a functional form to glycoconjugates in muscle extracellular matrix (Klagsbrun, 1990). However, neither we (unpublished data) nor other investigators (MacBride and Przybylski, 1980) have observed any effect of added L-14 on muscle cell proliferation. Furthermore, the doubling time of leader-L-14 expressing myoblast clones was not significantly altered. This is in marked contrast to a similar fusion of fibroblast growth factor behind a functional secretion signal, which causes neoplastic transformation of transfected 3T3 fibroblasts (Rogelj et al., 1988).

In other tissues where L-14 is prominent, it might function to modulate cell interaction with laminin or it might influence cell interactions with other glycoconjugates. For instance, L-14 expressed in motor and sensory neurons (Regan et al., 1986; Hynes et al., 1990) might influence laminin promotion of neurite outgrowth (Sephel et al., 1989). In placenta, which is rich in L-14, fibronectin is poly(lactosaminylated) (Zhu and Laine, 1985) and might functionally interact with the lectin. In chick embryonic skin a poly(lactosamine)-proteoglycan has been identified as a potential L-14 ligand (Oda and Kasai, 1984). Also, while we have observed inhibitory effects of L-14 on myoblast adhesion, it remains possible that in some tissues or under some conditions L-14, being divalent, might act as a bridge between matrix and cell surface glycoconjugates, thereby promoting cell adhesion. Thus, while the present results indicate a role for L-14 in promoting substrate detachment of differentiating myoblasts, regulated expression of L-14 and cognate glycoconjugates might influence cell behavior in many ways depending on tissue and developmental state.

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