CHARACTERIZATION OF THE EXTERNAL PROTEINS OF HAMSTER FIBROBLASTS

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ABSTRACT

The results of metabolic labeling studies and enzymatic treatments followed by analysis on polyacrylamide gels show that the external proteins of hamster fibroblast cell lines, which have been identified by lactoperoxidase-catalyzed iodination, do not contain sulphated mucopolysaccharides or hyaluronic acid and are probably unrelated to collagen. Several of the iodinated species comigrate with carbohydrate-containing molecules. In particular, the major iodine-labeled polypeptide of normal fibroblasts appears to be a glycoprotein. This glycoprotein is absent or much reduced in virus-transformed cells, as detected both by iodination and by metabolic labeling. We conclude that the major iodinated polypeptide is not detected on transformed cells because it is absent rather than because it is masked. Approximate molecular weights of the external proteins are also reported.

INTRODUCTION

Evidence has been presented elsewhere that the technique of lactoperoxidase-catalysed iodination (1, 2), when applied to mammalian fibroblasts grown in tissue culture, labels only, or predominantly, proteins which are accessible from the outside of the cell (3). In the case of fibroblasts showing density-dependent inhibition of growth, these external proteins comprise one high molecular weight polypeptide which is very heavily iodinated (band 1) and a number of less heavily labeled and lower molecular weight polypeptides. The polypeptide which migrates as band 1 on polyacrylamide gels is not available for iodination in virus-transformed derivatives of these "normal" fibroblasts, whereas the other external proteins appear to be unaffected by transformation.

Most of the external proteins detected by iodination are removed by mild treatments with trypsin (3) which confirms their external location. Band 1 polypeptide is particularly sensitive to proteolysis (3) which is consistent with the idea that its absence in transformed cells may be due to the action of proteolytic enzymes produced by these cells (3–8). However, it is equally possible that band 1 polypeptide is not synthesized by transformed cells or that it is present but masked so that it cannot be labeled by lactoperoxidase. We wish to report here results of experiments to investigate further the nature of the proteins detected by iodination, and evidence suggesting that band 1 polypeptide is actually absent from transformed cells, not merely unavailable for iodination.

MATERIALS AND METHODS

Cells and Culture Methods

Two cell types were used: a fibroblastic cell line, NIL8, from hamsters (9) and a derivative (NIL8.HSV6) transformed by hamster sarcoma virus (10) and isolated by its ability to grow in soft agar (11). Cells were grown

in plastic petri dishes in Dulbecco's modification of Eagle's medium plus 10% calf serum and subcultured by trypsinization. For labeling with [^{14}C]leucine, the level of leucine in the medium was reduced to 10% of the normal concentration, and for labeling with [^{14}C]glucosamine, the glucose level was reduced to 30% of normal.

Lactoperoxidase-Catalysed Iodination and Preparation of Samples

This was performed on cell monolayers as described previously (3). Cells were washed three times with phosphate-buffered saline, pH 7.2, and labeled in the same buffer plus 5 mM glucose, 400 μCi/ml carrier-free sodium [^{125}I]iodide, 20 μg/ml lactoperoxidase, and 0.1 U/ml glucose oxidase. Incubation was carried out for 10 min at room temperature. The reaction was stopped by addition of phosphate-buffered sodium iodide and the monolayers were washed twice more with the same buffer. The cells remained viable during this procedure and continued to grow if returned to culture medium. For analysis, the cells were scraped into phosphate-buffered iodide containing the protease inhibitor, phenyl methyl sulfonyl fluoride (2 mM), concentrated by centrifugation and dissolved by boiling in electrophoresis sample buffer containing 2% sodium dodecyl sulphate (SDS) and 2 mM phenyl methyl sulfonyl fluoride. Samples were made 0.1 M dithiothreitol and boiled again before electrophoresis.

Polyacrylamide Gel Electrophoresis

This was performed in buffers containing sodium dodecyl sulphate (12) either in vertical slabs or in cylindrical gels. In the former case, gels were dried down onto paper and radioactivity was detected by autoradiography on Kodirex X-ray film, Kodak, London (13), Cylindrical gels were run with 10% glycerol in all buffers, frozen in dry ice, and sliced into 1-mm slices. Gel slices were counted in a Nuclear Enterprises (Edinburgh II, Scotland) gamma counter (^{125}I) or dissolved in hydrogen peroxide and counted, using a Triton-toluene scintillant, in a Nuclear-Chicago (G. D. Searle, Bucks, England) scintillation counter (^{3}H and ^{14}C). Gels were stained with Coomassie brilliant blue R250 if desired.

