QUANTITATION OF A TRANSFORMATION-SENSITIVE, ADHESIVE CELL SURFACE GLYCOPROTEIN

Decrease on Several Untransformed Permanent Cell Lines

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ABSTRACT

We have quantitated the transformation-sensitive, cell surface LETS glycoprotein on many untransformed cell types. By SDS-polyacrylamide gel electrophoresis, this trypsin-sensitive iodinatable glycoprotein comprises 1–3% of total cellular protein of the seven early passage cell types tested. In contrast, it constitutes less than 0.15% of the protein in four of six continuous cell lines. This decrease is reflected in alterations both in [^{14}C]glucosamine labeling and in the immunofluorescent staining of early passage vs. these four permanent cell lines. These results help to clarify previous experiments in which CSP, a purified LETS protein, partially restored a fibroblastic phenotype to cells transformed by tumor viruses. These findings also indicate that a major decrease in this cell surface glycoprotein can occur in the establishment of a continuous cell line without resulting in cellular transformation.

KEY WORDS cell surface glycoprotein · cell adhesion · transformation · cell lines · LETS protein · cell surface protein

A high molecular weight glycoprotein, "LETS" protein, can be detected on the surfaces of untransformed fibroblasts by several procedures which label cell surface proteins by means of radioactive compounds or specific antibodies. This protein is usually decreased or absent after neoplastic transformation (7, 9–11, 17, 19, 21), and this decrease generally correlates with tumorigenicity (3). The LETS protein from chick embryo cells has been designated CSP. We have isolated CSP in a highly purified form and reattached it to transformed cells (25, 28, 30). Such reconstitution experiments indicate that CSP is a protein that increases cell-cell and cell-substratum adhesion, and thereby helps control overall cell shape and cell surface morphology, as well as promoting parallel alignment of cells and inhibiting overlapping of cells. CSP does not appear to regulate growth rate or nutrient transport (25–30).

We have now measured and compared the amounts of this LETS- or CSP-like glycoprotein on various fibroblastic cells from several species. We find that it constitutes 1–3% of total cell protein of early passage cells, but that it is present in substantially decreased amounts in four of six continuous untransformed cell lines and in slightly decreased amounts in the other two continuous lines. Therefore the LETS protein appears to be present in highest amounts in primary cell cultures, in more intermediate amounts in established nontumorigenic (or nontransformed) cell lines, and in lowest amounts after transformation.

MATERIALS AND METHODS

Protein Quantitation

Cells were cultured in 10% heat-inactivated calf serum in Dulbecco-Vogt medium as described previously (25), and the medium was changed every other day. Three days after reaching confluence, and 1 day after the last medium change, cultures were rinsed four times with Dulbecco's phosphate-buffered saline (PBS), then either (*a*) iodinated and homogenized according to Hynes (10) by means of 400 μCi/ml carrier-free ^{125}I-Na (New England Nuclear, Boston, Mass.), or (*b*) not labeled, and homogenized directly in the dish with 2% SDS (28), to eliminate the possibility that either the iodination proce-

dure or the subsequent manipulations were artifactually decreasing the protein. In each case, half of the cultures were trypsinized with 10 μg/ml crystalline trypsin (Worthington Biochemical Corp., Freehold, N. J.) in PBS for 10 min at 37°C, then rinsed twice in PBS containing the protease inhibitor phenylmethylsulfonyl fluoride at 2 mM, and homogenized.

Samples containing 40 μg of protein by the procedure of Lowry et al. (15) with bovine serum albumin (BSA) standards were electrophoresed on 5% polyacrylamide SDS gels, stained with Coomassie blue, photographed, and then processed for autoradiography as described (28). Differences in Coomassie-blue staining in different lanes were determined by absorbance at 550 nm with a Joyce-Loebl densitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England) and expressed as percent of total protein staining. The only detectable changes in staining after trypsinization were in the LETS/CSP region. The amount of trypsin-sensitive protein was not significantly different in iodinated vs. non-iodinated cultures; these values have therefore been averaged.

Molecular weight standards included chicken gizzard filamin (240,000), rabbit skeletal muscle myosin (200,000), RNA polymerase (150,000 and 160,000), phosphorylase a (94,000), BSA (68,000), and ovalbumin (43,000).

