Developmental regulation of neuraminidase-sensitive lectin-binding glycoproteins during myogenesis of rat L₆ myoblasts

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Intact monolayers of L₆ myoblasts were treated with neuraminidase, with the aim of selectively removing sialic acid residues of cell-surface glycoproteins. Neuraminidase treatment unmasked binding sites for *Ricinus communis* agglutinin I and peanut agglutinin, thus allowing the identification of the major binding proteins for these lectins. For *Ricinus communis* agglutinin I these neuraminidase-sensitive glycoproteins had apparent M_r values of 136 000, 115 000, 87 000, 83 000 and 49 000. For peanut agglutinin the major neuraminidase-sensitive glycoproteins had apparent M_r values of 200 000, 136 000, 87 000 and 83 000. We found highly reproducible, developmentally regulated, changes in the lectin-binding capacity of certain of these glycoproteins as L₆ myoblasts differentiated into myotubes. Coincident with myoblast fusion there was a co-ordinate decrease in *Ricinus communis* agglutinin I binding by glycoproteins of apparent M_r of 136 000 and 49 000. There was also a co-ordinate shift in mobility of the broad band of glycoprotein, centred at an apparent M_r of 115 000 in myoblasts, to a new average apparent M_r of 107 000 in mid-fusion cultures and myotube cultures. Peanut agglutinin binding by the major protein of apparent M_r 136 000 also decreased at the mid-fusion stage of myogenesis, and was barely detectable in 7-day-old fused cultures. These developmentally regulated changes in neuraminidase-sensitive glycoproteins were all inhibited by growth of myoblasts in 6.4 μ M-5-bromo-2'-deoxyuridine, indicating that they are associated with myoblast differentiation. In contrast, an increase in fibronectin was seen in mid-fusion cultures, which was not inhibited by growth of myoblasts in 5-bromo-2'-deoxyuridine. This initial increase in fibronectin is, therefore, unlikely to be directly related to myoblast fusion or differentiation.

During muscle differentiation, mononucleate muscle precursor cells, termed myoblasts, fuse to form multinucleate myotubes. The events surrounding these early stages of muscle differentiation have been intensively investigated in a number of laboratories. The transition from myoblast to myotube is normally associated with the activation of genes coding for proteins characteristic of the adult muscle phenotype (Devlin &

Emerson, 1978). Most emphasis to date has, therefore, been placed upon the co-ordinate synthesis of muscle contractile proteins as a model for the regulation of gene expression during eukaryotic cellular differentiation. Relatively little is known of the changes occurring at the surface membrane of myoblasts as they fuse to form myotubes.

Because of their well-documented role in cell-cell recognition, adhesion, and other cell-surface phenomena, it has been suggested that cell-surface glycoproteins may be directly involved in mediating myoblast alignment and fusion (Sanwal, 1979). Indirect evidence in support of this suggestion has come from studies showing that tunicamycin inhibits the fusion of chick embryonic myoblasts (Olden *et al.*, 1981) and rat L₆ myoblasts (Gilfix & Sanwal, 1980). To date, however, we have little

Abbreviations used: RCA I, *Ricinus communis* agglutinin I; PNA, peanut agglutinin; SDS, sodium dodecyl sulphate.

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knowledge of the glycoprotein composition of the myoblast or myotube plasma membrane, and whether such molecules are developmentally regulated.

Attempts have been made to identify changes in cell-surface glycoproteins during myogenesis using lactoperoxidase-catalysed iodination of exposed tyrosine residues (Hynes *et al.*, 1976; Moss *et al.*, 1978; Pauw & David, 1979; Cates & Holland, 1978, 1980; Walsh & Phillips, 1981). From these studies only one ^{125}I -labelled protein has been clearly shown to correspond to a cell-surface glycoprotein, namely fibronectin. With one exception (Chen, 1977) there is general agreement that fibronectin increases in amount at the cell surface either at, or immediately following, the time that myoblast fusion is initiated. In the G8-1 mouse muscle cell line it has been further demonstrated that as early myotubes mature there is then an overall decrease in cell-surface fibronectin (Walsh & Phillips, 1981). There is disagreement over the significance of fibronectin accumulation to the overall process of myogenesis. Exogenous fibronectin has been reported both to stimulate (Chen *et al.*, 1978) and to inhibit (Podleski *et al.*, 1979) myogenesis.

