Surface-Labeling Studies on Skeletal-Muscle Cells *in vitro*

HETEROGENEITY OF IODINATED CELL-SURFACE PROTEINS

George A. CATES and Paul C. HOLLAND*

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

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1. Two distinct classes of protein were detected at the surface of chick-embryo skeletal-muscle cells after iodination of the cells in monolayer culture. 2. The two classes of iodinated proteins differed in their ability to co-purify with a vesicular plasma-membrane fraction prepared from surface-labelled cells. 3. One class consisted of predominantly high-molecular-weight glycoproteins that co-purified with the plasma-membrane fraction, but showed no significant qualitative or quantitative alterations in labelling with ^{125}I and lactoperoxidase during myogenesis. 4. A second class of predominantly lower-molecular-weight proteins showed reproducible quantitative alterations in ^{125}I -labelling during myogenesis but failed to co-purify with the plasma-membrane fraction. 5. This second class of proteins may represent matrix proteins involved in intercellular adhesion or adhesion of cells to the substratum. They are unlikely to be directly required for the process of plasma-membrane fusion during myogenesis, since they do not co-purify with a vesicular plasma-membrane fraction known to be capable of Ca^{2+} -dependent fusion *in vitro*.

Differentiation of skeletal muscle *in vivo* and *in vitro* involves the formation of multinucleate syncytia, or myotubes, by the fusion of mononucleate myoblasts. This process must involve the interaction of surface-membrane components; however, the actual components involved and the manner in which they interact are as yet unknown.

Lactoperoxidase-catalysed iodination of exposed tyrosine residues has been used as a probe by several workers in attempts to detect cell-surface alterations during myogenesis. These studies have used the rat skeletal-muscle cell line L8 (Hynes *et al.*, 1976; Chen, 1977) and primary cultures of chick-embryo skeletal muscle (Moss *et al.*, 1978). The results of these studies are in conflict. Hynes *et al.* (1976) and Chen (1977) concluded that no significant differences in the pattern of labelling of cell-surface proteins with ^{125}I could be detected between pre-fusion and mid-fusion myoblasts. The only significant alteration in surface labelling seen during myogenesis (Hynes *et al.*, 1976) was an increase, after fusion, in the labelling of a high-molecular-weight glycoprotein with similar properties to the large external transformation-sensitive glycoprotein of fibro-

blast surface membranes (Yamada & Olden, 1978). In contrast, Moss *et al.* (1978) found both quantitative and qualitative changes in the pattern of labelling of a number of cell-surface proteins during myogenesis. Many of these alterations were detectable before or concomitant with fusion. Moss *et al.* (1978) therefore suggested that a major structural reorganization of surface-membrane protein occurs concomitant with the onset of fusion as well as after fusion. In a previous study on the biosynthesis of plasma-membrane proteins during myogenesis of chick-embryo skeletal muscle *in vitro* we used lactoperoxidase-catalysed iodination of cell-surface proteins as a marker of myotube plasma-membrane purification (Cates & Holland, 1978). Considerably fewer labelled proteins were observed in the plasma-membrane-enriched fraction in our study than were reported by either Moss *et al.* (1978) or Hynes *et al.* (1976) when whole-cell homogenates were analysed after surface labelling with ^{125}I . One of the major quantitative changes associated with myogenesis noted by Moss *et al.* (1978) in chick-embryo myoblasts was a marked decrease in the degree of iodination of protein of apparent mol.wt. 72 000. We had reported (Cates & Holland, 1978) that a plasma-membrane protein of apparent mol.wt. 70 000 showed increased synthesis and accumula-

Abbreviation used: SDS, sodium dodecyl sulphate.

* To whom correspondence should be addressed.

tion in a plasma-membrane-enriched fraction concomitant with the onset of myoblast fusion, but we were unable to detect any iodination of protein in this region after surface labelling of myotube cultures. In the present paper we present an explanation for the apparent discrepancy between our previous findings (Cates & Holland, 1978) and those obtained by Moss *et al.* (1978). We show that when skeletal-muscle cells are iodinated in monolayer culture two distinct classes of protein are labelled. One class consists of predominantly high-molecular-weight glycoproteins that co-purify with the plasma-membrane fraction. The second class of iodinated proteins tend to be of lower molecular weight and do not co-purify with the plasma membrane. Significant and reproducible alterations in the pattern of iodination of proteins that are associated with normal myogenesis or are observed with fusion-inhibited and normal fused cultures are confined to the latter group of proteins. The significance of these findings is discussed.

