Biosynthesis of Plasma-Membrane Proteins during Myogenesis of Skeletal Muscle *in vitro*

By GEORGE A. CATES and PAUL C. HOLLAND

Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan
S7N 0W0, Canada

(Received 14 March 1978)

1. Surface labelling of plasma-membrane proteins with ^{125}I , catalysed by lactoperoxidase, and radioactive L-fucose incorporation into glycoprotein were used as plasma-membrane markers for skeletal-muscle cells in culture. 2. Plasma membranes were prepared at various stages of myogenesis *in vitro* and rates of synthesis and accumulation of proteins in the membranes were compared. 3. Increased synthesis and accumulation of a protein of apparent mol.wt. 70000 occurred in the plasma-membrane fraction concomitant with the onset of myoblast fusion. 4. In cultures in which fusion of myoblasts was inhibited by 5'-bromo-2-deoxyuridine, synthesis and accumulation of the protein of apparent mol.wt. 70000 was selectively inhibited. 5. It is suggested the protein of apparent mol.wt. 70000 may be involved in the process of myoblast fusion.

During the differentiation of skeletal muscle, mononucleate myoblasts fuse to form multinucleate syncytia or myotubes (Yaffe, 1969). This process occurs with increased synchrony in cell cultures of neonatal or embryonic muscle and the molecular events preceding and following fusion of myoblasts *in vitro* have been the subject of intense investigation. There have been many reports concerned with the relationship between the process of myoblast fusion and the onset of terminal differentiation of skeletal-muscle cells (Holtzer *et al.*, 1972; Tarikas & Schubert, 1974; Keller & Nameroff, 1974; Emerson & Beckner, 1975; Holland & MacLennan, 1976); however, the molecular mechanisms involved in the fusion process itself are largely unknown. A number of studies indicate that alterations in the surface membrane of myoblasts do occur during myogenesis *in vitro*.

Increases in the glycolipid content of myoblasts, associated with early stages of cell contact, have been observed by Whatley *et al.* (1976) and McEvoy & Ellis (1977). The existence of a β -D-galactosyl-specific lectin in myoblasts, first reported by Teichberg *et al.* (1975), has also been reported by Gartner & Podleski (1975) and Den *et al.* (1976). It has been suggested by Gartner & Podleski (1975) that this lectin may play a role in the fusion process.

Cell-surface alterations during myogenesis in the rat skeletal-muscle cell line (L8) originally isolated by Yaffe (1969) were reported by Hynes *et al.* (1976) and Chen (1977). Chen (1977) used indirect immunofluorescence with antibody against the large external transformation-sensitive protein to show

that the pattern of distribution of this protein at the cell surface was altered after myoblast fusion. Hynes *et al.* (1976), using external labelling methods, detected an increase at the cell surface during myogenesis in the amount of a glycoprotein of similar molecular weight to the large external transformation-sensitive glycoprotein. However, this cell-surface alteration could only be detected after the rapid phase of myoblast fusion was complete and no changes could be detected at the cell surface before or concomitant with the onset of fusion.

In the present study, cell surfaces were labelled by reaction with ^{125}I in the presence of lactoperoxidase, and by incorporation of radioactive fucose into membrane glycoproteins. Cultured skeletal-muscle cells labelled in this way were fractionated by the method of Schimmel *et al.* (1973) and the radioactive markers were used to assess the purity of plasma-membrane fractions. Relative rates of synthesis and accumulation of proteins in myoblast plasma membranes were estimated at various stages of myogenesis in differentiating cultures of embryonic-chick skeletal muscle and in fusion-inhibited cultures. A plasma-membrane-specific protein that shows altered rates of synthesis and accumulation, coincident with the onset of the rapid phase of myoblast fusion, is identified. It is suggested that this protein may play a role in mediation of the fusion process.

Experimental

Materials

Fertile eggs from White Leghorn chickens were obtained from the Department of Poultry Science,

Abbreviations used: ATPase adenosine triphosphatase; SDS, sodium dodecyl sulphate.

University of Saskatchewan, Saskatoon, Saskatchewan, Canada. L-[4,5-³H]Leucine, L-[6-³H]fucose and ¹²⁵I were obtained from Amersham Corporation, Ontario, Canada. Medium 199, leucine-free minimum essential medium, foetal calf serum and chick-embryo extract were obtained from GIBCO Canada (c/o Micro Canada Research Ltd., Box 484, Calgary, Alberta, Canada). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Bio-Rad Laboratories, Ontario, Canada, or Fisher Scientific Co. Edmonton, Alberta, Canada.