Analysis for Hydroxyproline Incorporation

NIL8 cells labeled for 24 h with [^{3}H]proline in the presence of ascorbic acid (see Fig. 4) were run on cylindrical gels together with a trace of iodinated cells. Slices containing the major ^{125}I-labeled peak were eluted with 0.05% SDS, 5 mM $NaHCO_3$ overnight at 37°C (70% elution of ^{125}I cpm). The eluate was lyophilized, hydrolyzed with 6 N HCl, dried down, and run on thin layer cellulose plates with marker proline and hydroxyproline. Development of the plates was carried out in chloroform:methanol:ammonia, 2:2:1 (14). The plates were scraped and counted in a toluene scintillant.

Enzymes and Chemicals

Calf collagen (acid soluble) and lactoperoxidase, Calbiochem (London, England); glucose oxidase, hyaluronidase, and collagenase (pure), Worthington Biochemicals; trypsin 2 times crystallized, Koch-Light Laboratories Ltd. (Buckinghamshire, England) or Sigma Chemical Co. (London, Surrey); chondroitinase ABC, Seikagaku Kogyo Co. Ltd., Tokyo; neuraminidase, Behringwerke AG, Marburg-Lahn, W. Germany.

Radiochemicals

Carrier-free sodium [^{125}I]iodide, L-[5-^{3}H]proline (>5 Ci/mmol), L-[U-^{14}C]leucine (342 Ci/mol), D-[U-^{14}C]glucosamine (318 Ci/mol), and sodium [^{35}S]sulphate (carrier-free), were all obtained from the Radiochemical Centre, Amersham, Buckinghamshire. L-[1-^{14}C]fucose (52 Ci/mol) was obtained from Calbiochem.

RESULTS

Labeling with Carbohydrate Precursors

Normal (NIL8) or transformed (NIL8.HSV6) hamster fibroblasts were cultured for 2–3 days in medium containing [^{14}C]glucosamine or [^{14}C]fucose. When the cultures reached confluence, cells were harvested for SDS-polyacrylamide gel electrophoresis as described in Materials and Methods, either with or without a prior treatment with a low level of trypsin (10 μg/ml, 10 min). The tryptic digestion was the same as that shown previously (3) to remove all but one of the major iodinatable surface proteins (see also Figs. 3 and 5). During digestion there was no rounding up or detachment of cells from the monolayer, and the cells remained viable. Results are shown in Figs. 1 and 2. Both glucosamine and fucose were incorporated by normal cells into a high molecular weight band which comigrated with band 1 of the iodination profile (Figs. 1 *b*, 2 *b*, 2 *f*). Transformed cells did not show labeling of this band in either case (Figs. 1 *d*, 2 *d*), a result which parallels that obtained by iodination (3). In addition, these precursors were incorporated into a number of lower molecular weight bands which did not appear to differ between normal and transformed cells. These lower molecular weight sugar-labeled bands tended not to be sharply defined on the gels. The reason for this lack of definition is not understood, but it precludes identification of these bands with iodine-labeled bands of similar mobility.

When normal NIL8 cells labeled with fucose or glucosamine were treated with trypsin before pro-

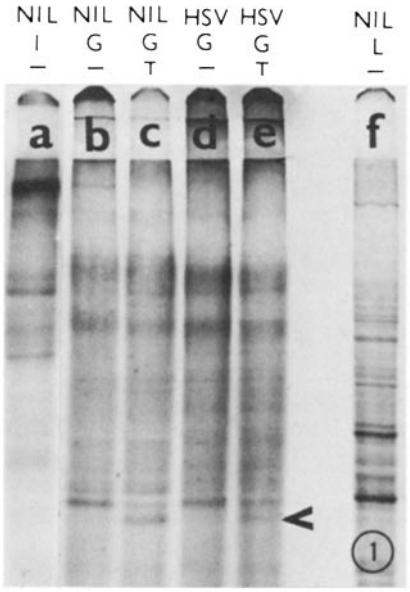

FIGURE 1 Labeling of NIL8 and NIL8.HSV6 cells with various isotopes: autoradiogram of a 7.5% SDS-polyacrylamide gel of cells labeled with either [^{14}C]glucosamine (*G*) 5 μCi/ml for 3 days; [^{14}C]leucine (*L*), 1 μCi/ml for 3 days; or by lactoperoxidase-mediated [^{125}I]-iodination (*I*) and treated where indicated with 10 μg/ml trypsin (*T*) for 10 min at room temperature before harvesting for electrophoresis. The arrow marks the position of the glucosamine-labeled band which appeared after trypsin treatment of both cell types. Electrophoresis in this and all similar figures was from top to bottom.