Experimental

Materials

Fertile eggs from White Leghorn chickens were obtained from the Department of Poultry Science, University of Saskatchewan, Saskatoon, Sask., Canada. [125 I]iodide and D-[6- 3 H]glucosamine were obtained from Amersham Corp., Oakville, Ont., Canada. Medium 199, Earle's salt solution, 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, Mississauga, Ont., 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/ml in Falcon 100mm-diameter tissue-culture dishes pre-coated with 0.1% gelatin. The standard growth medium was medium 199 supplemented with 10% (v/v) foetal calf serum, 2% (v/v) chick-embryo extract and 50 μ g 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.

Lactoperoxidase-catalysed iodination

Skeletal-muscle-cell monolayers were cultured for the indicated times in standard growth medium. The

growth medium was removed and the cultures were washed twice with 10ml of ice-cold Earle's salt solution. Each plate of cells was covered with 2ml of 0.15M-NaCl/10mM-sodium phosphate/1mM-CaCl₂ medium, pH 7.0, containing 100 μ Ci of [125 I]iodide (specific radioactivity 13–17 mCi/ μ g of I), 0.16 nmol of lactoperoxidase (16 munits) and 0.2 μ g of glucose oxidase (12 munits) (the enzyme units being μ mol of substrate transformed/min). The reaction was started by the addition of 50 μ l of 0.8M-glucose per culture plate and was allowed to proceed at 4°C for 15 min. The labelling medium was removed and the cultures were washed with 3×10 ml of ice-cold Earle's salt solution.

Preparation of total cellular membrane fraction

Cells were collected by scraping in 3ml of ice-cold Earle's salt solution per culture dish and were sedimented by centrifugation at 1100g for 5 min at 4°C in a model B20A centrifuge (Damon/IEC; rotor type 870, r_{av} 7.3 cm). The cell pellet was suspended in 0.25M-sucrose/1mM-triethanolamine hydrochloride/0.5mM-phenylmethanesulphonyl fluoride medium, pH 7.4 (15 ml/g wet wt. of cells), and homogenized in a Dounce homogenizer with 15 strokes of the B pestle. The homogenate was centrifuged at 150000g for 40 min at 4°C (IEC B60 centrifuge; rotor type A-321; r_{av} 5.4 cm). The membrane pellet was suspended in 0.2–0.5 ml of 1mM-triethanolamine hydrochloride, pH 7.4, and stored at –20°C until analysed.

Preparation of plasma membranes

Plasma membranes were prepared as described by Schimmel *et al.* (1973), with minor modifications as described by Cates & Holland (1978). Phenylmethanesulphonyl fluoride was included at a concentration of 0.5 mM in the homogenization buffer to inhibit proteolysis.

SDS/polyacrylamide-slab-gel electrophoresis

SDS/polyacrylamide-slab-gel electrophoresis was performed with 1.5 mm-thick slab gels (3% stacking gel and 8% separating gel) with the discontinuous buffer system of Laemmli (1970). A 30 μ g sample of protein was solubilized in the sample buffer and immersed in boiling water for 3 min. Electrophoresis was carried out with a constant power of 6 W for 4 h. The gel was fixed and stained for protein with Coomassie Blue as described by Fairbanks *et al.* (1971). 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 (200000); phosphorylase *a* (94000); bovine serum albumin (68000); ovalbumin (43000); trypsin (23300).

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 instrument. Densitometer scans were normalized to account for small changes in total radioactivity between samples obtained from cultures of different ages.

