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Identification and Partial Characterization of Five Major Membrane Glycoproteins of BHK Fibroblasts

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Summary. Five major membrane glycoproteins of the BHK-B4 hamster fibroblast plasma membrane have been identified by binding specific rabbit antibodies to the cell surface and by recovering the detergent solubilized immunocomplexes with Protein A-Sepharose immunoadsorption. These glycoproteins, designated as gp45, gp65, gp95, gp130 and gp140, are exposed at the cell surface since: (i) they were accessible to antibodies in intact viable cells; (ii) they were radioiodinated by the lactoperoxidase-glucose oxidase procedure; and (iii) they were cleaved by proteolytic enzymes in conditions affecting only the cell surface. Among these glycoproteins the gp130 is the predominant component and its exposed portion is characterized by lack of sensitivity to trypsin cleavage. Glycoproteins of different molecular weight, but immunologically related to the major hamster membrane glycoproteins, have been detected at the surface of both rat and mouse fibroblasts.

In contrast with the well-defined structure of the erythrocyte membrane proteins (for review, *see* [16]) the available information about the plasma membrane of nucleated cells are poor and uncoordinated. As far as fibroblasts are concerned, few plasma membrane proteins have already been identified and characterized. One of these is fibronectin, the major cell surface glycoprotein of subunit mol wt 250K [30] which is known to function in cell-cell and cell-substratum adhesive phenomena [8,18,30]. Two other glycoproteins of mol wt 95K and 75K have also been studied in some detail [12, 26]. Since their synthesis is stimulated by glucose starvation, they have been named Glucose Regulated Proteins (GRP) and a role in glucose transport has been suggested for both proteins [20, 23]. Several other proteins have been described by laboratories involved in the study of the alteration induced by the neoplastic transformation (for review, *see* [11]). However, since discrepancies exist between the data of different groups, it is difficult at present to draw any definitive conclusion on the identity, chemical nature, and localization of these proteins. In this study we have identified by immunological means five major glycoproteins exposed at the surface of BHK fibroblast plasma membrane.

Materials and Methods

Cells and Culture Conditions

All fibroblast lines used in these experiments were grown to subconfluency in Dulbecco modified Eagle's medium supplemented with 10% calf serum (Gibco), 50 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. Avian cells were grown in the same medium except that the calf serum was replaced by 4% fetal calf serum, 1% chicken serum, and 10% triptose phosphate broth. The following three sublines derived from BHK-21 Syrian hamster fibroblasts [25] were used: BHK-B4, BHK-C13, BHK-Py [29]. Most of the work was performed on BHK-B4 cells, but the data reported applies to the other two lines as well. NIL is a clone established from a line of hamster embryo fibroblasts [5]. XC is a line derived from a rat sarcoma [27]. 3T3-B77(1) and 16Q(17) are fibroblast lines of mouse and quail origin, respectively.

Metabolic Labeling

Methionine labeling was performed by incubating cell monolayers in medium containing one fifth of its normal methionine content and 25 μCi/ml of ^{35}S-methionine (Amersham, 1050 Ci/mmol). Labeling of glycoproteins was performed in complete medium containing 125 μCi/ml of D(6-^{3}H)glucosamine (Amersham, 38 Ci/mmol). Incubations were carried out for periods of 6 or 12 hr.

Radioiodination of Surface Components

The lactoperoxidase-glucose oxidase catalyzed radioiodination procedure was used [10, 19]. Cell monolayers were rinsed briefly with phosphate buffered saline (PBS) and incubated with 2 ml of PBS, and 2.5 mM glucose, to which 40 μg of lactoperoxidase, 0.4 units of glucose oxidase, and 250 μCi of carrier-free ^{125}I sodium iodide were added. The reaction was carried out for 15 min at room temperature. The cell monolayer was then washed with iced PBS and processed as described below for immunoprecipitation of surface proteins.