An extensive analysis of changes in cellular glycoproteins in the G8-1 mouse muscle cell line has been reported by Walsh & Phillips (1981). These authors used ^{125}I -labelled concanavalin A and wheat germ agglutinin to identify total cellular glycoproteins following their separation on SDS/polyacrylamide gels. Although a number of differences between myoblasts and myotubes were noted for the major concanavalin A and wheat germ agglutinin binding proteins in that study, it cannot be deduced whether the glycoproteins involved were present at the cell surface or intracellularly. Their precise relevance to differentiation of the cell surface is, therefore, uncertain.

In the present study we report the use of neuraminidase to unmask binding sites for lectins (Gordon & Pena, 1982) on L_6 myoblasts and myotubes. This procedure allowed identification of putative cell-surface glycoproteins, against the background of total cellular glycoprotein, and enabled us to demonstrate developmental regulation of certain of these glycoproteins, sensitive to inhibition of differentiation by growth of cells in 5-bromo-2'-deoxyuridine.

Experimental

Materials

Biotinylated RCA I and PNA, avidin and biotinyl-(horseradish peroxidase) were obtained from Vector Laboratories, Burlingame, CA, U.S.A. Neuraminidase (*Vibrio cholerae*) and goat

antiserum to chicken fibronectin were obtained from Calbiochem-Behring, San Diego, CA, U.S.A. Nitrocellulose paper and all electrophoresis reagents were obtained from Bio-Rad Laboratories (Canada), Mississauga, Ontario, Canada. All reagents for tissue culture were obtained from GIBCO (Canada), Burlington, Ontario, Canada. Rat L_6 myoblasts were obtained from Dr. B. D. Sanwal, University of Western Ontario, London, Ontario, Canada, and were a subclone, L6-9 (Ball *et al.*, 1979), of the line originally isolated by Yaffe (1968). 5-Bromo-2'-deoxyuridine was obtained from PL Laboratories Inc., Milwaukee, WI, U.S.A. All other reagents used were obtained from Sigma.

Cultures

Rat L_6 myoblasts were maintained in culture at 37°C and 5% CO_2 in Dulbecco's-modified Eagle's medium supplemented with 10% (v/v) horse serum. Cells were routinely plated at an initial density of 6×10^4 cells/60 mm diameter plate. Propagation of cells by subculture was carried out by detachment of cells, 3 days after plating, with 0.1% trypsin in Ca^{2+} - and Mg^{2+} -free Earle's balanced salt solution, and by replating in the standard growth medium described above. When present, 5-bromo-2'-deoxyuridine was added to a final concentration of $6.4 \mu\text{M}$ on the first day of culture. All studies reported here were carried out on cells before passage 10 of the stock obtained from Dr. Sanwal.

Neuraminidase treatment of L_6 monolayers

At various times after plating, monolayers of L_6 cells in 60 mm plates were rinsed twice with 5 ml of Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954), warmed to 37°C . They were then incubated with a solution of neuraminidase (40 units/ml) in 0.05 M-sodium acetate buffer, pH 5.5, containing 0.15 M-NaCl, at a temperature of 37°C . After 30 min incubation the cells were rinsed twice with 5 ml of Dulbecco's phosphate-buffered saline and were solubilized for 5 min at 100°C in 0.125 M-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol and 0.01% Bromophenol Blue. Control cells were treated identically except that neuraminidase was omitted from the sodium acetate buffer.

Protein

Protein concentration in the samples was determined by the method of Lowry *et al.* (1951), following the precipitation of protein with 5 vol. of cold acetone to remove SDS. Samples corresponding to $60 \mu\text{g}$ of protein were then subjected to electrophoresis.

Electrophoresis

SDS/polyacrylamide-slab-gel electrophoresis was performed with 1.5 mm slab gels (5% stacking gel and 10% separating gel) with the discontinuous buffer system of Laemmli (1970). Gels were electrophoresed for 4 h at a constant current of 30 mA/gel. They were then either fixed and stained for protein, by the method of Fairbanks *et al.* (1971), or the proteins were electrophoretically transferred to nitrocellulose, without prior fixation, as described below.