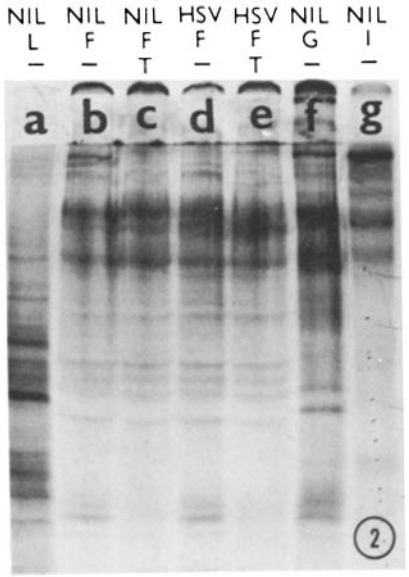

FIGURE 2 Labeling of NIL8 and NIL8.HSV6 cells with various isotopes: autoradiogram of an 8.0% SDS-polyacrylamide gel of cells labeled with either [^{14}C]fucose (*F*), 3 μCi/ml for 3 days; [^{14}C]glucosamine (*G*), 5 μCi/ml for 3 days; [^{14}C]leucine (*L*), 1 μCi/ml for 3 days; or lactoperoxidase-mediated [^{125}I]iodination (*I*) and treated where indicated with 10 μg/ml trypsin (*T*) for 10 min at room temperature before harvesting for electrophoresis.

cessing for gels, labeling of the band comigrating with band 1 was absent (Figs. 1 *c*, 2 *c*). The other major alteration to the profile of glucosamine labeling which was produced by trypsin treatment was the generation of a new band at a molecular weight of about 40,000 (arrow, Fig. 1). This was observed with both normal and transformed cells (Fig. 1 *c*, *e*). No other changes were detectable after this trypsin treatment, although more extensive digestion will remove up to 40% of the incorporated sugars (unpublished data and references 15, 16). A similar difference between normal, trypsinised, and transformed cells was detected after labeling with [^{14}C]leucine (data not shown).

Thus there is a glycoprotein containing fucose and amino sugars which (*a*) comigrates with band 1 polypeptide detected by iodination, (*b*) is sensitive to transformation, and (*c*) is removed by the mild trypsin treatment. This glycoprotein therefore shares three characteristics with the major external polypeptide detected by iodination.

NIL8 cells were labeled with [^{35}S]sulphate and harvested for SDS-polyacrylamide gel electrophoresis after treatment with various enzymes. The results are shown in Fig. 3 *f–i*. Virtually all the radioactivity remained on top of the gel or at the interface between the stacking and separation gels or ran with the dye front. None comigrated with the iodinated polypeptides. [^{35}S]sulphate is incorporated by cultured cells into sulphated mucopolysaccharides (16–18). In NIL8 cells, [^{35}S]sulphate is incorporated into chondroitin and heparitin sulphates (E. A. Davidson, personal communication). So these results indicate that none of the iodinatable species detected in the separation gel contain significant quantities of these sulphated mucopolysaccharides. Fig. 3 *c* also demonstrates that all the iodinated species detected on the gels are TCA precipitable and therefore macromolecular.

In order to test for possible synthesis of collagen and iodination of this secreted product, NIL8 cells

were labeled with [^{3}H]proline and 3T6 cells known to be synthesising collagen (R. Laskey, personal communication) were labeled in parallel as a positive control. Replicate dishes of each cell line without [^{3}H]proline were iodinated for comparison. All samples were analysed on cylindrical gels, sliced for counting, and the iodinated samples were also analysed on slab gels by autoradiography. The results are shown in Fig. 4. For comparison a [^{14}C]leucine-labeled profile of NIL8 cells from a different experiment is included. The profiles of [^{14}C]leucine- and [^{3}H]proline-labeled NIL8 cells were similar (Fig. 4 *c*, *d*). There was no large proline-rich peak suggestive of collagen synthesis. In contrast, a large proportion of the [^{3}H]proline radioactivity in the case of 3T6 cells was trapped at the top of the gel and at the interface between stacking and separation gels (Fig. 4 *b*). The ^{125}I profile of 3T6 cells (not shown) was similar to that of NIL8 cells (Fig. 4 *a*). Thus the proline counts were not trapped nonspecifically on the top of the gel, but presumably represent cross-linked collagen which is too large to enter the gel (β-aminopropionitrile was not included to inhibit cross-linking). The major iodine-labeled peaks (band 1) of 3T6 and NIL8 cells did not comigrate with major proline-labeled peaks. There was, as expected, a small peak labeled with both [^{3}H]proline and [^{14}C]leucine in this region (Fig. 4 *c*, *d*). In double label experiments (incorporation of [^{3}H]proline, [^{3}H]glycine, and [^{14}C]leucine) the ratio of proline plus glycine to leucine in the region of the gel containing band 1 was, at most, twofold higher than the average (data not shown). The ratios for α1 and α2 collagens are 18.7- and 10-fold higher, respectively, than the ratio for average proteins (19, 20).