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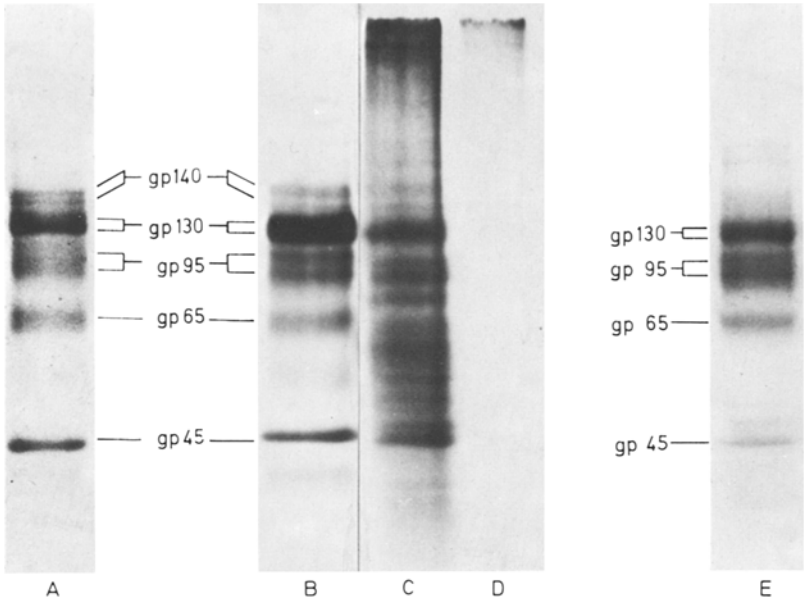

Fig. 1. Membrane proteins exposed at the BHK-B4 fibroblast surface. Fluorogram of the electrophoretic pattern obtained in SDS gradient slab gel. Column *A*: membrane proteins immunoprecipitated by the anti-BHK-B4 serum from BHK-B4 fibroblasts labeled for 6 hr with ^{35}S-methionine. Column *B*: same as *A*, except that cells were labeled with ^{3}H-glucosamine. Column *C*: SDS extract of ^{3}H-glucosamine-labeled BHK-B4 fibroblasts. Column *D*: same as *B* but nonimmune rabbit serum was used as control. Column *E*: membrane proteins immunoprecipitated by the anti-BHK-B4 serum from enzymatically radioiodinated BHK-B4 fibroblasts

Antiserum and Immunoprecipitation of Membrane Proteins

The anti-BHK-B4 serum was produced in rabbits primed by intramuscular injection of 2.5×10^7 BHK-B4 cells in complete Freund's adjuvant and boosted twice at 20-day intervals. Cells, grown in subconfluent monolayers in plastic petri dishes, were carefully washed with PBS and incubated for 15 min at 37 °C in PBS with 5 mM ethylendiamine tetraacetic acid (EDTA). After this treatment the cells were easily detached from the dish by gentle pipetting. Cell viability was unaffected by this treatment, as judged by refractility of the cells under the phase-contrast microscope and trypan blue exclusion. All the following steps were then carried out in ice to minimize proteolytic degradation: The cells were washed three times with culture medium containing 10% calf serum and incubated for 1 hr with saturating amounts of the anti-BHK-B4 serum or equivalent volumes of nonimmune rabbit serum as control. The unbound immunoglobulins were removed by repeated washings with culture medium, and the antigen-antibody complexes were solubilized from the membranes by extracting the cell pellet with 50 mM Tris (hydroxymethyl) aminomethane pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 0.5% Nonidet P40, and 0.5% sodium deoxycholate containing 2 mM phenylmethylsulphonyl fluoride as proteases inhibitor (lysing buffer). After 20 min of incubation with occasional "vortex" mixing, extracts were centrifuged at $10{,}000 \times g$ for 20 min and the supernatants were incubated with 50 µl of packed Sepharose beads to which nonimmune rabbit immunoglobulins were coupled. This step was necessary to adsorb and remove a number of proteins that were found to nonspecifically bind Sepharose. The immune-complexes were then adsorbed on 50 µl of packed Protein A-Sepharose CL-4B (Pharmacia). After 1 hr incubation with continuous mixing, Sepharose beads were pelletted by low speed centrifugation and washed three times with the lysing buffer. The Sepharose beads were then suspended in 50 µl of Laemmli buffer (*see* below) containing 2% sodium dodecyl sulphate (SDS) and 2% βmercaptoethanol, and the eluted material was analyzed by electrophoresis.

