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ENRICHMENT OF A 140 KD SURFACE GLYCOPROTEIN IN ADHERENT, DETERGENT-RESISTANT CYTOSKELETONS OF CULTURED HUMAN FIBROBLASTS

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SUMMARY: Radioactive surface and metabolic labeling techniques were used to study the surface glycoproteins which remain in detergent-resistant cytoskeletons of cultured human fibroblasts. A 140 kilodalton (kd), fucose-containing glycoprotein (gp) was enriched in the cytoskeletal preparations together with extracellular matrix fibronectin. The 140 kd gp resisted trypsin-treatment and was present as a major surface glycoprotein also in cytoskeletons of newly attached cells which had deposited only minimal quantities of extracellular matrix. In isoelectric focusing the protein was separated into three spots. The results suggest that the 140 kd gp is a cytoskeleton-associated surface glycoprotein which may play a role in the attachment of the cytoskeleton to the growth substratum.

Extraction of cultured cells with nonionic detergents removes most of the cellular proteins (1-7). Even after such a treatment, the cells remain tightly anchored to the growth substratum as ghost-like "detergent-resistant cytoskeletons" (3-7) which consist mainly of cytoskeletal filaments and a matrix-like nuclear residue. However, it is still unelucidated how the cytoskeletons are anchored to the growth substrate (4). Evidence from intact cells suggests a transmembrane linkage of cytoskeletal structures to the pericellular matrix (8-10). At the molecular level this would require the presence of one or several membrane components which span the membrane and connect the cytoskeleton to the extracellular matrix proteins (11-13). In this study we have used surface and metabolic labeling techniques to investigate whether there are membrane glycoproteins left in detergent treated and actomyosin depleted cells which could account for the anchorage of the cytoskeleton to the growth substratum.

Abbreviations: kd, kilodaltons; gp, glycoprotein; MW, molecular weight; NaB^3H_4, tritiated sodium borohydride; PMSF, phenylmethyl sulfonyl fluoride.

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MATERIALS AND METHODS

Cell culture and extractions: Human embryonic fibroblasts, obtained from a local source, were cultured on plastic Petri dishes in RPMI 1640 medium supplemented with 10 % fetal calf serum (Flow Laboratories, Irvine, Scotland) and antibiotics. To prepare cytoskeletons, cell cultures were treated with 0.5 % Triton X-100 (BDH Chemicals Ltd., Poole, England) in 50 mM Tris-HCl, pH 7.2 supplemented with 1 mM PMSF (Sigma Chemical Company, St. Louis, Mi.) at 0°C for 30 min. To dissociate the actomyosin system, the cells were further treated with low and high ionic strength solutions: first with the low ionic (60 mM KCl, 1 mM EGTA, 1 mM cysteine hydrochloride, 10 mM ATP and 40 mM imidazole, pH 7.0) followed by the high ionic solution (600 mM KCl, 1 mM EDTA, 10 mM ATP, 1 mM cysteine hydrochloride and 40 mM imidazole, pH 7.0) at 0°C for 30 min, both (7,14).
To detach the cells from the growth substratum, the cultures were treated with trypsin (250 ug/ml; Medica Pharmaceuticals, Helsinki, Finland) in NaCl-P buffer (140 mM NaCl, 10 mM sodium phosphate, pH 7.2) at 37°C for 15 min. Detached cells were pelletted by low speed centrifugation and washed once with the culture medium to inactivate trypsin. Thereafter, trypsinized cells were either seeded again onto culture dishes or washed twice with the NaCl-P buffer supplemented with Ca^{++} and used for surface labeling studies as described below. Cell viability test with trypan blue showed that practically all cells (98 %) excluded the dye. For phase contrast microscopy the cells were fixed in 3.5 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.2.