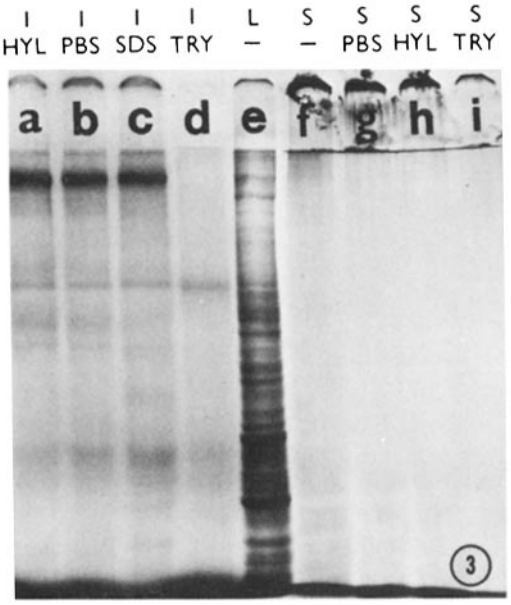

FIGURE 3 Labeling of NIL8 cells with [^{35}S]sulphate and by [^{125}I]iodination: effects of various treatments before harvesting for electrophoresis. Autoradiogram of a 7.5% SDS-polyacrylamide gel of cells labeled with either [^{32}S]sulphate (*S*), 100 μCi/ml for 2 days, or lactoperoxidase-mediated [^{125}I]iodination (*I*). Treatments for 10 min at room temperature were in either 100 μg/ml hyaluronidase (*HYL*); 10 μg/ml trypsin (*TRY*) or phosphate-buffered saline alone (*PBS*). Other samples were harvested directly (–), or in sodium dodecyl sulfate (*SDS*) followed by precipitation with trichloracetic acid to concentrate macromolecules. A [^{14}C]leucine (*L*) whole cell lysate was included for reference. Radioactivity at the bottom of the gel includes free iodide or sulphate. Lipids also migrate in this region.

Finally, proteins comigrating with [^{125}I]band 1 were analysed for incorporation of [^{3}H]proline into hydroxyproline (see Materials and Methods). All the radioactivity comigrated with the proline marker: none (less than 2.5% would have been detectable) comigrated with hydroxyproline. This evidence demonstrates that band 1 polypeptide is not a collagen-like molecule. Further evidence is presented below. Because of the heterogeneous nature of the [^{3}H]proline profiles, they do not represent evidence one way or the other concerning the identity of any of the other iodinated bands with collagen species.

Effects of Digestive Enzymes

In order to investigate further the chemical nature of the iodinated species, their susceptibility to various lytic enzymes was tested. As reported previously (3), the eight iodinated bands which migrate more slowly than serum albumin (i.e. have molecular weights greater than 70,000) can all be removed by trypsin except band 6 (Figs. 3 *d*, 5 *c*). The lower molecular weight bands were rather indistinct and variable and have not been studied further here because serum contaminants also migrate in this region (unpublished data).

Since the trypsin treatment which removes bands 1–5 and 7–8 is very mild (50-fold below that used routinely to subculture the cells), this is evidence supporting their external location. Band 6