Electrophoresis and Fluorography

Sodium dodecylsulphate polyacrylamide electrophoresis (SDS-PAGE) was carried out in 5–15% acrylamide slab gels using the procedure described by Laemmli [14]. Gels were processed for fluorography as described by Bonner and Laskey [2], dried and placed in contact with a Kodak X Omat R film which had been prefogged to achieve linearity [15]. The following radioiodinated mol wt markers were used: myosin (200K), phosphorylase A (94K), bovine serum albumin (68K), heavy (50K) and light (25K) chains of rabbit immunoglobulin G and ovalbumin (43K).

Proteolytic Cleavage of Surface Proteins

Cell monolayers, metabolically labeled with ^{35}S-methionine, were carefully washed with serum-free culture medium and incubated for 15 min at 37 °C with 2 ml of serum-free medium containing increasing amounts of tosylamido phenylethylchloromethylketone-treated trypsin (Worthington) or pronase (Calbiochem, grade A). Trypsin digestion was stopped by adding bovine pancreatic inhibitor (Worthington) at 1:1 (wt/wt) ratio. Pronase was blocked by 2 mM phenylmethanesulphonyl fluoride (Calbiochem) and instant chilling. Cells were then washed three times with culture medium containing 10% calf serum and subjected to the immunoprecipitation procedure.

Results

Immunoprecipitation of Membrane Proteins

The procedure used for selective immunoprecipitation of membrane proteins was based on the assumption that antibody molecules do not cross the plasma membrane barrier. Thus the incubation of the anti-BHK-B4 serum with intact viable cells allowed the binding – and subsequently the immunoprecipitation – of only those molecules exposing antigenic determinants at the cell surface [7].

In the experiments reported, the antibodies were allowed to bind either to cells' monolayers or to sus-

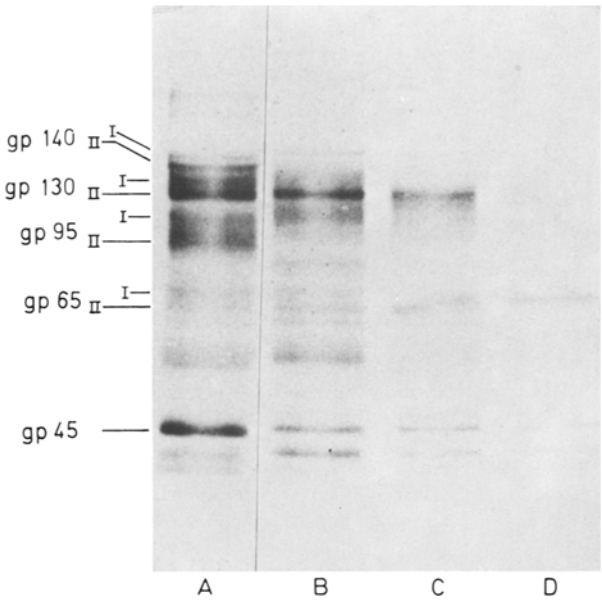

Fig. 2. Proteolytic cleavage of membrane proteins. Fluorography of the SDS electrophoretic pattern of membrane proteins immunoprecipitated by the anti BHK-B4 serum from pronase treated BHK-B4 fibroblasts. ^{35}S-methionine labeled subconfluent monolayers were incubated for 15 min at 37 °C with serum-free medium (column *A*) or medium containing 5 μg/ml of pronase (column *B*); 50 μg/ml of pronase (column *C*); 500 μg/ml of pronase (column *D*). Metabolic labeling was carried out for 12 hr, and samples of cells containing the same amount of acid precipitable radioactivity were used. The roman numbers *I* and *II* indicate the two bands of gp65, gp95, gp130, gp140 doublets