Protein

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

Results

Iodination profiles for total cell-membrane fractions isolated from surface-labelled cells at various stages of myogenesis are shown in Fig. 1. The stages of myogenesis selected represent pre-fusion (24 h), mid-fusion (48 h) and post-fusion (72 h). At all stages of myogenesis examined, proteins with a wide range of apparent molecular weights were iodinated. However, at later stages of myogenesis there was a reproducible tendency for certain proteins of lower apparent molecular weight (70 000 and below) to be iodinated to a smaller extent. The most dramatic quantitative alteration in the extent of iodination of a protein between pre-fusion and mid-fusion cells was a marked decrease in the labelling of a diffuse peak of protein in the 70 000-mol.wt. region of the gel. This tendency carried through to the post-fusion stage. The only reproducible alteration seen in the iodination of high-molecular-weight proteins was a marked increase, post-fusion, in the extent of iodination of a protein of apparent mol.wt. 255 000. This latter finding is in agreement with observations made on the L8 cell line of rat myoblasts (Hynes *et al.*, 1976) and primary cultures of chick myoblasts (Moss *et al.*, 1978). This protein is believed to be similar to, if not identical with, the high-molecular-weight protein fibronectin (Yamada & Olden, 1978) found on surface membranes of cultured fibroblasts. When skeletal-muscle-cell cultures were surface-labelled with ^{125}I and a plasma-membrane-enriched fraction was prepared by the method of Schimmel *et al.* (1973), not all the iodinated proteins seen on analysis of the total cell-membrane fraction co-purified with the plasma-membrane fraction (Fig. 2). In particular, several iodinated proteins of apparent mol.wt. 70 000 and below were present at a lower relative concentration in the plasma-membrane fraction than in the more crude total membrane fractions shown in Fig. 1. The plasma-membrane frac-

Fig. 1. Lactoperoxidase-catalysed iodination of surface proteins at various stages of myogenesis

Skeletal-muscle monolayers were grown in standard growth medium for (a) 72 h, (b) 48 h or (c) 24 h. The cultures were then surface-labelled with ^{125}I as described in the Experimental section and the total cell-membrane fraction was isolated. Equal quantities (30 μg) of membrane protein were subjected to SDS/polyacrylamide-slab-gel electrophoresis and quantitative fluorography. ^{125}I incorporation into protein was estimated by densitometric scanning of the fluorograms obtained.

tion shown in Fig. 2 was prepared from a fused culture maintained for 65 h in standard growth medium. Although there is less iodination of protein in the 70 000-mol.wt. region in fused cultures than in unfused cultures (Fig. 1), this time point best illustrates the difference between iodinated protein in this molecular-weight region and the protein of apparent mol.wt. 70 000 we have previously reported to accumulate in the plasma-membrane fraction during myogenesis (Cates & Holland, 1978). Thus comparison of Fig. 2(a) with Fig. 2(b) shows that there is no peak of iodination corresponding to the protein of apparent mol.wt. 70 000 despite the high concentration of this protein in the plasma-membrane fraction of fused cultures.

During purification of plasma membrane the

Fig. 2. Iodinated proteins found in the plasma-membrane-enriched fraction

Skeletal-muscle monolayers were grown for 65 h in standard growth medium. The cultures were then surface-labelled with ^{125}I as described in the Experimental section and a plasma-membrane-enriched fraction was prepared by the method of Schimmel *et al.* (1973). Membrane protein (30 μg) from this fraction was subjected to SDS/polyacrylamide-gel electrophoresis and quantitative fluorography. (a) Densitometric scan of fluorogram, showing ^{125}I incorporation into proteins of the plasma-membrane fraction; (b) densitometric scan of the Coomassie Blue-staining profile of the plasma-membrane preparation. Those iodinated proteins that can be seen to be increased in relative content in the plasma-membrane fraction, compared with the total membrane fractions shown in Fig. 1, are indicated with arrows. Their apparent molecular weights are: (i) 266 000; (ii) 217 000; (iii) 171 500; (iv) 149 500; (v) 123 000; (vi) 98 000; (vii) 85 000; (viii) 76 500.

protein of apparent mol.wt. 70 000 shown in Fig. 2(b) actually co-purifies with other plasma-membrane markers (Cates & Holland, 1978). The iodinated peak of protein in the 70 000-mol.wt. region seen in total membrane fractions of fused cultures (Fig. 1a) cannot therefore represent iodination of the plasma-membrane-associated protein of apparent mol.wt. 70 000. There is sufficient enrichment of this protein in the plasma-membrane fraction, over the crude total membrane fractions, that a large peak of iodinated material would have been seen in the 70 000-mol.wt. region of Fig. 2(a) had it been iodinated.