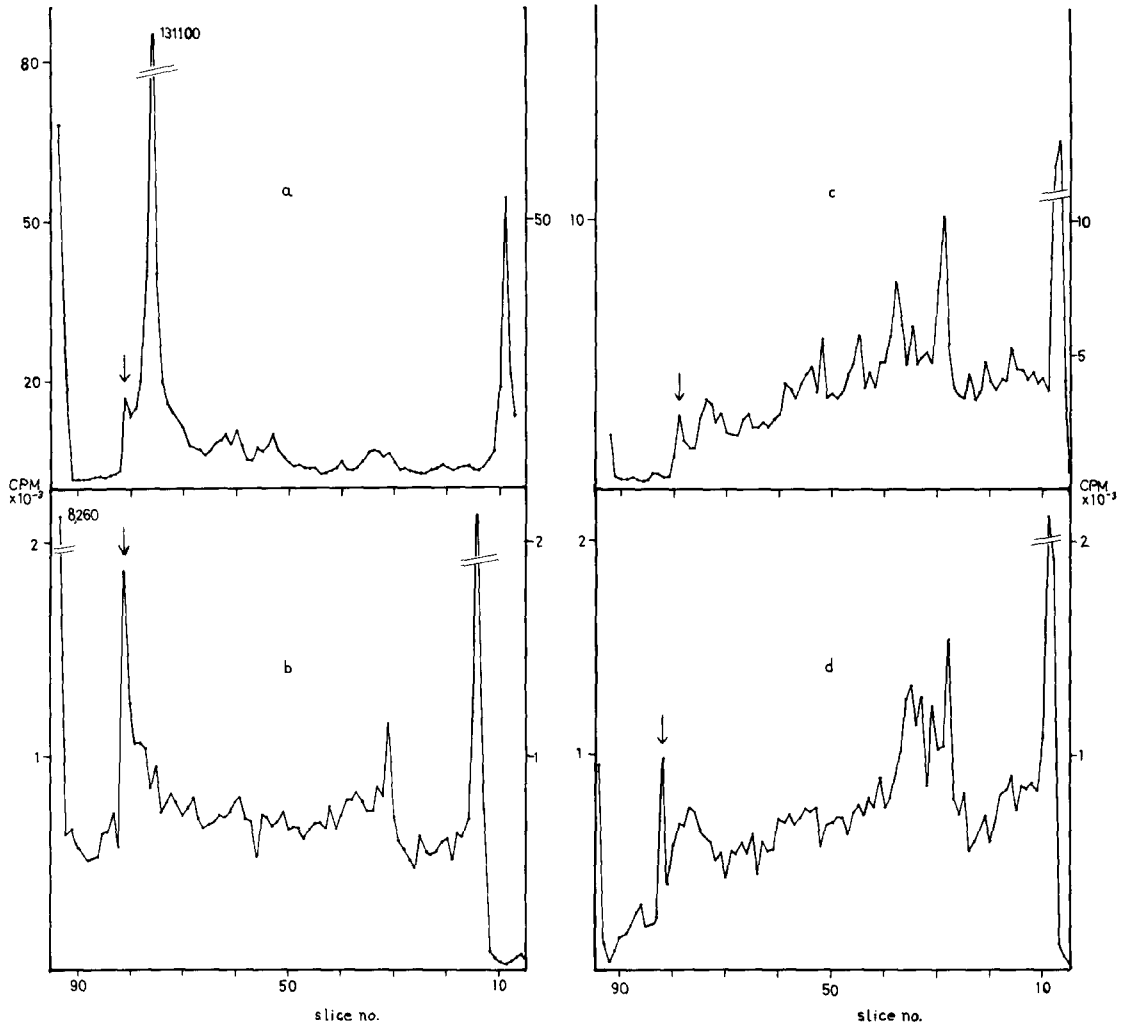

FIGURE 4 Radioactivity profiles of 7.5% SDS-polyacrylamide disk gels of NIL8 and 3T6 cells. The arrow in each panel marks the interface between stacking and separation gels. Electrophoresis was from left to right and the large peak at the right represents the dye front. Slices were 1 mm thick. (a) [^{125}I]iodinated NIL8 cells; (b) 3T6 cells labeled with 5 μCi/ml [5-^{3}H]proline plus 50 μg/ml ascorbic acid for 24 h; (c) NIL8 cells labeled with 0.1 μCi/ml [^{14}C] leucine for 3 days; (d) NIL8 cells labeled with [5-^{3}H]proline as in (b).

appeared to be completely resistant to trypsin; neither its intensity nor its mobility was changed. Of the sensitive proteins, bands 1 and 8 were most readily removed (e.g. 1 μg/ml trypsin for 5 min), whereas the others required more extensive treatments (3). The trypsin effect was inhibited by soyabean trypsin inhibitor (Fig. 5 *b*), and the end result was the same whether digestion was carried out before (Fig. 5 *c*) or after (Fig. 3 *d*) iodination (see also reference 3). Thus, the effect was due to proteolysis and not merely to conformational changes affecting accessibility of the proteins to iodination. This is in contrast with the results of Phillips and Morrison on erythrocytes (21). No new bands were generated, i.e. there were no trypsin-resistant cores remaining with the cells after this treatment, unless their molecular weights were below about 25,000 daltons, in which case they would migrate in the dye front on the gels we have used. Large fragments derived from band 1 polypeptide are detectable in the digest medium (unpublished data).

Treatment of the cells, either before or after iodination, with collagenase caused splitting of band 1 into a doublet and appearance of a new iodinated band at about 40,000 daltons (Fig. 5 *e*).

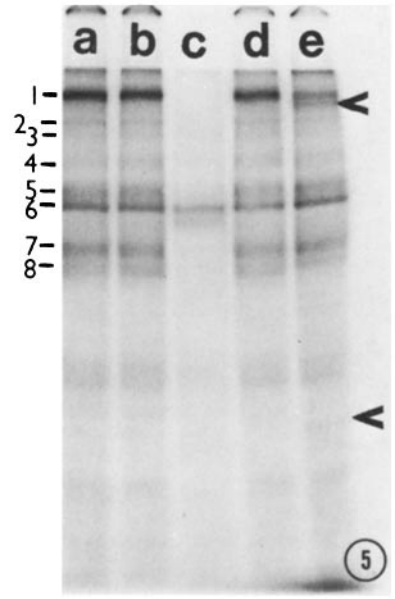

FIGURE 5 Treatment of NIL8 cells with enzymes before [^{125}I]iodination: autoradiogram of a 7.5% SDS polyacrylamide gel. Before iodination and harvesting for electrophoresis, cell monolayers were washed and variously treated for 10 min at room temperature as follows: (*a*) phosphate-buffered saline; (*b*) 10 μg/ml trypsin and 20 μg/ml soyabean trypsin inhibitor; (*c*) 10 μg/ml trypsin, reaction terminated by addition of soyabean inhibitor; (*d*) 5 U/ml chondroitinase ABC; (*e*) 100 μg/ml collagenase; arrows mark new bands generated by this treatment. Major iodinated bands numbered on left.