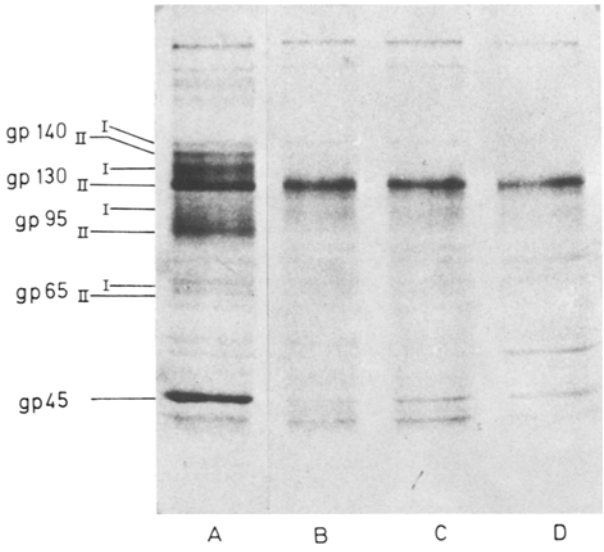

Fig. 3. Proteolytic cleavage of membrane proteins. Fluorography of the SDS electrophoretic pattern of membrane proteins immunoprecipitated by the anti-BHK-B4 serum from trypsin-treated BHK-B4 fibroblasts. Untreated control cells (column *A*). Cells treated with: 5 μg/ml of trypsin (column *B*); 50 μg/ml of trypsin (column *C*); 500 μg/ml of trypsin (column *D*). Labeling and digestion conditions were as in Fig. 2

pensions of viable cells harvested from the plate by EDTA treatment. The results obtained by either procedure were identical. The antigen-antibody complexes were then solubilized from the plasma membrane by detergents in conditions reported to be effective in solubilizing integral membrane components [13] and recovered with the protein A-Sepharose adsorbent. A pre-adsorbtion of the solubilized material with Sepharose beads coupled with nonimmune rabbit immunoglobulins was always performed. It was noted in fact that four major methionine labeled proteins of approximate mol wt 45K, 58K, 200K, and 250K were invariably bound to Sepharose beads. These proteins have a mol wt coincident to that of actin, tubulin, myosin, and fibronectin, and they may represent a poorly soluble complex of the major cytoskeleton proteins. The pre-adsorption step quantitatively eliminated these proteins, and the only material bound to the protein A-Sepharose were immunocomplexes. Under these conditions, the immunoprecipitated material accounted for about 0.25% of the total acid precipitable ^{35}S-methionine.

Identification of the Major Membrane Proteins of BHK Fibroblasts

The electrophoretic pattern of ^{35}S-methionine labeled proteins recognized by the anti-BHK-B4 serum at the surface of BHK-B4 cells is shown in Fig. 1 (column *A*). Five major bands with apparent mol wt of 45K, 65K, 95K, 130K and 140K were resolved, the pattern being very reproducible in different experiments.

The 45K component always migrated as a sharp electrophoretic band, while the 65K, 95K, 130K and 140K components generated diffuse bands and were often resolved in two closely migrating bands identified as I and II (Figs. 2 and 3). This fact, and the experiments with proteolytic enzymes described below, indicate that each of these components is likely to be generated by more than one protein.

Four additional bands of mol wt close to 35K, 55K, 160K and 180K were also recognized by the antiserum even if – due to the low level of radioactivity – they were detectable only after longer exposure of the fluorogram. By changing the time of incubation with ^{35}S-methionine, only small variations in the amount of radioactivity incorporated by the membrane proteins recognized by the antiserum were observed. The only exception was the 65K component; the radioactivity associated with the 65K band was in fact high when the labeling was carried