Electrophoretic transfer of proteins to nitrocellulose sheets

This was carried out essentially as described by Towbin *et al.* (1979). Transfer was performed at 75 mA for 18 h in 0.025 M-Tris/0.192 M-glycine, pH 8.3.

Staining of glycoproteins on nitrocellulose with biotinyl-lectins and biotinyl-antibodies

This was carried out as described by Gordon & Pena (1982) for biotinyl-lectins. For the detection of fibronectin with goat anti-(chicken fibronectin), nitrocellulose blots were blocked with excess bovine serum albumin and were incubated overnight with antiserum diluted 200-fold in 137 mM-NaCl/2.7 mM-KCl/0.9 mM-CaCl₂/0.5 mM-MgCl₂/30 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4 (HBS). After being washed four times with HBS for 30 min at room temperature, the nitrocellulose blots were then reacted for 2 h at room temperature with biotinyl rabbit anti-(goat immunoglobulin G) diluted 100-fold in HBS. The nitrocellulose blots were then washed again with HBS as described above and were incubated with preformed avidin-biotinyl-peroxidase complexes and stained with 3,3'-diaminobenzidine as described by Gordon & Pena (1982).

M_r markers

Apparent M_r values of glycoproteins were calculated with reference to the mobility of the following proteins, included in each SDS/polyacrylamide-gel electrophoresis run: myosin heavy chain (200 000); phosphorylase *b* (97 500); bovine serum albumin (68 000) and ovalbumin (43 000).

Results

Effects of neuraminidase treatment on the protein composition of L₆ homogenates

We first examined the effect of neuraminidase treatment of L₆ cell monolayers, at various stages of myogenesis, on the protein profile of homogenates to be sure that this was not significantly

Fig. 1. Protein composition of L₆ cells at various stages of myogenesis

Rat L₆ myoblasts were plated in standard growth medium and harvested at 2 days (pre-fusion), 5 days (mid-fusion) and 7 days (post-fusion) after plating. The cells were either harvested directly, or were treated with neuraminidase before harvesting as described in the Experimental section. Samples (60 µg of protein) were then subjected to SDS/polyacrylamide-slab-gel electrophoresis and were fixed and stained for protein by the method of Fairbanks *et al.* (1971). Lanes (a), (c) and (e) correspond to 2, 5 and 7 day neuraminidase-treated cultures respectively. Lanes (b), (d) and (f) correspond to 2, 5 and 7 day cultures respectively, harvested directly into sample buffer without neuraminidase treatment. The numbers on the left indicate the apparent M_r values of the markers described in the Experimental section.

altered by contaminating proteinases in the neuraminidase. Monolayers were treated with neuraminidase at 2, 5 and 7 days of culture. These time points correspond to the pre-fusion, mid-fusion and early post-fusion stages of myogenesis respectively. Analysis of protein profiles by Coomassie Blue staining revealed no detectable alteration at any stage of myogenesis as a consequence of neuraminidase treatment (Fig. 1). Neuraminidase-treated cells also remained viable, as judged by

Fig. 2. Detection of glycoproteins by RCA I binding to proteins of neuraminidase-treated cells

Cultures of L₆ myoblasts were harvested 2, 5 and 7 days after plating in standard growth medium. Samples (60 µg of protein) were subjected to SDS/polyacrylamide-gel electrophoresis, and were transferred directly to nitrocellulose. The nitrocellulose blots were then incubated sequentially with biotinyl-(RCA I), avidin-biotinyl-peroxidase complexes and 3,3'-diaminobenzidine, as described in the Experimental section. Lanes (a), (c) and (e) correspond to 2, 5 and 7 day neuraminidase-treated cultures respectively. Lanes (b), (d) and (f) correspond to 2, 5 and 7 day cultures respectively, which were solubilized directly in sample buffer without prior neuraminidase treatment. The numbers on the left indicate apparent M_r values, calculated with reference to the markers described in the Experimental section.

their ability to exclude the dye Trypan Blue (results not shown).