The trace of iodinated material in this region that does carry over to the plasma-membrane fraction actually migrates slightly faster than the major peak of protein in the plasma-membrane fraction. Patterns of labelling identical with that shown in Fig. 2(a) were obtained when plasma-membrane fractions were prepared from cultures iodinated at earlier stages of differentiation. Even at 24 h in culture, when iodination of protein in the 70 000-mol.wt. region was most marked in the crude total membrane fraction (Fig. 1c), we found only trace amounts of this iodinated protein in the purified plasma-membrane fraction. Proteins in the 100 000 *g* supernatant of cell homogenates prepared from iodinated cultures at the pre-fusion, mid-fusion and post-fusion stages were also analysed for incorporation of ^{125}I by SDS/polyacrylamide-slab-gel electrophoresis and fluorography. No labelling of these cytoplasmic proteins was detected at any stage of myogenesis examined, demonstrating that the cells remained impermeable to the iodination probe during the labelling procedure, and that labelling was specific for proteins at the cell surface. Since cytoplasmic proteins were unlabelled, those iodinated proteins that did not co-purify with the plasma-membrane fraction cannot represent cytoplasmic proteins exposed during the iodination process and subsequently adsorbed on the membranes.

Comparison of Figs. 1 and 2 reveals that the iodinated protein of apparent mol.wt. 255 000, suggested to be fibronectin, was present at a higher concentration in the total membrane fraction than in the plasma-membrane-enriched fraction. This indicates that this matrix protein (Furcht *et al.*, 1978; Hedman *et al.*, 1978) is detached from the plasma membrane and lost during the preparation of plasma-membrane vesicles from cultured muscle cells by sucrose-density-gradient centrifugation.

The major iodinated proteins found in the purified plasma-membrane fraction were glycosylated, as indicated by the incorporation of D- ^3H glucosamine (Fig. 3). A similar pattern of glycosylation was observed when L- ^3H fucose was incubated with the cells (results not shown). The lower-molecular-weight iodinated proteins, which did not co-purify with the plasma-membrane fraction, also did not incorporate D- ^3H glucosamine or L- ^3H fucose.

Cells were also incubated in media with EGTA added to lower the free Ca^{2+} concentration to values inhibitory to fusion. There was no marked qualitative or quantitative change in the pattern of iodination of proteins that have been shown to co-purify with the plasma-membrane fraction. There was, however, a slight decrease, compared with controls, in the extent of iodination of some of the lower-molecular-weight iodinated proteins shown not to co-purify with the plasma-membrane fraction (Fig. 4).

Fig. 3. Comparison of iodinated proteins of the plasma-membrane-enriched fraction with the pattern of D-[^3H]-glucosamine incorporation into plasma-membrane proteins

Skeletal-muscle monolayers were grown for: (a) 65 h in standard growth medium and then surface-labelled with ^{125}I as described in the Experimental section; (b) 56 h in standard growth medium and then for 9 h in standard growth medium supplemented with $5\mu\text{Ci}$ of D-[^3H]-glucosamine/ml. Plasma-membrane-enriched fractions were prepared from these cultures by the method of Schimmel *et al.* (1973), and incorporations of ^{125}I and [^3H]-glucosamine into $30\mu\text{g}$ samples of membrane protein were analysed by fluorography after SDS/polyacrylamide-slab-gel electrophoresis.

Fig. 4. Effect of inhibition of fusion, by growth in EGTA-containing medium, on the iodination of cell-surface proteins

Skeletal-muscle monolayers were grown in: (a) standard growth medium for 48 h; (b) standard growth medium for 24 h, then standard growth medium containing 1.85 mM-EGTA for 24 h. The cultures were then surface-labelled with ^{125}I , and the total membrane fraction was isolated and analysed for ^{125}I incorporation into proteins as described in Fig. 1 legend. At the time of iodination, cultures grown in EGTA-containing medium were entirely unfused, whereas the control cultures were at the midfusion stage.