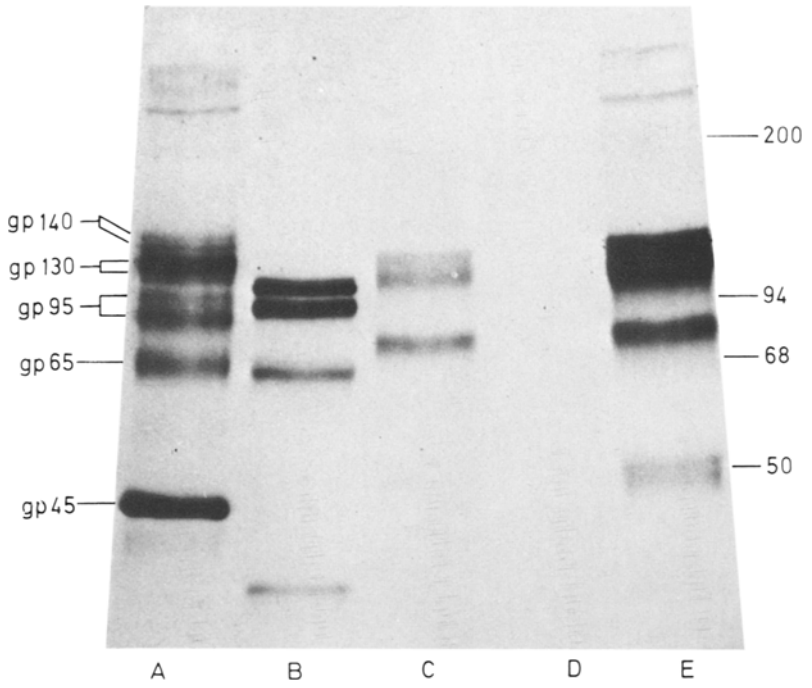

Fig. 4. Immunological cross-reaction of membrane glycoproteins among fibroblasts of different animal species. Fluorogram of the SDS gel electrophoretic pattern of membrane glycoproteins immunoprecipitated by the anti-BHK-B4 serum from the surface of ^{3}H-glucosamine labeled cells. Column *A*: BHK-B4 hamster fibroblasts; Column *B*: XC rat fibroblasts; Column *C*: 3T3-B77 mouse fibroblasts; Column *D*: 16Q quail fibroblasts. The immunoprecipitations were carried out with samples of cells containing the same amount of acid-precipitable radioactivity in order to demonstrate the relative degree of cross-reaction. Column *E*: same as *C* except that a higher amount of radioactivity was used to show the presence of gp50. The numbers on the right indicate mobilities of proteins with known molecular weight

out for 6 hr (Fig. 1, column *A*) and much lower when longer incubation periods were used (Fig. 2, column *A*).

In order to establish the chemical nature of the proteins recognized by the antiserum, cells were labeled with ^{3}H-glucosamine. The electrophoretic pattern of ^{3}H-glucosamine labeled glycoproteins immunoprecipitated by the anti-BHK-B4 serum is shown in Fig. 1 (column *B*). Five major bands corresponding to the components of mol wt 45K, 65K, 95K, 130K and 140K previously detected by the ^{35}S-methionine labeling, were detected. These glycoproteins comigrated with the major ^{3}H-glucosamine-labeled glycoproteins of the whole cell extract (Fig. 1, column *C*), indicating that the antibodies did not select for quantitatively minor, but highly antigenic, membrane proteins. These surface components were thus named gp45, gp65, gp95, gp130 and gp140, where the letters gp stand for glycoprotein and the number indicates the apparent mol wt expressed in kilodalton (K).

Localization of Membrane Glycoproteins by Radioiodination

To further confirm the cell surface localization of the membrane glycoproteins recognized by the anti-BHK-B4 serum, enzymatic radioiodination was performed by the lactoperoxidase-glucose oxidase system known to selectively label surface molecules. The electrophoretic pattern of the ^{125}I labeled components of the BHK-B4 surface was similar to that obtained by others [10, 19], the major labeled components being a 250K band, two clusters of bands spread over a mol wt range of 65–100K and 120–130K and a group of lower mol wt bands of 30–50K (not shown).