Ricinus communis agglutinin I receptors

In control cells, not treated with neuraminidase, there was very little binding of RCA I to cellular glycoproteins at any stage of myogenesis examined (Figs. 2b, 2d and 2f). The major RCA I binding protein in 2 day L₆ myoblasts had an apparent M_r of 49 000 (Fig. 2b). This binding markedly decreased in extent in 5-day mid-fusion cultures and 7 day cultures (Figs. 2d and 2f). An increase in the extent of RCA I binding by a glycoprotein doublet at an apparent M_r of approx. 220 000 was also seen at the mid-fusion time point in untreated cells (Fig. 2d).

On treatment of intact monolayers with neuraminidase, major RCA I-binding proteins of apparent M_r values 136 000, 115 000, 87 000 and 83 000

were unmasked (Figs. 2a, 2c and 2e). We have tentatively assigned these glycoproteins a cell-surface location. The previously mentioned proteins of apparent M_r 220 000 and 49 000 may also be cell-surface glycoproteins, since their binding of RCA I was enhanced significantly by neuraminidase treatment of intact cells (compare Fig. 2a with Fig. 2b, and Fig. 2c with Fig. 2d). In neuraminidase-treated cells the major band of RCA I-binding protein, with an average apparent M_r of 150 000, was broad, indicating a heterogeneity of glycoproteins within this band. Comparison of neuraminidase-treated pre-fusion and early post-fusion cultures (Figs. 2a and 2e) reveals a distinct shift in the mobility of this heterogeneous band of glycoprotein, to an average apparent M_r of 107 000. This was accompanied by an increase in RCA I binding by the band at M_r approx. 220 000, and loss of RCA I-binding proteins of apparent M_r values 136 000 and 49 000. Other RCA I-binding proteins were essentially invariant.

Peanut agglutinin receptors

In the absence of neuraminidase treatment, L₆ cells at all stages of myogenesis examined had many PNA-binding proteins (Fig. 3). No reproducible or significant change in these glycoproteins could be detected in association with myoblast differentiation.

After neuraminidase treatment of 2-day cultures, a major PNA-binding protein of apparent M_r 136 000, and less prominent proteins of apparent M_r values 200 000, 87 000 and 83 000 were unmasked (Figs. 2a and 2b). Of these only the band of apparent M_r 136 000 appeared to be developmentally regulated. Lectin binding in this M_r region decreased in association with the transition from the pre-fusion to the mid-fusion stage of myogenesis (compare Fig. 3a with Fig. 3c). Faint staining of a band of protein at an apparent M_r of 220 000 was also seen in neuraminidase-treated 5-day and 7-day cultures.

All neuraminidase-sensitive and developmentally regulated changes in the lectin-binding capacity of the glycoproteins described above were reproducible in six repeat experiments on different sets of cultures, using both lectins.

Effect of 5-bromo-2'-deoxyuridine on lectin-binding glycoproteins

L₆ myoblasts were grown continuously from the time of plating in standard growth medium containing 6.4 µM-5-bromo-2'-deoxyuridine. Under these conditions myoblast fusion was completely inhibited (Stockdale *et al.*, 1964). Comparison of control and 5-bromo-2'-deoxyuridine-treated cells stained with RCA I and PNA is shown in Figs. 4 and 5 respectively. It can be seen

Fig. 3. Detection of glycoproteins by PNA binding to proteins of neuraminidase-treated cells

Cultures of L₆ myoblasts were harvested 2, 5 and 7 days after plating in standard growth medium. Samples (60 µg of protein) were subjected to SDS/polyacrylamide-gel electrophoresis, and were transferred directly to nitrocellulose. The nitrocellulose blots were then incubated sequentially with biotinyl-PNA, avidin-biotinyl-peroxidase complexes and 3,3'-diaminobenzidine, as described in the Experimental section. Lanes (a), (c) and (e) correspond to 2, 5 and 7 day neuraminidase-treated cultures respectively. Lanes (b), (d) and (f) correspond to 2, 5 and 7 day cultures respectively, which were solubilized directly in sample buffer without prior neuraminidase treatment. The numbers on the left indicate apparent M_r values, calculated with reference to the markers described in the Experimental section.