Skeletal-muscle cell cultures

Breast muscle from 11-day chick embryos was mechanically dissociated by vortex mixing (Tepperman *et al.*, 1975). The dispersed cells were filtered through lens paper to remove myotubes and cell aggregates and plated at a density of 1.2×10^7 cells in Falcon 100mm-diameter tissue-culture dishes precoated with 0.1% gelatin. The standard growth medium was medium 199 supplemented with 10% foetal calf serum, 2% chick-embryo extract and 50 µg of gentamycin sulphate/ml. Cultures were incubated at 37°C in a humidified air atmosphere containing 5% CO₂. The medium was changed 48h after initial plating and every 24h thereafter.

Preparation of plasma membranes

Plasma membranes were prepared as described by Schimmel *et al.* (1973) with minor modification. All procedures were performed at 0–4°C. Some 20–30 culture dishes were washed twice with ice-cold Earle's salt solution and cells were collected by scraping in 3ml of cold Earle's salt solution per culture dish. The cells were sedimented by centrifugation for 5 min at 1100g and 4°C in a model B20A centrifuge (Damon/IEC; rotor type 870, r_{av} . 7.3 cm). The pellet was suspended in 0.25M-sucrose/1mM-triethanolamine hydrochloride, pH 7.4 (15ml/g wet wt. of cells), and homogenized in a Dounce homogenizer with 15 strokes of the B pestle. The homogenate was centrifuged for 10 min at 1700g and 4°C (IEC rotor type 870; r_{av} . 7.3 cm). The supernatant was collected and the pellet was resuspended in one-half of the original homogenization volume of 0.25M-sucrose/1mM-triethanolamine hydrochloride, pH 7.4, and centrifuged for 10 min at 1700g and 4°C (IEC rotor type 870; r_{av} . 7.3 cm). The supernatant was combined with that of the previous step and centrifuged for 1 h at 33000g and 4°C in a Damon/IEC B60 centrifuge (rotor type A-192; r_{av} . 7.3 cm). The pellet was suspended in 1 ml of 0.25M-sucrose/1mM-triethanolamine hydrochloride, pH 7.4, and layered on a discontinuous sucrose density gradient of the following composition: 0.5 ml of 55% (w/w) sucrose, followed by 2.5 ml each of 40, 32, 27 and 20% (w/w)

sucrose. The gradients were formed in 12ml-capacity tubes and were centrifuged for 1.5 h at 200000g and 4°C (IEC B60 centrifuge; rotor type SB 283; r_{av} . 10.3 cm). Membranes at the interfaces of the sucrose layers were collected as follows: 8.3–20% (w/w) sucrose, fraction I; 20–27% (w/w) sucrose, fraction II; 27–32% (w/w) sucrose, fraction III; 32–40% (w/w) sucrose, fraction IV; 40–55% (w/w) sucrose, fraction V.

These fractions were diluted 10-fold with 1mM-triethanolamine hydrochloride, pH 7.4, and centrifuged for 40 min at 150000g and 40°C (IEC B60 centrifuge; rotor type A-321; r_{av} . 5.4 cm). The pellets were suspended in 0.1–0.2 ml of 1mM-triethanolamine hydrochloride, pH 7.4, and used immediately in experiments involving assay of enzyme activity. Samples to be analysed by electrophoresis were stored at –20°C if they were not analysed immediately. Phenylmethanesulphonyl fluoride was included at a concentration of 0.5 mM in the homogenization and storage buffers, to inhibit proteolysis in experiments where membrane fractions were to be analysed by electrophoresis.

Enzyme assays

Phenylmethanesulphonyl fluoride was not used in experiments where enzyme assays were to be performed on membrane fractions.

Ouabin-sensitive (Na⁺/K⁺)-dependent ATPase activity was determined in 0.1 ml of reaction mixture containing 30mM-imidazole/HCl (pH 7.5), 0.11M-NaCl, 15mM-KCl, 5mM-NaN₃, 0.5mM-EGTA, 4mM-MgCl₂, 3mM-ATP and 20 µg of protein. The reaction was left to proceed for 60 min at 37°C and was terminated by adding 0.3 ml of silicotungstic acid (Lindberg & Ernster, 1956). The P_i released was determined as described by Martin & Doty (1949). (Na⁺/K⁺)-dependent ATPase activity was calculated as the difference in ATPase activity in the presence and absence of 1mM-ouabain. 5'-Nucleotidase activity was determined in 0.1 ml of assay mixture containing 50mM-glycine/HCl (pH 8.7), 0.2mM-EDTA, 0.2 µg of SDS/µg of protein, 6mM-AMP and 30 µg of protein. The reaction proceeded for 60 min at 37°C and was terminated by adding 0.3 ml of silicotungstic acid. The P_i released was then determined as described by Martin & Doty (1949).