Immunofluorescence

Cells were plated on glass cover slips (25) and maintained in culture for 3 days after reaching confluence. Cultures were fixed for 1 h in 2% formaldehyde in PBS, stained for 1 h with 20 μg/ml affinity-purified goat anti-CSP, then stained with a 1:10 dilution of fluorescein-labeled anti-goat IgG rabbit IgG (Miles Laboratories, Elkhart, Ind.) for 1 h and examined on a Zeiss microscope equipped with a BG 12 exciter and a 500-nm barrier filter. Substitution of 20 μg/ml goat gamma globulin from control antiserum (29) for the anti-CSP resulted in negative immunofluorescent localization for all cell types. Cells were photographed with Polaroid type 107 film (ASA 3,000).

Cell Types

Chick embryo fibroblasts prepared from 10-day embryos according to Vogt (22) were used at passage no. 2 or 3; mouse embryo cells prepared similarly from mid-term Swiss mice, or purchased from Microbiological Associates (Bethesda, Md.), at passage no. 2; newborn Syrian hamster kidney cells and 6 day Wistar rat kidney cells (North American Biologicals, Inc., Miami, Fla., or Microbiological Associates) at passage no. 2; human WI38 embryonic lung fibroblasts (8; NIH media unit) at passages nos. 21-23; human HR6 embryonic lung fibroblasts (HEM Research, Inc., Rockville, Md.) at passages nos. 22-24; and human MA196 adult skin fibroblasts (Microbiological Associates) at passage no. 39. (For histories of the derivation of permanent cell lines Swiss 3T3 (20), BALB/c 3T3 (1), NIH 3T3 (13), BHK (18), NRK (2, 6), and Nil (5), see indicated references.)

RESULTS

We have utilized the following characteristics to identify this glycoprotein: (*a*) it has an apparent subunit mol wt of 220,000-250,000 on SDS gels; (*b*) it is heavily labeled when intact cells are iodinated with ^{125}I; (*c*) it is labeled when cells are incubated with [^{14}C]glucosamine; (*d*) it has a fibrillar extracellular distribution by immunofluorescent staining using anti-CSP antibodies; and (*e*) it is rapidly destroyed by trypsin (3, 7, 9-11, 17, 19, 21, 24, 28).

Quantitation by Protein Staining

Each cell type was iodinated using lactoperoxidase and glucose oxidase. After trypsinization with 10 μg/ml trypsin at 37°C for 10 min, the prominent LETS or CSP band was absent, whether the iodination reaction was carried out before or after trypsinization (Fig. 1). The amount of this glycoprotein was quantitated by determining the difference in Coomassie blue staining in the LETS/CSP region before and after trypsinization.

Homogenates of all early passage cells contained a broad, heavily staining protein band in the 220,000-250,000 mol wt region which was estimated by densitometry to constitute 1-3% of total cellular protein (Fig. 1; Table I). This band was also heavily labeled with ^{125}I, and this label was also removed by the trypsinization (Fig. 1). The early passage cells we tested included human, mouse, rat, hamster, and chicken cells at the passage numbers indicated in Materials and Methods. A major protein band adjacent to the LETS protein in gels stained with Coomassie blue had an apparent mol wt of 240,000 and was not affected by the trypsinization. This band is the intracellular protein filamin (4, 23).

In contrast to the results with early passage cells, in homogenates of the continuous cell lines Swiss 3T3, BALB/c 3T3, BHK, and NRK, there was no detectable alteration in the staining of the 220,000-250,000 region of the gel after trypsinization, even though all the iodinatable protein in this region was removed by the trypsin treatment (Fig. 1, Table I). Two other permanent cell lines, NIH 3T3 and Nil, contained substantial amounts of this protein. However, the amounts were moderately decreased compared to the original early