that the transition from the myoblast to the myotube phenotype (lanes c, g and k) was essentially prevented by growth in 5-bromo-2'-deoxyuridine for glycoproteins reactive with both lectins (lanes e, i and m). Comparison of Figs. 4 and 5 (lanes c and m) shows that the phenotype of 7-day 5-bromo-2'-deoxyuridine-treated cells (lanes m) is virtually identical with that of 2-day control cultures (lanes c) and quite distinct from that of 7-day control cultures (lane k), with the exception of the band of glycoprotein at an apparent M_r of 220 000. Lanes (a) and (b) of Figs. 4 and 5 demonstrate the trypsin susceptibility of the major neuraminidase-sensitive RCA I and PNA-binding proteins. Although this is consistent with a cell-surface location for these glycoproteins, trypsin

treatment of intact cells may, under some conditions, stimulate the release of secretory glycoproteins (Jett & Jamieson, 1973), and caution must be used in the interpretation of this particular result.

Fibronectin

The doublet of RCA I-binding protein at an apparent M_r of 200 000 corresponds in mobility to fibronectin. This protein reacts only weakly with PNA. It was verified that this glycoprotein is fibronectin by its binding of anti-fibronectin antibody (Fig. 6). This also provides confirmation that, in contrast with the other developmentally regulated glycoproteins detected in this study, the increase in fibronectin associated with early stages of myoblast differentiation was not inhibited by 5-bromo-2'-deoxyuridine. In fact, a moderate but reproducible enhancement of fibronectin accumulation was seen in 5-day and 7-day 5-bromo-2'-deoxyuridine-treated cultures, compared with untreated controls. The diffuse and irregular staining of all tracks seen at an M_r of approx. 70 000 in Fig. 6 is due to non-specific staining of the nitrocellulose blot. This band of non-specific staining was also observed on immunochemical staining of samples with monospecific antisera against chick skeletal myosin heavy chain and against 2,4-dinitrophenol (results not shown).

Discussion

With the exception of the increase in fibronectin concentration at the mid-fusion stage of myogenesis, all developmentally regulated changes in lectin-binding proteins were inhibited by growth in 5-bromo-2'-deoxyuridine. This suggests that these changes are a consequence of myoblast differentiation.

Although growth of myoblasts in 5-bromo-2'-deoxyuridine did not prevent the increase in fibronectin accumulation normally seen at mid-fusion, it did reproducibly increase the overall amount of fibronectin accumulated by 7-day cultures (Figs. 4 and 6). Some enhancement of fibronectin accumulation has also been observed on 5-bromo-2'-deoxyuridine treatment of primary cultures of chick embryonic muscle (Chiquet *et al.*, 1981). The possible contribution of fibroblasts, in primary muscle cultures, to the overall pool of fibronectin complicates the interpretation of such data. Nevertheless, these observations are consistent with our findings in the cloned L₆ muscle cell line.

We conclude that the initial increase in fibronectin accumulation associated with the mid-fusion stage of myogenesis (Hynes *et al.*, 1976; Moss *et al.*, 1978; Cates & Holland, 1978, 1980; Pauw &

Fig. 4. Effect of 5-bromo-2'-deoxyuridine on the accumulation of RCA I-binding glycoproteins during myogenesis. Rat L₆ myoblasts were plated in standard growth medium. When present, 5-bromo-2'-deoxyuridine was added to a final concentration of 6.4 μ M at the time of plating. At various times after plating, the cultures were harvested by direct solubilization in electrophoresis sample buffer. Samples (60 μ g of protein) were subjected to SDS/polyacrylamide electrophoresis and were then transferred to nitrocellulose. RCA I-binding proteins were detected by incubation with biotinyl-(RCA I) as described in the legend to Fig. 2. Lane (a), 2-day neuraminidase-treated cultures, incubated with 0.1% trypsin for 10 min prior to harvesting; lane (b), 2-day cultures incubated with 0.1% trypsin for 10 min prior to harvesting; lane (c), 2-day neuraminidase-treated cultures; lane (d), 2-day cultures; lane (e), 2-day neuraminidase-treated cultures grown in 5-bromo-2'-deoxyuridine; lane (f), 2-day cultures grown in 5-bromo-2'-deoxyuridine; lane (g), 5-day neuraminidase-treated cultures; lane (h), 5-day cultures; lane (i), 5-day neuraminidase-treated cultures grown in 5-bromo-2'-deoxyuridine; lane (j), 5-day cultures grown in 5-bromo-2'-deoxyuridine; lane (k), 7-day neuraminidase-treated cultures; lane (l), 7-day cultures; lane (m), 7-day neuraminidase-treated cultures grown in 5-bromo-2'-deoxyuridine; lane (n), 7-day cultures grown in 5-bromo-2'-deoxyuridine. The numbers on the left indicate apparent M_r values calculated with reference to the markers described in the Experimental section.