This effect did not occur with all batches of collagenase, and a similar result is obtained after digestion with 1 μg/ml of trypsin for 1 min (3), so it is probably caused by a trace of contaminating protease in certain batches of collagenase (contamination with 0.1–1% trypsin equivalents would be sufficient). None of the other bands was affected by collagenase treatment.

Hyaluronidase and chondroitinase ABC (22) were without any effect on the profile of iodination of NIL8 cells (Figs. 3 *a*, 5 *d*). Since the pH of 7.2 was suboptimal for hyaluronidase, it was also tested against [^{35}S]sulphate-labeled cells. Table I shows that a treatment which removed 25% of the incorporated sulphate removed no iodine label. These results supplement those in the previous section showing that none of the iodinated species contains sulphated mucopolysaccharide and also argue against their containing hyaluronic acid. A neuraminidase digestion (50 U/ml, 1 h, 37°C) which removed 45% of the total cell sialic acid had no effect on the labeling or mobility of band 1 (unpublished data).

The results described so far in this section all concern NIL8 cells. Similar experiments were done with NIL8.HSV cells with similar results. Digestion of NIL8.HSV cells with collagenase, hyaluronidase, or chondroitinase before iodination did not lead to any alteration in profile (Fig. 6). In particular, these pretreatments did not lead to iodination of a polypeptide comigrating with band 1, nor did a pretreatment with neuraminidase (see above) which removed 30% of the total sialic acid from NIL8.HSV6 cells (unpublished data). These results suggest that failure to iodinate band 1 polypeptide on transformed cells is not due to its

TABLE I

Hyaluronidase Resistance of Iodinated and Sulphated Material

Label	Treatment after labeling	TCA precipitable cpm/μg protein	Percent of control
^{125}I	Control, no incubation	345	100
^{125}I	Hyaluronidase 100 μg/ml in PBS, 10 min	360	104
^{125}I	PBS, 10 min	409	118
$^{35}SO_4$ 10 μCi/ml	Control, no incubation	24	100
$^{35}SO_4$ 10 μCi/ml	Hyaluronidase 100 μg/ml in PBS, 10 min	18	75
$^{35}SO_4$ 10 μCi/ml	PBS, 10 min	23	96
$^{35}SO_4$ 100 μCi/ml	Control, no incubation	224	100
$^{35}SO_4$ 100 μCi/ml	Hyaluronidase 100 μg/ml in PBS, 10 min	165	74
$^{35}SO_4$ 100 μCi/ml	Collagenase 100 μg/ml in PBS, 10 min	217	97

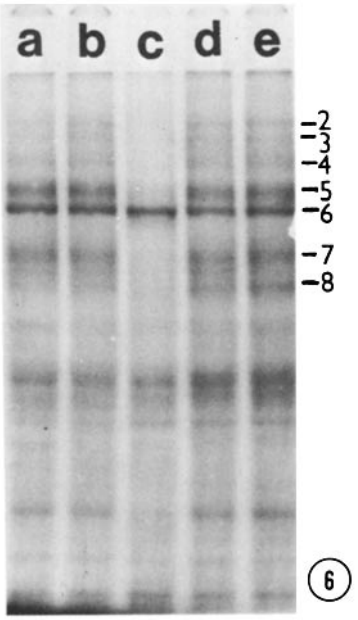

FIGURE 6 Treatment of NIL8.HSV6 cells with enzymes before [^{125}I]iodination: autoradiogram of a 7.5% SDS-polyacrylamide gel. Procedures as in Fig. 5 according to the following track lettering; (*a*) collagenase; (*b*) trypsin plus soyabean inhibitor; (*c*) trypsin; (*d*) chondroitinase ABC; (*e*) 100 μg/ml hyaluronidase. Major iodinated bands are numbered on right.

being covered by collagen, mucopolysaccharides, or large quantities of sialic acid (see Discussion).

Molecular Weight Determinations for Iodinated Species and for Collagen

Iodinated cells were analysed by coelectrophoresis on 5% gels with molecular weight markers and collagen from calf skin or rat tail (acetic acid extract kindly provided by D. Piggott, Imperial Cancer Research Fund). α1, α2, β11, and β12 collagen were clearly identified and were of a similar mobility in both collagen preparations. In addition, both had a number of faint bands which only just entered the separation gel. Bands 1–4 did not comigrate with any collagen band, band 1 being slower than the β-doublet and bands 2–4 being faster. Bands 5 and 6 comigrated with α1 and α2, respectively, and 7 and 8 migrated faster than any collagen bands. Approximate molecular weights as determined by mobilities in SDS gels (23) are given in Table II. These values are not intended to be accurate estimates owing to the large size of several of the molecules and the anomalous migration of both collagen (24) and glycoproteins (25). They do serve to compare the iodinated polypeptides with collagen species.