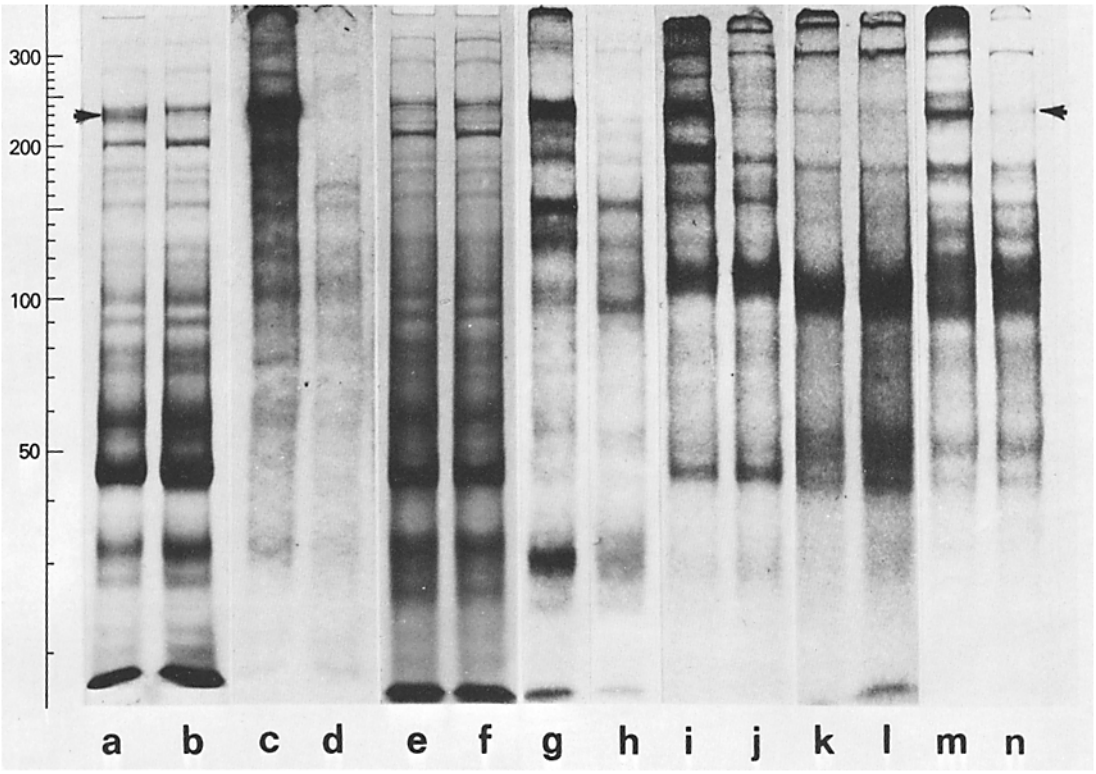

FIGURE 1 Protein staining, iodination, and glucosamine labeling of early passage cells compared with established cell lines. Cells were cultured, labeled, homogenized in SDS, and electrophoresed in 5% polyacrylamide SDS gels as indicated in Materials and Methods. (*a–h*) ^{125}I-labeled cultures, 40 μg protein per lane. (*a*) Coomassie blue-stained gel of homogenate of iodinated Swiss mouse embryo fibroblasts. (*b*) Same as Fig. 1 *a*, except cells were treated with 10 μg/ml trypsin for 10 min at 37°C after iodination. (*c* and *d*) Autoradiographs of Fig. 1 *a* and *b*. (*e*) Coomassie blue-stained gel of homogenate of iodinated BALB/c 3T3 cells. (*f*) Same as Fig. 1 *e*, except cells were treated with 10 μg/ml trypsin for 10 min after iodination. (*g* and *h*) Autoradiographs of Fig. 1 *e* and *f*. (*i–n*) Autoradiographs of gels of cells labeled with [^{14}C]glucosamine. Cultures were labeled with 1 μCi/ml [^{14}C]glucosamine (New England Nuclear, 237.7 mCi/mmol) in regular culture medium for 24 h, then homogenized and electrophoresed using 40 μg per lane. (*i*) Swiss mouse embryo fibroblasts. (*j*) Mouse embryo fibroblasts after trypsinization as above. (*k*) BALB/c 3T3 mouse cells. (*l*) Trypsinized BALB/c 3T3 cells. (*m*) Human embryonic lung WI38 fibroblasts. (*n*) Trypsinized WI38 fibroblasts. Arrows indicate LETS/CSP band.

passage Swiss mouse and hamster cells (Table I). The Nil cells were tested at passage 36; even at passage 66, they still contained comparable amounts of LETS protein.