NADPH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities were measured spectrophotometrically as described by Sottocasa *et al.* (1967).

SDS/polyacrylamide-slab-gel electrophoresis

SDS/polyacrylamide-slab-gel electrophoresis was performed with 1.5 mm slab gels (3% stacking gel and 9% separating gel) with the discontinuous buffer

system of Laemmli (1970). Either 25 or 40 μg of protein was solubilized in the sample buffer and immersed in boiling water for 3 min. Electrophoresis was carried out with a constant current of 30 mA for 4 h. The gel was fixed and stained for protein by the procedure of Fairbanks *et al.* (1971).

Quantitative fluorography of labelled proteins in polyacrylamide gels

^3H - or ^{125}I -labelled proteins in polyacrylamide slab gels were detected by the fluorographic method of Bonner & Laskey (1974). Pre-exposure of X-ray film as recommended by Laskey & Mills (1975) was routinely used. The fluorographs obtained were scanned densitometrically with a Joyce-Loebl Chromoscan 200.

Incubation of cultures with [^3H]leucine

Cells to be labelled were gently rinsed once with 10 ml of Ca^{2+} -free phosphate-buffered saline at 37°C (2.67 mM-KCl/1.47 mM- KH_2PO_4 /4.9 mM- MgCl_2 /0.136 M-NaCl/6.5 mM- Na_2HPO_4) and incubated for exactly 1 h at 37°C in a humidified atmosphere containing 5% CO_2 , with 5 ml of leucine-free minimum essential medium supplemented with $5\mu\text{Ci}$ of L-[4,5- ^3H]leucine/ml (specific radioactivity $50\mu\text{Ci}/\text{mmol}$; Amersham/Searle). The labelling medium was then removed and the dishes were washed twice with ice-cold Earle's salt solution. Cells were collected by scraping into 3 ml of ice-cold Earle's salt solution per dish and plasma membranes were isolated as previously described.

Protein

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Apparent molecular weights

Molecular weights of membrane proteins were calculated by reference to the mobility of the following polypeptides, included as molecular-weight standards in each SDS/polyacrylamide-gel-electrophoretic analysis; myosin heavy chain (200 000); phosphorylase *a* (94 000); bovine serum albumin (68 000); ovalbumin (43 000); cytochrome *c* (11 700).

Results and Discussion

Characterization of membrane preparation

Schimmel *et al.* (1973) demonstrated that their method for plasma-membrane isolation from skeletal-muscle cell cultures worked equally well with fused

3-day cultures and 3-day cultures in which myoblast fusion had been inhibited with 5'-bromo-2-deoxyuridine, indicating that the method is applicable to myoblasts and early myotubes. The purity of membrane fractions obtained from these cells was estimated from the activity of selected marker enzymes in the membranes and the binding of ^{125}I -labelled α -bungarotoxin to nicotinic acetylcholine receptors in the membrane fractions. Since the purity and reproducibility of the plasma-membrane preparation used is crucial to the interpretation of the present study, we used selected subcellular marker enzymes to assess whether the method of Schimmel *et al.* (1973) could be used for the isolation of plasma membranes from skeletal-muscle cultures at various times after the initial plating of cells. Membrane fractions were isolated from cultures at 40, 64, 88 and 128 h after the initial plating of cells and the relative distribution of marker enzyme activities in each fraction was found to be essentially unaltered by the time spent in culture and the degree of development of the cultures (Table 1).

The specific activities and recovery of the marker enzymes assayed in membrane fractions obtained from the sucrose discontinuous density gradient were reproducible and were in close agreement with those originally reported by Schimmel *et al.* (1973). Insufficient amounts of protein were obtained in fraction I for analysis. In all cases membranes banding between 20% and 27% (w/w) sucrose (fraction II) or between 27 and 32% (w/w) sucrose (fraction III) had the highest ouabain-sensitive (Na^+/K^+)-dependent ATPase activity and were free of mitochondrial contamination, as indicated by the absence of detectable succinate-cytochrome *c* reductase activity in the membranes.

Some separation of the putative plasma-membrane marker enzyme 5'-nucleotidase from (Na^+/K^+)-dependent ATPase activity was observed. Thus 5'-nucleotidase activity tended to be fairly evenly distributed over the gradient and to peak in fractions III and IV. Separation of 5'-nucleotidase activity from (Na^+/K^+)-dependent ATPase activity in plasma-membrane-derived vesicles prepared from cultured skeletal muscle was originally observed by Schimmel *et al.* (1973) and has also been reported by Vandenburgh (1977). Failure of 5'-nucleotidase activity to be co-purified with other plasma-membrane markers has been noted by Gahmberg & Simons (1970), Warley & Cook (1973) and Thom *et al.* (1977), who have suggested that 5'-nucleotidase may be a poor marker for the plasma membranes of tissue-culture cells.