David, 1979) is not inhibited by 5-bromo-2'-deoxyuridine, and is not directly related to myoblast differentiation. In contrast a stabilization of fibronectin levels, which normally occurs at later stages of myogenesis (Gardner & Fambrough, 1983; Walsh & Phillips, 1981; Podleski *et al.*, 1979) is prevented by growth in 5-bromo-2'-deoxyuridine, and this may therefore represent a specific, differentiation-related, adaptation of the myotube surface membrane.

We have detected glycoproteins in the present study by their ability to bind lectins. Developmentally regulated changes in the lectin-binding capacity of individual glycoprotein bands could be due to changes in their concentration, or in their extent of glycosylation, or in the accessibility of the glycoprotein to exogenous neuraminidase. Regardless of the precise molecular mechanism behind the altered lectin-binding observed, the present results suggest that the biological activity of these glycoproteins is regulated during myogenesis.

It must also be noted that although the addition of exogenous neuraminidase to intact cells *in vitro* should cleave sialic acid only from cell-surface glycoproteins, we cannot entirely exclude the possibility that some of the enzyme may enter the cells. Our assignment of neuraminidase-sensitive glycoproteins to the cell surface can therefore be only tentative at present. Greater than 97% of the cells excluded Trypan Blue following neuraminidase treatment, ruling out significant influx of the enzyme as a consequence of cell damage. Baumann & Doyle (1979) have presented evidence in support of the use of neuraminidase as an agent for the selective modification of cell-surface glycoproteins. These authors found that two pools of neuraminidase-sensitive glycoprotein existed in rat hepatoma cells. One pool was readily modified by neuraminidase treatment of intact cells, and was deduced to be present at the cell surface. The second pool of glycoprotein was resistant to even prolonged digestion of intact cells with neurami-

Fig. 5. Effect of 5-bromo-2'-deoxyuridine on the accumulation of PNA-binding glycoproteins during myogenesis. Rat L₆ myoblasts were plated in standard growth medium. When present, 5-bromo-2'-deoxyuridine was added to a final concentration of 6.4 μ M at the time of plating. At various times after plating, the cultures were harvested by direct solubilization in electrophoresis sample buffer. Samples (60 μ g of protein) were subjected to SDS/polyacrylamide-slab-gel electrophoresis and were then transferred to nitrocellulose. PNA-binding proteins were detected by incubation with biotinyl-PNA as described in the legend to Fig. 2. Lane (a), 2-day neuraminidase-treated cultures, incubated with 0.1% trypsin for 10 min prior to harvesting; lane (b), 2-day cultures incubated with 0.1% trypsin for 10 min prior to harvesting; lane (c), 2-day neuraminidase-treated cultures; lane (d), 2-day cultures; lane (e), 2-day neuraminidase-treated cultures grown in 5-bromo-2'-deoxyuridine; lane (f), 2-day cultures grown in 5-bromo-2'-deoxyuridine; lane (g), 5-day neuraminidase-treated cultures; lane (h), 5-day cultures; lane (i), 5-day neuraminidase-treated cultures grown in 5-bromo-2'-deoxyuridine; lane (j), 5-day cultures grown in 5-bromo-2'-deoxyuridine; lane (k), 7-day neuraminidase-treated cultures; lane (l), 7-day cultures; lane (m), 7-day neuraminidase-treated cultures grown in 5-bromo-2'-deoxyuridine; lane (n), 7-day cultures grown in 5-bromo-2'-deoxyuridine. The numbers on the left indicate apparent M_r values calculated with reference to the markers described in the Experimental section.

dase and was only modified upon incubation of the isolated glycoproteins with neuraminidase *in vitro*. This was therefore deduced to represent intracellular glycoprotein, protected from exogenous neuraminidase in the intact cell.