Glucosamine Incorporation

We compared glucosamine labeling of this glycoprotein in various cell types to evaluate the possibility that the apparent decreases in protein staining were due to some unusual alteration in accessibility, e.g., a loss of sensitivity to trypsin of all but a very short portion of the molecule that could still be iodinated and removed by trypsin. Cells were incubated for 24 h in culture medium containing 1 μCi/ml [^{14}C]glucosamine, homogenized, and electrophoresed on SDS-polyacrylamide gels. BHK, NRK, and BALB/c 3T3 cells incorporated substantially less glucosamine into the LETS region when compared with early passage mouse embryo fibroblasts, chick embryo fibroblasts, and human fibroblasts (Fig. 1). Total glucosamine labeling was not decreased in the permanent cell lines.

Immunofluorescence

The decreases in this glycoprotein in several

TABLE I
Percent of Total Cell Protein in LETS Band

Cell type	Cell protein	Final cell density
	% of total	*cells/cm²*
Chick embryo fibroblasts	3.1	3.3×10^5
Mouse embryo fibroblasts	1.0	2.2×10^5
Swiss 3T3-4*	<0.15	4.8×10^4
BALB/c 3T3*	<0.15	5.1×10^4
NIH 3T3*	0.7	1.8×10^5
Hamster kidney cells	0.4	5.0×10^4
BHK*	<0.15	7.6×10^5
Rat kidney cells	2.0	1.2×10^5
NRK*	<0.15	1.5×10^5
Hamster embryo fibroblasts	0.8	3.2×10^5
Nil*	0.6	7.6×10^4
Human embryonic lung fibroblasts WI38	0.6	2.2×10^5
Human embryonic lung fibroblasts HR6	2.5	4.0×10^5
Human adult skin fibroblasts MA196	2.1	2.0×10^5

* Asterisks indicate permanent cell lines; others were early passage cells. All cells were assayed as described in Materials and Methods 3 days after reaching confluence.

continuous cell lines as determined by protein staining and glucosamine labeling are also confirmed by the immunofluorescent staining of these cells. Antibody to isolated, electrophoretically purified CSP (29) intensely stains all early passage cells tested (Fig. 2 *a–c*). The protein is distributed in complex fibrillar patterns similar to those reported by Wartiovaara et al. (24) and Chen et al. (3) for several cell types in vitro as well as in vivo (14). The continuous cell lines BALB/c 3T3, Swiss 3T3, NRK, and BHK, however, show weaker staining. The fibrils surrounding BHK and both lines of 3T3 cells were more slender, and overall staining was less intense, than on freshly explanted cells (Fig. 2 *d* and *e*). Clusters of thicker fibrils were present (Fig. 2 *e*), but these were infrequent and isolated. NRK cells contained even fewer, very weakly staining fibrils. Nil cells, as reported by Hynes et al. (12), stain in prominent