NADPH-cytochrome *c* reductase, an enzyme frequently used as a marker for endoplasmic reticulum, was found in all fractions obtained from the discontinuous gradient. The specific activity of NADPH-cytochrome *c* reductase was similar in

Table 1. *Enzyme activities in membrane preparations*

Skeletal-muscle cell monolayers were grown in standard growth medium for the times indicated. Cells were collected by scraping, homogenized in 0.25 M-sucrose/1 mM-triethanolamine hydrochloride, pH 7.4, and fractionated on a sucrose discontinuous density gradient as described in the Experimental section. Fractions were collected at the interfaces of the gradient as follows: II [20–27% (w/w) sucrose]; III [27–32% (w/w) sucrose]; IV [32–40% (w/w) sucrose]; V [40–55% (w/w) sucrose]. The membrane fractions were used immediately for enzyme assay. Activities are expressed as nmol/min per mg of protein. The values shown are means $\pm$ S.E.M. The numbers of different preparations assayed to obtain these values are indicated in parentheses.

Fraction no.	Age of culture (h)	Activity (nmol/min per mg)			
		... 40	64	84	128
Ouabain-sensitive (Na ⁺ /K ⁺)-dependent ATPase					
II		67.7 ± 2.3 (3)	63.0 ± 3.4 (4)	69.3 ± 17.8 (3)	64.0 ± 15.3 (3)
III		45.7 ± 2.3 (3)	66.8 ± 8.4 (4)	71.0 ± 16.0 (3)	42.3 ± 4.5 (3)
IV		23.0 ± 4.0 (3)	37.8 ± 3.5 (4)	18.3 ± 9.2 (3)	22.3 ± 4.7 (3)
V		11.0 ± 3.0 (3)	23.5 ± 7.1 (4)	22.0 ± 3.6 (3)	18.0 ± 10.1 (3)
5'-Nucleotidase					
II		8.2 ± 2.1 (3)	4.3 ± 1.6 (3)	3.2 ± 1.5 (3)	6.0 ± 1.5 (3)
III		8.2 ± 0.6 (3)	5.8 ± 2.6 (3)	5.5 ± 1.9 (3)	17.0 ± 3.6 (3)
IV		9.1 ± 1.5 (3)	5.7 ± 1.0 (3)	5.8 ± 0.7 (3)	17.0 ± 3.6 (3)
V		8.3 ± 1.5 (3)	3.3 ± 0.7 (3)	2.9 ± 0.6 (3)	15.3 ± 3.3 (3)
NADPH-cytochrome c reductase					
II		1.92 ± 0.25 (2)	1.84 ± 0.16 (2)	5.60 ± 1.10 (3)	2.40 ± 0.60 (2)
III		1.55 ± 0.03 (2)	1.95 ± 0.16 (2)	6.10 ± 0.50 (3)	1.90 ± 0.90 (2)
IV		1.41 ± 0.17 (2)	2.07 ± 0.16 (2)	5.30 ± 0.10 (3)	2.00 ± 0.60 (2)
V		1.34 ± 0.06 (2)	1.65 ± 0.11 (2)	4.00 ± 0.70 (3)	1.30 ± 0.80 (2)
Succinate-cytochrome c reductase					
II		0 (2)	0 (2)	0 (3)	0 (2)
III		0 (2)	0 (2)	0.59 ± 0.32 (3)	0 (2)
IV		0.73 ± 0.06 (2)	1.14 ± 0.20 (2)	2.44 ± 0.60 (3)	0.76 ± 0.76 (2)
V		0.57 ± 0.06 (2)	0.93 ± 0.01 (2)	2.44 ± 0.54 (3)	3.25 ± 0.72 (2)

fractions II–V, indicating that these fractions contained similar proportions of membranes originating from the endoplasmic reticulum. Because of the presence of endoplasmic-reticulum vesicles in all fractions, changes in the synthesis or accumulation of a protein found in the plasma-membrane-enriched fraction can only be taken to reflect changes occurring in the plasma membrane if that protein is associated specifically with the plasma membrane, and is absent from, or present at lower concentrations in, the other fractions obtained from the gradient.