Discussion

There are several possible explanations for the existence of two distinct classes of iodinated cell-surface proteins in cultured skeletal-muscle cells. Iodinated proteins may represent intrinsic membrane proteins, extrinsic membrane proteins, matrix proteins involved in cell adhesion and cell-cell recognition (Culp, 1976) or adsorbed serum components (Kinzel & Kubler, 1978). During purification of plasma membrane loosely bound extrinsic membrane proteins, matrix proteins and adsorbed serum components may detach from the plasma membrane and be separated from plasma-membrane vesicles prepared by sucrose-density-gradient centrifugation, as in the present study. For example, it has been shown that the matrix protein fibronectin is not retained in preparations of plasma-membrane vesicles from cultured fibroblasts unless the membranes are first fixed with ZnCl_2 (Marciani & Bader, 1975). In the present study iodinated protein of apparent mol.wt. 255 000, believed to be fibronectin, was lost during

the preparation of the plasma-membrane fraction, demonstrating that this matrix protein is also readily detached from the plasma membrane of cultured muscle cells. It is also possible that some or all of the iodinated polypeptides seen in Fig. 1 that did not copurify with the plasma-membrane fraction could represent proteolytic fragments of high-molecular-weight iodinated proteins that are able to adhere to the crude membrane fractions shown in Fig. 1, but are removed during the washing and centrifugation procedures required for purification of the plasma membrane. To minimize this possibility we routinely included the proteolytic enzyme inhibitor phenylmethanesulphonyl fluoride in the buffer used to homogenize iodinated cells. The fact that we obtained identical iodination profiles for purified plasma-membrane fractions from pre-fusion, mid-fusion and post-fusion cultures, where most of the iodinated proteins were of high molecular weight, tends to argue against differences in proteolytic enzyme activity being responsible for the different iodination profiles seen in Fig. 1.

Another possible explanation is that the plasma-membrane vesicles prepared in the present study do not contain a representative sample of all the intrinsic and extrinsic proteins present in the myoblast or myotube plasma membrane. Thus the plasma membrane may contain specialized domains, of a peculiar protein composition, or such domains may be induced during the plasma-membrane isolation procedure. Vesicles derived from such domains of the membrane may be preferentially isolated during purification of plasma membrane and may consequently be unrepresentative of the entire polypeptide composition of the intact cell membrane.

Iodination of cell-surface proteins during myogenesis of cells in culture has been studied with the primary objective of detecting alterations in the presence or orientation of proteins at the cell surface that may be involved in the mediation of myoblast fusion (Hynes *et al.*, 1976; Chen, 1977; Moss *et al.*, 1978). In the present study we have shown that there are no significant or reproducible alterations, before or concomitant with fusion, in the pattern of iodination of a class of cell-surface proteins that co-purify with a vesicular plasma-membrane fraction. As outlined above, the results obtained in this study do not enable us to define whether those iodinated proteins that do not co-purify with the plasma-membrane fraction, and are altered during myogenesis, represent intrinsic plasma-membrane proteins, peripheral proteins or adsorbed serum components. However, the vesicular plasma-membrane preparation that we have used has been demonstrated to undergo Ca^{2+} -dependent fusion *in vitro*, dependent on the state of maturation of the myoblast membranes used to prepare the plasma-membrane vesicles (Dahl *et al.*, 1978). Regardless of the origin of those iodinated proteins that do not co-purify with the plasma-membrane fraction, and regardless of alterations observed in the iodination of such proteins during myogenesis, we can therefore conclude that these proteins are not required for the fusion process. In particular, the failure of iodinated protein of apparent mol.wt. 70000 to co-purify with the plasma-membrane fraction demonstrates that this iodinated protein is not the same protein observed to increase in synthesis and accumulation in the myoblast plasma membrane concomitant with fusion (Cates & Holland, 1978).

Since the precise origin of those iodinated proteins that did not co-purify with the vesicular plasma-membrane fraction is not known, the significance of alterations in the pattern of iodination of these proteins during myogenesis is also unknown. It is likely that many of these proteins represent matrix proteins, as with fibronectin, which may be involved in cellular adhesion and cell-cell recognition.

A number of studies (Yamada *et al.*, 1976; Pearlstein, 1976; Furcht *et al.*, 1978) indicate that myoblasts are more adhesive than myotubes. Associated with myotube formation there is a decrease in the amount of extracellular filamentous matrix associated with the cells (Furcht *et al.*, 1978). The loss of this matrix may be reflected in the decrease in iodination of many cell-surface proteins, shown not to co-purify with the plasma-membrane fraction, which was observed in the present study concomitant with and after myoblast fusion. Although the present study indicates that such proteins are not required for the fusion of plasma-membrane vesicles *in vitro* (Dahl *et al.*, 1978), they may still play a role in facilitating fusion in the intact cultured cell.

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