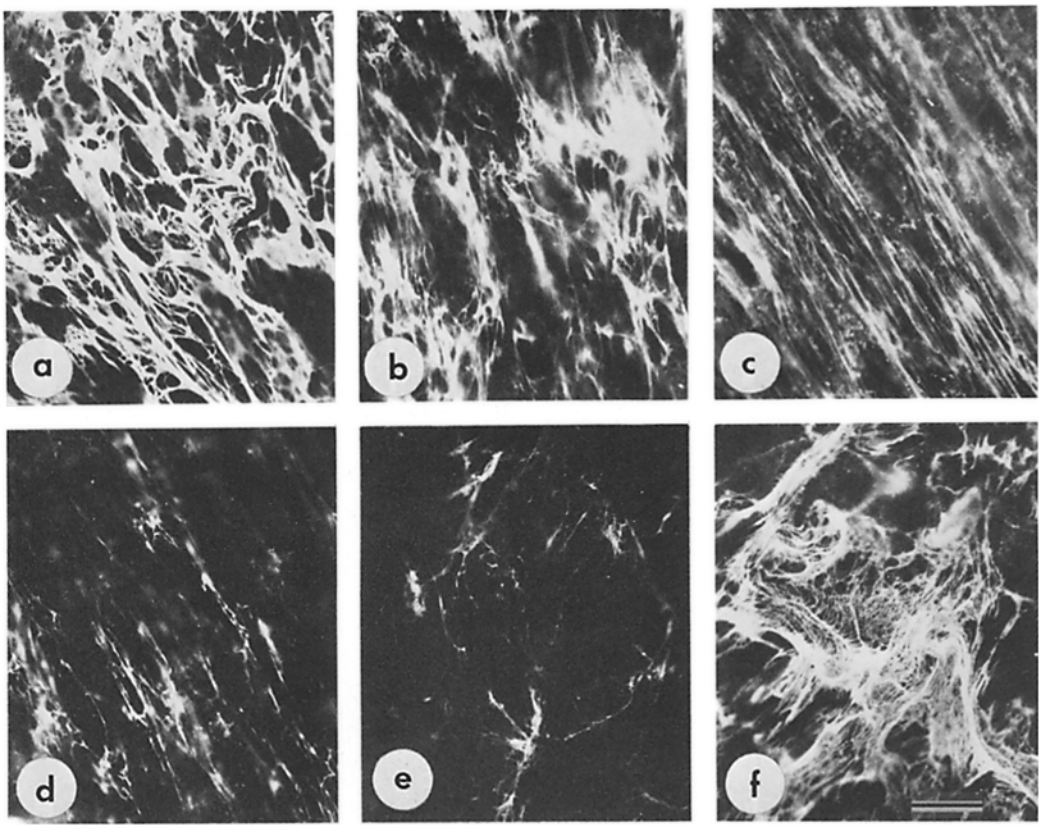

FIGURE 2 Immunofluorescent staining of early passage cells vs. permanent cell lines using anti-CSP antibody. (*a*) Chick embryo fibroblasts; (*b*) Swiss mouse embryo fibroblasts; (*c*) human lung fibroblasts, HR6; (*d*) BHK; (*e*) BALB/c 3T3; and (*f*) Nil. Cells were photographed with exposures of 5 s for (*c–f*), which were reduced to 2 s for Fig. 2 *a–b* due to their greater fluorescence. Bar, 25 μm.

fibrillar networks (Fig. 2*f*); NIH 3T3 cells also stain heavily.

DISCUSSION

Our results indicate that early passage adult and embryonic fibroblastic cells produce large amounts of CSP or other LETS proteins, but that much less is found at the surface of four of six widely used, "untransformed" permanent cell lines. Whether this decrease reflects decreased synthesis, increased degradation, or failure of retention at the cell surface remains to be determined.

Although our results show a decrease of severalfold after establishment of several permanent cell lines, we wish to emphasize that neoplastic transformation generally decreases the quantity of this glycoprotein further as determined by surface labeling or immunofluorescence techniques (7, 9, 10, 17, 19, 21, 24, and our unpublished results).

The presence of large amounts of LETS protein on primary explanted cells from human, mouse, rat, and hamster sources suggests that this protein could play an adhesive role in these cells similar to that postulated for CSP on chick cells (25–30). However, the decreased quantities of the protein in four of six continuous cell lines raises the possibility that this glycoprotein might play a quantitatively less important role in the behavior of some of these permanent cell lines, and that their cell-to-substratum adhesion might depend on additional adhesive glycoproteins (16).

Our results help clarify several previous apparently anomalous results. In reconstitution experiments (25, 30), CSP was added back to transformed cells in amounts which resulted in the accumulation of quantities of CSP similar to those found on untransformed primary cells (2–3% of total protein). Transformed chick cells regained the flattened morphology and parallel alignment of cells characteristic of untransformed chick fibroblasts. Transformed 3T3 and NRK cells also responded to this treatment by becoming flattened and elongated. However, in morphology the latter cells were more similar to freshly explanted fibroblasts than to the parental BALB/c 3T3 or NRK cells, which had a distinct polygonal or epithelioid shape (30). In addition, the parental cells remained polygonal when they became confluent, whereas several of these CSP-treated cell lines displayed marked alignment at confluence (25, 30). Moreover, when the untransformed parental BALB/c 3T3 and NRK cells (which have decreased amounts of LETS protein) were treated with CSP, they also became partially elongated and aligned (30).

CONCLUSION

The results presented here are consistent with the notion that CSP and other LETS proteins are partially responsible for the flattened elongated morphology and parallel alignment of freshly explanted fibroblasts, and that reconstitution of CSP on transformed cells partially restores this particular phenotype.

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