Lactoperoxidase-catalysed iodination of membranes

The use of enzyme markers as indicators of the purity of membrane fractions may be criticized on the grounds that few enzymes are known to be entirely specific for a given membrane. This is particularly true in developmental studies, where the activity or concentration of an enzyme in a particular membrane fraction may change as a result of differentiation of the membrane. We therefore used lactoperoxidase-catalysed ^{125}I incorporation into cell-surface proteins, as a marker for cell-surface-derived membranes. Subsequent to iodination, membranes were prepared by the method of Schimmel *et al.*

(1973) and ^{125}I incorporation into proteins of the membranes was analysed by quantitative fluorography of samples subjected to SDS/polyacrylamide-slab-gel electrophoresis (Fig. 1). The qualitative pattern of iodination was identical in fractions II and III, where proteins of apparent mol.wt. 174000, 144000, 123000, 90000, 60000 and 54000 were labelled. In the more dense fractions IV and V only two proteins of apparent mol.wts. 60000 and 54000 were iodinated. Analysis of proteins in the 100000g supernatant of cell homogenates showed that cytoplasmic proteins were not iodinated, demonstrating that the method was specific for proteins at the cell surface.

The presence of two iodinated proteins in the more dense fractions of the gradient and the separation of the plasma-membrane marker enzymes 5'-nucleotidase and (Na^+/K^+)-dependent ATPase may reflect surface-membrane heterogeneity. Possible sources of surface-membrane heterogeneity for cells growing in tissue culture have been discussed by Neville (1976).

Incorporation of L-fucose into membrane fractions

L-Fucose has been shown to be preferentially incorporated into glycoproteins associated with the

Fig. 1. Lactoperoxidase-catalysed iodination of plasma-membrane proteins

Skeletal-muscle cell monolayers were cultured for 66 h in standard growth medium. The growth medium was removed and the cultures were washed twice with 10 ml of ice-cold Earle's salt solution. Each plate of cells was covered with 2 ml of 0.154 M-NaCl/10 mM-sodium phosphate, pH 7.0, containing 100 μ Ci of 125 I, 0.16 nmol of lactoperoxidase (16 munits) and 0.2 μ g of glucose oxidase (12 munits). The reaction was started by the addition of 50 μ l of 0.8 M-glucose per culture plate and was left to proceed at 4°C for 15 min. The labelling medium was removed and the cultures were washed three times with 10 ml of ice-cold Earle's salt solution. Cells were collected by scraping and were homogenized and membrane fractions prepared on a sucrose discontinuous density gradient as described in the Experimental section. Samples of each fraction obtained corresponding to 20 μ g of protein were subjected to SDS/polyacrylamide-slab-gel electrophoresis. 125 I incorporation into proteins was estimated by quantitative fluorography of electrophoretograms and densitometric scanning. (a) Fraction II [20–27% (w/w) sucrose]; (b) fraction III [27–32% (w/w) sucrose]; (c) fraction IV [32–40% (w/w) sucrose]; (d) fraction V [40–55% (w/w) sucrose]; (e) 100 000g supernatant of cell homogenate. Electrophoresis was from left (–) to right (+). The apparent molecular weights of the iodinated proteins, indicated with arrows, are: (i) 174 000; (ii) 144 000; (iii) 123 000; (iv) 90 000; (v) 60 000; (vi) 54 000.

plasma membrane of eukaryotic cells (Bennett & Leblond, 1970; Atkinson & Summers, 1971; Bennett *et al.*, 1974; Atkinson, 1975; Hudson & Johnson, 1977). When myoblast cultures were incubated with radioactive L-fucose and membrane fractions were prepared from the labelled cells by the method of Schimmel *et al.* (1973), fraction II membranes were the most heavily labelled. The pattern of incorporation of L-fucose into specific proteins of the membrane fractions is shown in Fig. 2. Similar qualitative

Fig. 2. [3 H]Fucose incorporation into the glycoproteins of membrane fractions obtained from the discontinuous sucrose density gradient

Skeletal-muscle monolayer cultures were grown for 55 h in standard growth medium and were then transferred to standard growth medium (5 ml per plate) supplemented with 5 μ Ci of L-[6- 3 H]fucose/ml. The labelling medium was removed 12 h later and the cultures were washed twice with 10 ml of ice-cold Earle's salt solution and were scraped, homogenized and fractionated on a discontinuous sucrose density gradient as described in the Experimental section. Samples of each fraction obtained, corresponding to 25 μ g of protein, were subjected to SDS/polyacrylamide-slab-gel electrophoresis and quantitative fluorography. Radioactive fucose incorporation into membrane glycoproteins was estimated by densitometric scanning of the fluorograms. (a) Fraction II; (b) fraction III; (c) fraction IV; (d) fraction V. Electrophoresis was as described in Fig. 1. Molecular-weight markers are: M, myosin (200 000); P, phosphorylase (94 000); BSA, bovine serum albumin (68 000); OA, ovalbumin (43 000).

patterns of incorporation of L-fucose were seen in all membrane fractions, although the total amount of label incorporated into fractions IV and V was much lower than in fractions II and III. One dominant peak of fucose incorporation was seen at an apparent mol.wt. of 80000. The majority of the remaining radioactively labelled proteins were of higher apparent molecular weight.