When surface proteins immunoprecipitated by the anti-BHK-B4 serum from enzymatically radioiodinated cells were analyzed by SDS-PAGE, four bands corresponding to the above described gp45, gp65, gp95 and gp130 were resolved (Fig. 1, column *E*). The gp140, however, was not labeled by iodine. Since the localization at the cell surface of this component is supported by its susceptibility to proteases as described below, it should thus be concluded that gp140 exposes only a few surface tyrosine residues accessible to the lactoperoxidase.

Proteolytic Cleavage of Major Surface Glycoproteins of BHK Fibroblasts

To test the susceptibility to proteolytic enzymes of the major surface glycoproteins of BHK-B4 fibroblasts, monolayers of metabolically labeled cells were subjected to the action of pronase or trypsin, as described in detail in the methods section. No gross damage of the cell membrane permeability was observed after treatment with as much as 500 μg/ml of pronase or trypsin, as judged by cell refractility

under phase-contrast microscope and trypan blue exclusion, indicating that the proteolytic cleavage was restricted at the cell surface. Under these conditions all the major surface glycoproteins described were cleaved with the exception of gp130 which was sensitive to pronase but not to trypsin digestion (Figs. 2 and 3).

Using lower concentrations of pronase, different susceptibility to proteolytic degradation among different glycoproteins was observed. Incubation of BHK-B4 fibroblasts with 5 μg/ml of pronase for 10 min at 37 °C caused the complete cleavage of the second component of gp95 and of gp140 (gp95 II and gp140 II) as shown by the electrophoretic pattern of the immunoprecipitated proteins (Fig. 2, column *B*). Gp45 was only partially cleaved, judging by the decreased intensity of the corresponding band, while the other glycoproteins were not significantly affected. By increasing the pronase concentration to 50 μg/ml, complete digestion of gp95 I, gp130 I and gp140 I was obtained (Fig. 2 column *C*). Gp130 II was still detectable in small amounts, 500 μg/ml of enzyme being necessary to achieve its complete digestion (Fig. 2, column *D*).

Different concentrations of trypsin were also used. This enzyme was much more active than pronase, and 5 μg/ml were enough to obtain complete digestion of gp45, gp65 I and II, gp95 I and II, gp130 I, and gp140 I and II. The gp130 II, however, was not affected by this treatment (Fig. 3, column *B*). Moreover, no significant digestion of gp130 II was observed even when trypsin concentration was raised to 500 μg/ml (Fig. 3, column *D*).

Immunological Cross-Reaction of Major Membrane Glycoproteins Among Fibroblasts of Different Species

Other hamster fibroblast lines, such as BHK-C13, NIL, and BHK-Py, were subjected to immunoprecipitation with the anti-BHK-B4 serum, and the electrophoretic patterns of the major membrane glycoproteins were compared to that of BHK-B4 cells. The five major glycoproteins described were present at the surface of all three cell lines, and no differences were observed among control and virally transformed cells except for small variation in the relative intensity of the bands corresponding to gp65, gp95 I, and gp140 (not shown).

The anti-BHK-B4 serum was also tested for its ability to precipitate ^{3}H-glucosamine-labeled membrane glycoproteins from fibroblasts of different species such as rat, mouse, and quail. Cross-reaction was observed among hamster, rat, and mouse surface glycoproteins, but no cross-reaction was found between surface glycoproteins of hamster and avian fibroblasts. Four glycoproteins gp30, gp62, gp98 and gp115 were immunoprecipitated by the antiserum from rat plasma membrane (Fig. 4, column B); a gp75, gp120, gp135, and low amount of a gp50 were immunoprecipitated from mouse cells (Fig. 4, columns *C* and *E*). These data show that glycoproteins of different mol wt, but immunologically related, are exposed at the cell surface of rodent fibroblasts.