TABLE II

Molecular Weight Estimates from Polyacrylamide Gels

Band Refer to Figs. 5 and 6	Nominal molecular weight ($\times 10^{-3}$) as determined from mobility in SDS-polyacrylamide gels.*
1	250–265
2	200–210
3	185–190
4	160–165
5	130–140
6	120–125
7	90–105
8	85–90
Collagen β_{11}‡	235–240
Collagen β_{12}‡	220–230
Collagen α_1‡	130–135
Collagen α_2‡	115–120

* Relative to the following markers: *E. coli* RNA polymerase, 165, 155, 90; lactoperoxidase, 84; glucose oxidase, 75; bovine serum albumin, 69 × 10^3. Values are given as the range of results from at least three gels in each case.

‡ Migration of collagen is anomalous (24) and these values are overestimates but are given here for comparison with the nominal molecular weights of iodine-labeled bands.

DISCUSSION

Normal hamster fibroblasts synthesize and accumulate a glycoprotein containing amino sugars and fucose, which comigrates with the major iodinatable protein of these cells on polyacrylamide gels (Figs. 1 and 2). Both the glycoprotein and the iodinated protein are sensitive to low doses of trypsin, and neither is detected in transformed cells. This identity of behavior according to several criteria suggests that the major polypeptide detected by iodination (band 1 polypeptide) is a glycoprotein. However, until one is isolated and shown to be identical with the other, the identity of these two proteins remains unproven. Assuming it to be true, this evidence, then, suggests that not only is this glycoprotein inaccessible from outside transformed cells but that it is not present in masked form within these cells. Further indirect evidence consistent with this idea comes from the fact that pretreatment of NIL8.HSV6 cells with

enzymes which digest mucopolysaccharides or collagen does not expose band 1 polypeptide to iodination (Fig. 6). It is known that some transformed cells secrete hyaluronic acid which obscures sites on the membrane which can be exposed by hyaluronidase treatment (26): this does not appear to be the case here. Warren and his co-workers (27) have shown that a variety of transformed cells bear on their surfaces glycopeptides which are enriched in sialic acid as compared with their counterparts on normal cells. It is therefore conceivable that extensive sialylation could block iodination by lactoperoxidase. However, our results show that removal of a large fraction of the total sialic acid does not allow labeling of any more proteins. In summary, it appears that band 1 polypeptide is absent from the surfaces of transformed hamster cells, not merely masked. It remains to be discovered whether this glycoprotein is absent because it is not synthesized or because it turns over rapidly in transformed cells and therefore does not accumulate in detectable quantities.

Glucosamine and fucose are also incorporated into glycoproteins of mobilities similar to those of the other iodinated polypeptides, but this is insufficient to establish that the latter are glycoprotein in nature.

The results of labeling with [^{35}S]sulphate indicate that none of the proteins detected by iodination contains sulphated mucopolysaccharides (Fig. 3): sulphate-labeled material is trapped at the top of the gel, consistent with a large molecular size for mucopolysaccharide-protein complexes. Failure to affect the labeling or mobility of the iodinated bands by treatment with chondroitinase or hyaluronidase (Figs. 3, 5, and 6) is also consistent with this conclusion and extends it to include hyaluronic acid, which is, in any case, not covalently attached to protein. The lack of effect of neuraminidase on band 1 suggests that the glycoprotein is not rich in sialic acid.

In considering the possible identity of the iodinated polypeptides with collagen-like molecules, it is necessary to consider them in three groups. Band 1 is very heavily labeled with iodine and is, therefore, tyrosine-rich or present outside the cells in large quantity. Proline labeling of NIL8 cells (Fig. 4) failed to detect accumulation of a large amount of collagen with this or any other mobility. In 3T6 cells which were synthesizing collagen, the major accumulation of proline label was at the top of the gel. This is presumably due to the fact that collagen becomes covalently cross-linked (19) and, therefore, has a very high molecular weight. Band 1 does not comigrate with any of the polypeptides present in acid-soluble collagen (α1, α2, β11, and β12), but precursor forms of collagen with higher molecular weights have been reported (28–31). Procollagen is a trimer of proα chains which are 25–30% longer than α chains (29–31). This trimer is held together by disulphide bonds and is dissociated by dithiothreitol treatment before electrophoresis (30, 31). The mobility of band 1 could only be consistent with a dimer of proα chains covalently joined by bonds other than disulphide links, a structure which has not been reported. The amino acid labeling data reported here make it very unlikely that band 1 polypeptide is related to collagen. The proline plus glycine to leucine ratio is 5- to 10-fold lower than would be expected for collagen (19, 20), and there is no hydroxyproline detectable in this region of the gels. The sensitivity of band 1 polypeptide to some batches of collagenase (Fig. 5 *e*) remains to be explained. As discussed in Results, this sensitivity could well be due to contamination of the collagenase with a low level of protease.