By the methods used above to estimate the degree of enrichment of fractions II–V for plasma-membrane-derived material we confirmed the conclusion of Schimmel *et al.* (1973) that fraction II membranes are the most plasma-membrane enriched, although in our hands the various markers used indicated that fraction III membranes also contained a large amount of plasma-membrane-derived material and closely though not exactly resembled fraction II membranes in protein composition.

Protein composition of membrane fractions

Membrane fractions obtained by the method of Schimmel *et al.* (1973) from 3-day cultures were subjected to SDS/polyacrylamide-slab-gel electrophoresis and stained for protein.

The protein composition of the fractions is shown in Fig. 3. Several proteins were found in fraction II membranes, which were present in progressively lower concentrations in fractions III–V. The protein profiles of the various membrane fractions obtained were extremely reproducible between preparations. The proteins iodinated by ^{125}I and lactoperoxidase (Fig. 1) appeared to correspond to proteins present at quite low concentrations in the membranes. Similarly those proteins in the membrane which incorporate L-fucose were difficult to identify unequivocally, since they also appeared to correspond to regions of the gel which stained only lightly for protein. Assignment of the labelled bands to particular proteins on the gel was further complicated by the fact that soaking of gels in dimethyl sulphoxide before fluorography causes considerable diffusion of the protein stain in the gel.

Leucine incorporation into fraction II membrane proteins

Fraction II membranes were prepared from cell cultures pulse-labelled with radioactive leucine at various stages of myogenesis in standard growth medium. Rates of synthesis of membrane proteins were then estimated by quantitative fluorography of membranes subjected to SDS/polyacrylamide-slab-gel electrophoresis (Fig. 4). Leucine incorporation into membrane proteins was measured at 15, 40, 64 and 88 h after the initial plating of cultures. The process of myoblast fusion in cultures grown in

Fig. 3. Protein composition of membrane fractions obtained from the discontinuous sucrose density gradient

Skeletal-muscle cell cultures were grown for 68 h in standard growth medium. Membrane fractions were prepared from the cells as described in the Experimental section. Samples ($20\mu\text{g}$ of protein) of each fraction obtained from the discontinuous sucrose density gradient were subjected to SDS/polyacrylamide-slab-gel electrophoresis and the gel was stained for protein and scanned densitometrically. (a) Fraction II; (b) fraction III; (c) fraction IV; (d) fraction V. Electrophoresis and molecular-weight markers were as described in Fig. 2. Proteins most prominent in fraction II, decreasing through to fraction V, are indicated with arrows. Apparent molecular weights are: (i) 70 000; (ii) 45 000; (iii) 31 300; (iv) 28 500.

standard growth medium always began 35–40 h after the time of initial plating of the cultures. Between 15 and 40 h in culture, slightly increased rates of synthesis of a number of proteins in fraction II membranes could be observed. A marked and reproducible increase of the rate of leucine incorporation into a protein of apparent mol.wt. 70000 occurred over this time period. This protein is a major constituent of fraction II membranes prepared from 3-day-old fused cultures and shows considerable specificity for fraction II (Fig. 3). The protein can also be seen to increase in total concentration in the membrane between 15 and 40 h in culture (results not shown). Repeated washing of fraction II membranes with 1.0M-KCl, 0.6M-LiBr or 0.1mg of sodium deoxycholate/mg of membrane protein did not remove this protein from the membranes. In fact salt washing

Fig. 4. [^3H]Leucine incorporation into the proteins of fraction II membranes at various stages of myogenesis

Skeletal-muscle cell monolayers were grown in standard growth medium for: (a) 15h; (b) 40h; (c) 64h; (d) 88h. The cultures were then pulse-labelled with [^3H]leucine and fraction II membranes were prepared from the labelled cultures as described in the Experimental section. Samples of fraction II membranes corresponding to 20 μg of protein were subjected to SDS/polyacrylamide-slab-gel electrophoresis and quantitative fluorography. [^3H]Leucine incorporation into membrane proteins was estimated by densitometric scanning of the fluorograms obtained. Electrophoresis and molecular-weight markers were as described in Fig. 2.

actually increased the relative concentration of the protein in the membranes. Preparation of membranes from cells homogenized in a hypo-osmotic medium (1mM-triethanolamine hydrochloride, pH7.4) yielded fraction II membranes of almost identical composition with those prepared from cells homogenized in the iso-osmotic medium normally used. It is unlikely therefore that the protein of apparent mol.wt. 70000 is a cytoplasmic contaminant. Analysis of soluble protein from cell homogenates of cultures pulse-labelled with [^3H]leucine at 15, 40 and 64h after initial plating showed that although some protein was present in the soluble fraction at a mol.wt. close to 70000 no increase in its synthesis or accumulation occurred over this time period, demonstrating that it is not the same protein as that found in the plasma-membrane fraction.