Metabolic and surface labeling and electrophoretic analysis: For metabolic labeling the cells were incubated at an initial density of 0.5 x 10^6 cells/ml with L-[^{3}H] fucose (10 uCi/ml; 27 Ci/mmol, Radiochemical Centre, Amersham, England) in RPMI medium depleted of glucose and supplemented with sodium pyruvate, for 48 hours. For surface labeling, the neuraminidase/galactose oxidase/NaB^3H_4 method of Gahmberg and Hakomori (15) was used. Briefly, the cells were treated with neuraminidase (10 U/ml; Behringwerke, Marburg-Lahn, FRG) and galactose oxidase (5 U/ml; Kabi, Stockholm, Sweden) in NaCl-P buffer supplemented with Ca^{++} at 37°C for 30 min. After washing twice in NaCl-P buffer, the cells were treated with NaB^3H_4 (2.5 mCi/ml; 9.8 Ci/mmol; Radiochemical Centre) in NaCl-P buffer at 23°C for 30 min. The enzyme treatments were performed at a density of 4 x 10^6 cells/ml either with cells attached on culture vessels or with cells brought to suspension with rubber policeman or trypsin treatment. Practically all the cells excluded trypan blue when tested prior to or after the treatments. After labeling, the cells were extracted as above and dissolved in electrophoresis sample buffer. In some experiments the enzyme treatments and the labeling were done after the extractions. The experiments, in which the cells were treated with NaB^3H_4 without pretreatment with the enzymes (15) showed the lack of unspecific labeling of the cells.
Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to Laemmli (16) using 5 % and 8 % slab gels. Isoelectric focusing was carried out using the two-dimensional gel system of O'Farrell (17) with 8 % slab

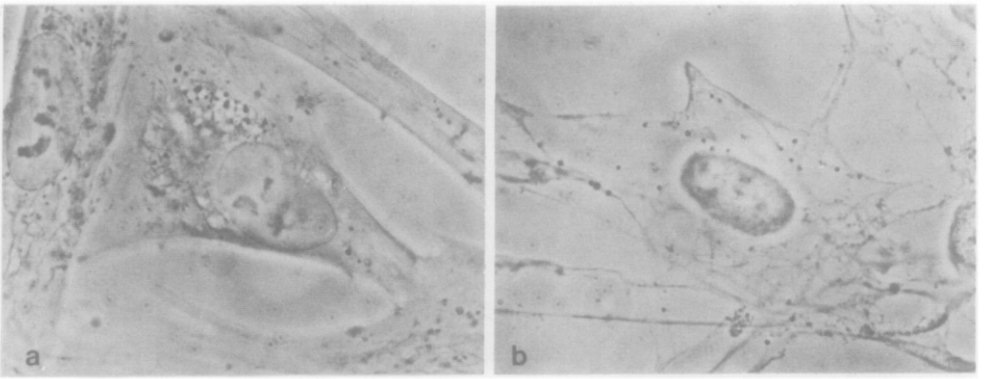

Fig. 1.
Phase contrast micrograph of (a) untreated, well spread cells and (b) extracted cells (x 560).

gels in the second dimension. After the electrophoresis, the gels were processed for fluorography according to Bonner and Laskey (18).

RESULTS

Phase contrast microscopy: Fibroblasts cultured for four days appeared well spread and had a dense cytoplasm (Fig. 1a). After the Triton X-100 and the low and high ionic actomyosin extractions, ghost-like structures were left. The cytoskeletal preparations remained tightly anchored to the growth substratum and could not be detached even by vigorous agitation (Fig. 1b). The cytoplasmic region of the extracted cells appeared pale and lacked cell organelles while the nuclei became distinctly visualized.

Electrophoretic analysis: Polyacrylamide gel electrophoresis of the neuraminidase/galactose oxidase/NaB^3H_4-labeled cultured fibroblasts revealed several polypeptides in the MW region of 30 kd to 250 kd (Fig. 2, track 1). After the detergent and actomyosin extractions, only two major polypeptides remained in the surface labeled cells: a 220 kd and a 140 kd polypeptides with some faintly labeled polypeptides in the MW region of 80 kd and 50-60 kd (Fig. 2, track 2). If the cells were first extracted and then surface labeled, the 140 kd polypeptide remained distinct while the 220 kd polypeptide could be hardly detect-