Bands 5 and 6 do migrate on polyacrylamide gels close to α1 and α2 collagen, but they are not removed by digestion with collagenase (100 μg/ml, 10 min), and band 5 is removed by trypsin (10 μg/ml, 10 min). Both these facts argue against their being collagen as does failure to detect prominent proline-labeled peaks in this area of the gels. However, since bands 5 and 6 are not heavily iodinated, this latter evidence is not conclusive. There is, thus, no compelling evidence for the identity of bands 5 and 6 with the α-collagens, but it cannot be completely ruled out. All the other bands, 2–4 and 7–8, are insensitive to collagenase (Figs. 5 and 6) and show mobilities different from those of known collagen species (Table II). It is therefore unlikely that they are collagen polypeptides.

Evidence has been presented elsewhere that the iodinated species are probably not bound serum proteins (3). This conclusion is particularly clear for band 1, since it is present on cells grown in the absence of serum (3). The results reported here show metabolic labeling of a glycoprotein which appears to be identical with the band 1 iodinated polypeptide, further arguing against its being a bound serum protein. Band 8 migrates close to

lactoperoxidase (Table II) and, at this point, it is not possible to decide whether it is distinct from the enzyme or not. Iodinated proteins of lower molecular weight are detected on the cells (Figs. 3, 5, and 6) but are rather variable. We have not analyzed these further since serum proteins which adhere to dishes (and perhaps to cells) migrate in this molecular weight range (unpublished data).

The results of cell fractionation experiments demonstrate that all the iodinated species and the majority of the radioactivity are associated with the microsomal fraction of cells after homogenization: nuclei, mitochondria, and soluble proteins have very little iodine label (unpublished data with J. M. Graham, Imperial Cancer Research Fund), providing further evidence that the iodination procedure does not label internal proteins of the cell. However, this does not identify the iodinated proteins as plasma membrane components, merely as external. The distinction between a true membrane protein and an extracellular protein is a vague one. Two differentiated products of fibroblasts, which are usually described as extracellular collagen and mucopolysaccharides, have been investigated here. The evidence suggests that the iodinated species are not identical with either of these, but there may well be other differentiated products. This question is of particular interest with respect to band 1 polypeptide which is absent from transformed cells. One wishes to know whether this absence is a causal event in the transformation process perhaps involving alteration of membrane proteins, or merely a secondary effect of transformation leading to loss of a differentiated function. Further work will be needed to decide upon this.

Other workers have reported differences in glycoprotein components of normal and transformed cells. Bussell and Robinson (32) found that transformation by Rous sarcoma virus (RSV) led to a marked reduction in levels of a 145,000-dalton membrane protein in the membranes of chicken embryo fibroblasts. This protein was not trypsin-sensitive. Working with baby hamster kidney (BHK) cells and virus-transformed derivatives, Chiarugi and Urbano (33) detected a glucosamine-labeled component of molecular weight 135,000 daltons which was reduced in the membranes of transformed cells but was present in the medium from these cells. In contrast, Sakiyama and Burge (34) detected a glucosamine-labeled component of about 150,000 daltons in the medium of 3T3 cells which was not found in the case of transformed SV 3T3 cells. They were unable to detect any differences between the membrane fractions of these two cell types. In these papers, no evidence was presented as to whether the proteins concerned were external or not, and it is not clear how they relate to one another or to the band 1 polypeptide discussed in the present paper.

Wickus et al. (35), using lactoperoxidase-catalysed iodination, have obtained results showing that transformation by RSV of chicken embryo fibroblasts leads to the disappearance of a major external protein. This protein is equivalent to the band 1 polypeptide described here (Hynes and Wyke, unpublished results). Wickus et al. (35) also reported transformation sensitivity of a methionine-labeled protein of similar molecular weight. A recent paper, concerning another external labeling technique, reported a large external glycoprotein in hamster cells which was absent in cells transformed by polyoma virus (36). This is probably also equivalent to the iodinated band 1 polypeptide. In an earlier study in which lactoperoxidase-catalyzed iodination was used, no differences were detected between normal and transformed cells (37). However, this latter paper concerned trypsinized cells and, as shown here and elsewhere (3, 35), this would eliminate any differences between the cell types.

Finally, one is left with the question as to the reason for the absence of a major external glycoprotein in transformed cells. The evidence presented here suggests that it is not due to masking. It could therefore be due to failure to synthesize, turnover into the medium (cf. 33), or proteolysis by enzymes produced by the transformed cells (4–8). Work in progress is directed towards investigating these possibilities.

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