Fig. 5. Effect of inhibition of myoblast fusion by 5'-bromo-2-deoxyuridine upon the polypeptide composition of fraction II membranes

Skeletal-muscle cell monolayers were grown for 41h in (a) standard growth medium or (b) standard growth medium containing 6.4 μM 5'-bromo-2-deoxyuridine. Fraction II membranes were prepared from the cultures as described in the Experimental section. Samples of fraction II membranes corresponding to 20 μg of protein were subjected to SDS/polyacrylamide-slab-gel electrophoresis and stained for protein. Electrophoresis and molecular-weight markers were as described in Fig. 2.

By 64h in culture the rapid phase of myoblast fusion was essentially complete. By this time a decline in the rate of leucine incorporation into a large number of proteins in fraction II membranes was apparent, including the protein of apparent mol.wt. 70000.

Effect of 5'-bromo-2-deoxyuridine

5-Bromo-2-deoxyuridine is an inhibitor of differentiation in skeletal-muscle cells (Stockdale *et al.*, 1964) as well as in many other cell types (Rutter *et al.*, 1973). When cells were grown in medium supplemented with 6.4 μM 5'-bromo-2-deoxyuridine, myoblast fusion was almost completely inhibited. The protein composition of fraction II membranes prepared from 5'-bromo-2-deoxyuridine-treated cells closely resembled that of fraction II membranes prepared from untreated cells (Fig. 5). One noticeable difference, however, was a decrease in the amount of protein of apparent mol.wt. 70000 in the membranes of cells grown in 5'-bromo-2-deoxyuridine. When cells were pulse-labelled with [^3H]leucine and fraction II membranes were prepared from the labelled cells, a decrease in the rate of synthesis of protein of apparent mol.wt. 70000 was also apparent in 5'-bromo-2-deoxyuridine-treated cells (Fig. 6).

Fig. 6. Effect of inhibition of myoblast fusion by 5'-bromo-2-deoxyuridine upon $[^3\text{H}]$ leucine incorporation into fraction II membrane proteins

Skeletal-muscle cell monolayers were grown for 41 h in (a) standard growth medium or (b) standard growth medium containing $6.4\ \mu\text{M}$ 5'-bromo-2-deoxyuridine. The cultures were then pulse-labelled with $[^3\text{H}]$ leucine and fraction II membranes were prepared from the labelled cultures as described in the Experimental section. Samples of fraction II membranes corresponding to $20\ \mu\text{g}$ of protein were subjected to SDS/polyacrylamide-slab-gel electrophoresis and quantitative fluorography. $[^3\text{H}]$ Leucine incorporation into membrane proteins was estimated by densitometric scanning of the fluorograms obtained. Electrophoresis and molecular-weight markers were as described in Fig. 2.

A decrease in the rate of synthesis of the protein of apparent mol.wt. 70000 and the corresponding decrease in its concentration in fraction II membranes of 5'-bromo-2-deoxyuridine-treated cells were the only significant and reproducible differences observed between these membranes and fraction II membranes from non-treated cells.

Thus, of the proteins characteristic of fraction II membranes, the protein of apparent mol.wt. 70000 showed marked and reproducible alterations in synthesis and accumulation in the membrane temporally associated with the onset of myoblast fusion. This protein was not detected at the cell

surface by the technique of lactoperoxidase-catalysed iodination of surface proteins (Fig. 1). However, external labelling methods have generally failed to reveal any changes in myoblast plasma-membrane proteins which occur early enough in myogenesis to be associated in a causal manner with the fusion process. Thus Hynes *et al.* (1976), using the technique of lactoperoxidase-catalysed iodination of cell-surface proteins and galactose oxidase-catalysed labelling of surface glycoproteins with boro $[^3\text{H}]$ -hydride, detected an increase in the amount of large external transformation-sensitive protein accessible at the cell surface after myoblast fusion had occurred, but failed to detect any changes in the pattern of labelling of surface components before fusion. In the present study the proteins labelled by lactoperoxidase and ^{125}I correspond to proteins present at low concentrations in the plasma-membrane-enriched fraction, and no reproducible changes in the synthesis of these proteins occurred before or concomitant with fusion.

Although an increase in the synthesis and accumulation of a plasma-membrane protein concomitant with the onset of myoblast fusion, such as was seen in the present study for the protein of apparent mol.wt. 70000, suggests that the protein may play a role in the fusion process, further work is required to demonstrate a causal relationship between the two events. An increase in the synthesis and accumulation of a plasma-membrane protein may be due to some aspect of membrane differentiation mechanistically divorced from, but temporally associated with, the fusion process.