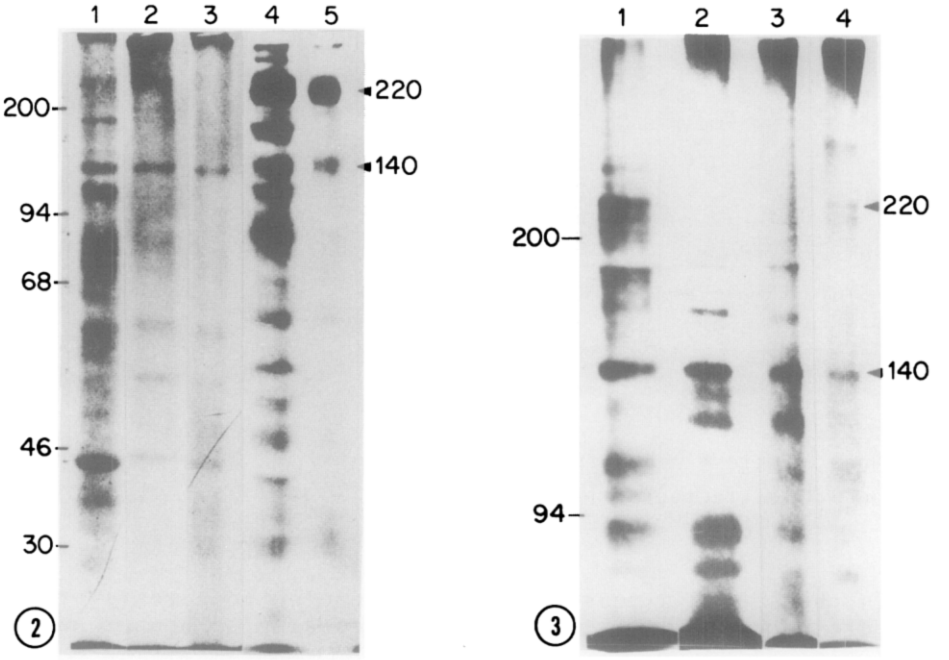

Fig. 2.
SDS polyacrylamide gel electrophoresis (8 % gel) of human embryonic fibroblasts cultured for 96 hours. (1) Adherent cells treated with neuraminidase and galactose oxidase, brought to suspension with a rubber policeman and reduced with NaB^3H_4; (2) adherent cells surface labeled as above and thereafter extracted first with 0.5 % Triton X-100 and then with the actomyosin extraction buffers; (3) adherent cells extracted with 0.5 % Triton X-100 and the actomyosin extraction buffers and thereafter surface labeled as above; (4) unextracted cells labeled with tritiated fucose for 48 hours and (5) adherent cells labeled with tritiated fucose for 48 hours and then extracted with 0.5 % Triton X-100 and the actomyosin extraction buffers. Note the distinct enrichment of the 140 kd and the 220 kd polypeptides in the extracted cells. As MW standards the ^{14}C-methylated protein mixture (Radiochemical Centre), consisting of myosin (200 kd), phosphorylase B (93 kd), bovine serum albumin (68 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd) and lysozyme (14 kd) was used. MW standards are indicated on the left-hand side.

Fig. 3.
SDS polyacrylamide gel electrophoresis (5 % gel) of (1) adherent cells cultured for 96 hours, treated with neuraminidase and galactose oxidase, brought to suspension with a rubber policeman and reduced with NaB^3H_4; (2) adherent cells cultured for 96 hours,

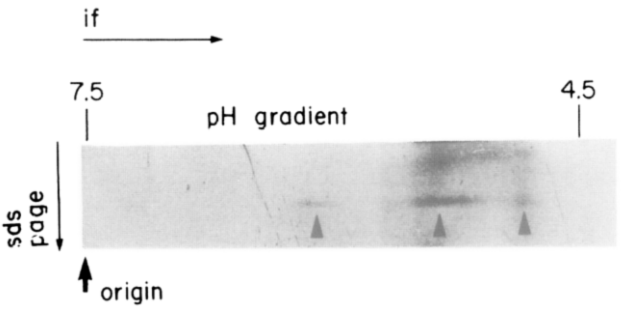

Fig. 4.
Isoelectric focusing of the 140 kd polypeptide. The sample was prepared as in Fig. 2, track 3. The 140 kd polypeptide is separated into three components in the pH range of about 4.8-6.3 with a major component with pI of about 5.3.