Three categories of developmental regulation of lectin-binding proteins can be recognized from the present study. These are: (1) proteins that increase in lectin-binding capacity around the time of myoblast fusion; (2) proteins that decrease in lectin-binding capacity around the time of fusion; (3) lectin-binding proteins that are invariant throughout myogenesis.

The only protein that fits clearly into category (1) is fibronectin. The shift in average M_r of the major RCA I-binding glycoprotein from an apparent M_r of 115000 in myoblasts to 107000 in myotubes (Figs. 2 and 4) could also reflect the appearance of a novel glycoprotein which could be placed in this category. It is not possible to tell from the present data whether this represents synthesis *de novo* of a

novel, fusion-related glycoprotein, or post-translational modification of a pre-existing pool of glycoprotein of slightly higher M_r .

Proteins that decreased in lectin-binding capacity during myogenesis had apparent M_r values of 136000 (PNA and RCA I) and 49000 (RCA I). Lectin-binding proteins of apparent M_r values 200000 (PNA), 87000 and 83000 (PNA and RCA I) were invariant during myogenesis.

Similar diverse modes of regulation of cellular glycoprotein accumulation have been reported by Walsh & Phillips (1981) in the mouse skeletal muscle G8-1 cell line. These authors used NaB³H₄/periodate to detect sialoglycoproteins that had similar apparent M_r values to the neuraminidase-sensitive glycoproteins detected in the present study (200000, 115000, 80000–82000, 49000). All these proteins were, however, present at all stages of myogenesis in G8-1 cells. This contrasts with our findings for the proteins of apparent M_r values 115000 and 49000, which shifted in mobility and

Fig. 6. Effect of 5-bromo-2'-deoxyuridine on fibronectin accumulation

Rat L₆ myoblasts were grown in standard growth medium. When present 5-bromo-2'-deoxyuridine was added to a final concentration of 6.4 μ M. Cells were harvested at various stages of myogenesis by direct solubilization in electrophoresis sample buffer. Samples (60 μ g of protein) were subjected to SDS/polyacrylamide-slab-gel electrophoresis and were transferred to nitrocellulose. Fibronectin was detected by the binding of goat anti-(chick fibronectin) to the nitrocellulose blots as described in the Experimental section. Lane (a), 2-day cultures; lane (b), 2-day cultures grown in 5-bromo-2'-deoxyuridine; lane (c), 5-day cultures; lane (d), 5-day cultures grown in 5-bromo-2'-deoxyuridine; lane (e), 7-day cultures; lane (f), 7-day cultures grown in 5-bromo-2'-deoxyuridine.

decreased in lectin-binding capacity respectively, during myogenesis of the rat L₆ muscle cell line. Walsh & Phillips (1981) also detected four sialoglycoproteins that appeared myotube-specific. With the exception of the shift in mobility of the RCA I-binding protein, from an average M_r of 115000 to 107000 in mid-fusion cultures, we saw no evidence of similar myotube-specific components in the L₆ cell line. This could be due to the higher degree of myotube differentiation attained in G8-1 cultures. However, the myotube-specific components detected by Walsh & Phillips (1981) were present at the earliest stages of myotube formation examined. It may be that the lectins used in the present study select for a sub-class of glycoproteins that tend to be invariant or to decrease throughout myogenesis.

At present we can only speculate on the significance of these changes in L₆ glycoproteins to the overall process of myogenesis. Other reports suggest that many myoblast cell-surface components decrease in concentration, or in their extent of surface labelling, during myogenesis (Grove *et al.*, 1981; Cates & Holland, 1980). Bischoff (1978) has suggested that the existence of an extensive glycocalyx on myoblasts could prevent the lipid bilayers of fusion-competent cells from coming into the close proximity required for membrane union. The decrease in mid-fusion cultures in lectin-binding proteins, seen in this study, could reflect such a need for removal of the bulk of the myoblast glycocalyx prior to fusion. Further investigation of the glycoproteins constituting the diffuse band of RCA I-binding protein at an apparent M_r of 115000 in myoblasts may prove profitable. The present study suggests that a novel molecular species of glycoprotein appears in this region at the mid-fusion stage of myogenesis. It remains to be established whether such changes may be directly associated with myoblast fusion, or with some other aspect of membrane differentiation.

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