Changes in the cell cycle have been shown to cause changes in the plasma-membrane properties of synchronized tissue culture cells (Pasternak, 1975). Myoblasts fuse only in the G1 phase of the cell cycle and fusion is preceded by entry of myoblasts into an extended G1 phase (Buckley & Konigsberg, 1974). After fusion the nucleus becomes incapable of DNA synthesis and is totally arrested in the G1 phase of the cell cycle. Changes in the composition of plasma membranes of myoblasts immediately before or concomitant with fusion may therefore reflect differentiation of the membrane dependent upon the entry of the nucleus into the G1 phase of the cell cycle.

We thank Ms. Jeanne Killens for expert technical assistance. This work was supported by the Muscular Dystrophy Association of Canada and the Medical Research Council of Canada.

References

- Atkinson, P. H. (1975) *J. Biol. Chem.* **250**, 2123-2134
- Atkinson, P. H. & Summers, D. F. (1971) *J. Biol. Chem.* **246**, 5162-5175

- Bennett, G. & Leblond, C. P. (1970) *J. Cell Biol.* **46**, 409–416
- Bennett, G., Leblond, C. P. & Haddad, A. (1974) *J. Cell Biol.* **60**, 258–284
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Buckley, P. A. & Konigsberg, I. R. (1974) *Dev. Biol.* **37**, 193–212
- Chen, L. B. (1977) *Cell* **10**, 393–400
- Den, H., Malinzak, D. A. & Rosenberg, A. (1976) *Biochem. Biophys. Res. Commun.* **69**, 621–627
- Emerson, C. P. & Beckner, S. K. (1975) *J. Mol. Biol.* **93**, 431–447
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Gahmberg, C. G. & Simons, K. (1970) *Acta Pathol. Microbiol. Scand. Sect. B* **78**, 176–182
- Gartner, T. K. & Podleski, T. R. (1975) *Biochem. Biophys. Res. Commun.* **67**, 972–978
- Holland, P. C. & MacLennan, D. H. (1976) *J. Biol. Chem.* **251**, 2030–2036
- Holtzer, H., Weintraub, H., Mayne, R. & Mochan, B. (1972) *Curr. Top. Dev. Biol.* **7**, 229–256
- Hudson, J. E. & Johnson, T. C. (1977) *Biochem. J.* **166**, 217–223
- Hynes, R. O., Martin, G. S., Shearer, M., Critchley, D. R. & Epstein, C. J. (1976) *Dev. Biol.* **48**, 35–46
- Keller, J. M. & Nameroff, M. (1974) *Differentiation* **2**, 19–23
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Lindberg, O. & Ernster, L. (1956) *Methods Biochem. Anal.* **3**, 1–22
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, J. B. & Doty, D. M. (1949) *Anal. Chem.* **21**, 965
- McEvoy, F. A. & Ellis, D. E. (1977) *Biochem. Soc. Trans.* **5**, 1719–1721
- Neville, D. M. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., ed.), pp. 27–54, Chapman and Hall, London
- Pasternak, C. A. (1975) in *Biological Membranes* (Parsons, D. S., ed.), pp. 55–68, Oxford University Press, London
- Rutter, W. J., Pictet, R. L. & Morris, P. W. (1973) *Annu. Rev. Biochem.* **42**, 601–646
- Schimmel, S. D., Kent, C., Bischoff, R. & Vagelos, P. R. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3195–3199
- Sottocasa, G. L., Kuylenskierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
- Stockdale, F., Okazaki, K., Nameroff, M. & Holtzer, H. (1964) *Science* **146**, 533–535
- Tarikas, H. & Schubert, D. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2377–2381
- Teichberg, V. I., Silman, I., Beitsch, D. D. & Resheff, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1383–1387
- Tepperman, K., Morris, G., Essien, F. & Heywood, S. M. (1975) *J. Cell. Physiol.* **86**, 561–566
- Thom, D., Powell, A. J., Lloyd, C. W. & Rees, D. A. (1977) *Biochem. J.* **168**, 187–194
- Vandenburgh, H. H. (1977) *Biochim. Biophys. Acta* **466**, 302–314
- Warley, A. & Cook, G. M. W. (1973) *Biochim. Biophys. Acta* **323**, 55–68
- Whatley, R., Ng, S. K., Rogers, J., McMurray, W. C. & Sanwal, B. D. (1976) *Biochem. Biophys. Res. Commun.* **70**, 180–185
- Yaffe, D. (1969) *Curr. Top. Dev. Biol.* **4**, 37–77