Discussion

By means of a specific antiserum, we have identified five major membrane glycoprotein components of BHK fibroblasts. These glycoproteins have been designated as gp45, gp65, gp95, gp130, and gp140. Their localization at the cell surface is supported by the following findings: (i) they are accessible to antibodies in intact viable cells; (ii) they are radioiodinated by the lactoperoxidase-glucose oxidase procedure; (iii) they are cleaved when intact viable cells are incubated with proteolytic enzymes. These results extend our previous studies on trinitrobenzene sulphonic acid-labeled cell surface proteins of BHK fibroblasts [4, 28].

It seems clear that among these components gp65, gp95, gp130 and gp140 do not contain a single molecular species, but most likely represent classes of closely migrating glycoproteins. This is suggested by the fact that the diffuse gp65, gp95, gp130 and gp140 bands are often resolved in doublets. Moreover, when the cell surface was subjected to digestion by pronase, different susceptibility of the two bands of each doublet was observed.

The second band of the gp130 doublet (gp130 II) is the predominant glycoprotein of the BHK plasma membrane, judging by the intensity of labeling with methionine and glucosamine as well as with enzymatic radioiodination. The possibility that the high recovery of this glycoprotein in the immunoprecipitation was due to a selective effect of the antiserum used is unlikely since gp130 is *per se* a major glycoprotein component of the whole cell.

A characteristic of this glycoprotein is its lack of sensitivity to tryptic digestion. Gp130 II, in fact, was digested by pronase but was virtually unaffected by trypsin, even at concentrations a hundred times higher than those effective in cleaving all other glycoproteins. Similar results were obtained by Hynes [10] who found, at the NIL fibroblast surface, a 130K membrane protein, labeled by enzymatic iodination, which was not affected by trypsin digestion. Since the antiserum used in our experiments recognizes a gp130 also on NIL fibroblasts, we believe that the

proteins are identical. This membrane glycoprotein is readily accessible in intact cells to antibodies, to lactoperoxidase, as well as to pronase; we thus conclude that its insensitivity to trypsin is due to the absence of cleavage sites on the exposed portion of the molecule. The peculiar behavior of the gp130 II toward trypsin is reminiscent of that of the anion channel Band 3, an integral erythrocyte membrane protein [16], which exposes at the external cell surface a glycosylated domain which is cleaved by pronase but not by trypsin [6, 24]. The intriguing possibility that the two proteins could be structurally or functionally related deserves further investigation.

Recently two glycoproteins of 75K and 95K had been described in rat, mouse, and chicken fibroblasts [12, 26]. These proteins have been named glucose regulated proteins (GRP) since their synthesis was modulated by the level of glucose in the culture medium [23]. Moreover, these proteins were found to be induced by drugs that inhibit protein glycosylation [20, 21]. Gp95 could tentatively correspond to the GRP95 since its expression is modulated in experimental conditions affecting glycosylation *(manuscript in preparation)*.

No appreciable amount of the 250K fibronectin was detected by the procedure used. This glycoprotein is known to be a major component of fibroblast plasma membrane as detected by a variety of different methods [3, 10, 22]. The absence of fibronectin in our immunoprecipitates could be explained in different ways, the simplest explanation being the lack of solubility of this protein in the detergents used [9]. As expected, a small amount of a 250K component was, in fact, present in the electrophoretic profile of BHK-B4 surface proteins enzymatically radioiodinated and solubilized with SDS.

The expression of the five major glycoproteins described does not depend on the viral transformation. All the glycoproteins recognized by the antiserum, in fact, are present in the plasma membrane of either control or virally transformed fibroblast lines such as BHK-Py, BHK-C13, and NIL.

Furthermore, the anti-BHK-B4 serum cross-reacts immunologically with a similar number of glycoproteins exposed at the cell surface of rat and mouse fibroblasts. No cross-reaction, on the contrary, was observed with surface glycoproteins of avian cells. Moreover, the glycoproteins immunoprecipitated from rat and mouse membranes have apparent molecular weights different from those of hamster membrane glycoproteins. These data indicate that proteins with different molecular weights – but structurally related – are present at the surface of rodent fibroblasts. This finding may explain the difficulties encountered in comparing the data on membrane composition obtained by different authors in different systems [11].

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