ed (Fig. 2, track 3). No major differences were found in the polypeptide profiles of the cells irrespective of whether the neuraminidase and galactose oxidase treatments were performed on attached cells or cells brought to suspension with the rubber policeman. Electrophoretic analysis of the ^{3}H-fucose-labeled cells showed several prominent polypeptides (Fig. 2, track 4). After the extractions only two major polypeptides, 140 kd and 220 kd, remained (Fig. 2, track 5).

The polypeptide profile of the trypsinized and surface labeled cells did not differ from that of untreated fibroblasts except the disappearance of the prominent 220 kd polypeptide and a more intensive labeling of the lower molecular weight components (Fig. 3, track 1 and 2). If the cells were allowed to adhere for three hours after trypsinization and analyzed electrophoretically, the 140 kd gp appeared to be the major surface glycoprotein while the 220 kd gp was hardly detectable (Fig. 3, track 3). Notably, the 140 kd gp was present as the major glyco-

trypsinized and surface labeled as above; (3) adherent cells cultured for three hours after trypsinization and surface labeled as above and (4) adherent cells cultured for three hours after trypsinization, surface labeled as above and extracted with 0.5 % Triton X-100 and the actomyosin extraction buffers. Note the disappearance of the 220 kd polypeptide after trypsinization and the persistence of the 140 kd polypeptide as the major polypeptide also after the extractions.

protein in the detergent-resistant cytoskeleton of these newly--attached cells (Fig. 3, track 4).
Two-dimensional gel electrophoresis of the sample produced as in Fig. 2, track 3, revealed three separate spots in the 140 kd region with approximate isoelectric points of 4.8 to 6.3 (Fig. 4).

DISCUSSION

In this study we used surface and metabolic labeling techniques to recognize the surface glycoproteins present in adherent, detergent-resistant cytoskeletons of cultured human embryonic fibroblasts. The neuraminidase/galactose oxidase/NaB^3H_4 method labels specifically the galactosyl and N-acetylgalactosylaminyl residues of surface glycoproteins and -lipids of intact cells (15,19-21). Fortuitous labeling of intracellular glycoproteins was ruled out in this study by trypan blue exclusion test (cf. 19). Also tritiated fucose, used for metabolic labeling, has been found to be preferentially a marker for surface glycoproteins (19,20,22). It could be used also for distinguishing between cell products and the fetal calf serum proteins which can be deposited at the cell surface and become detectable by the surface labeling technique (12).

Two major glycoproteins were revealed in the cytoskeletons of the cultured fibroblasts by both surface and metabolic labeling; a 220 kd and 140 kd glycoprotein. The 220 kd gp appeared to be trypsin-sensitive and it apparently corresponds to fibronectin which is an abundant pericellular glycoprotein in confluent fibroblast cultures (24,25). The 140 kd gp was, on the other hand, trypsin resistant. It was present as a major glycoprotein also in newly attached cells which had deposited only minimal quantities of pericellular matrix material, fibronectin and collagen (25) suggesting that it is rather a surface than a pericellular matrix protein. The non-collagenous nature of the 140 kd gp is supported also by its strong labeling with tritiated fucose (26,27) and by our finding that it resists treatment with bacterial collagenase (Lehto, to be published).

The results show the presence of a novel 140 kd surface glycoprotein which is enriched in the detergent resistant cytoskele-

tons of the cultured human fibroblasts. The trypsin- and detergent-resistance indicates that the protein is stongly associated with the plasma membrane. As it is the only major surface glycoprotein left in the cytoskeletons of the newly attached cells, we assume it to play major role in the anchorage of the cytoskeleton. It could be part of the specialized detergent resistant membrane areas which correspond to the cell attachment sites and are associated with filamentous cytoskeletal structures (5,28-31). Further studies are needed, however, to elucidate whether the 140 kd gp is an integral membrane protein which could be transmembraneously linked to cytoskeletal components.

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