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Isolation and Immunological Characterization of an Iron-Regulated, Transformation-Sensitive Cell Surface Protein of Normal Rat Kidney Cells

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We have analyzed the surface proteins of cultured normal rat kidney (NRK) cells and virus-transformed NRK cells subjected to iron deprivation. Such a treatment specifically induces two transformation-sensitive plasma membrane-associated glycoproteins with a subunit molecular weight of 160,000 (160 K) and 130,000 (130 K) daltons in NRK cells. In these cells the 160 K glycoprotein is readily available to lactoperoxidase-mediated iodination, and the 130 K is apparently inaccessible to iodination. Major differences were revealed when iodinated membrane proteins of normal and virus-transformed cells subjected to iron deprivation were compared. In Kirsten sarcoma virus-transformed NRK cells the 160 K glycoprotein was weakly labeled. In two clones of simian virus 40-transformed NRK cells the 160 K glycoprotein was weakly labeled or not at all. The 130 K glycoprotein was inaccessible to iodination in all the virus-transformed cell lines.

The 160 K and 130 K glycoproteins were isolated from plasma membranes of NRK cells using preparative SDS gel electrophoresis. Antibodies generated against these glycoproteins stained the external surfaces of NRK cells and induced antigen redistribution. Evidence presented suggests that 160 K and 130 K are plasma membrane-associated procollagen molecules. A possible interaction of these proteins with transferrin is also described. The data suggest that these proteins may have an important role in the sequence of events leading to transformation

Key words: viral transformation, iron starvation, membrane proteins, procollagen

Iron has been previously implicated in the control of cell growth in cultured cells [1–4]. Though its main effects have been determined, the molecular aspects of the suggested control function remains undefined. This element is important in a number of metabolic processes. It is essential in DNA synthesis as a cofactor of the enzyme ribonucleotide reductase. It is also required in collagen synthesis at the level of proline and

Abbreviations: NRK, normal rat kidney; SV, simian virus 40; K, Kirsten sarcoma virus; DME, Dulbecco-Vogts modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenyl methylsulfonylfluoride; PBS, phosphate-buffered saline.

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lysine hydroxylation. Experimental evidence that iron deprivation affects both of these processes in cultured cells has been presented [5, 6].

We have previously demonstrated that NRK cells express two membrane-associated glycoproteins in response to iron deprivation [7]. These membrane glycoproteins are of interest because their synthesis can be experimentally regulated by the cellular concentration of iron and because they can exist associated with the plasma membrane in two molecular forms, partially glycosylated and fully glycosylated [7]. These two proteins are decreased when cells are transformed by oncogenic viruses, and their decrease might correlate with the development of the transformed phenotype [7].

In this study, we have labeled external cell proteins by a lactoperoxidase-catalyzed iodination procedure to explore further the nature and location of the membrane glycoproteins with subunit molecular weight 160 K and 130 K induced by iron depletion. Evidence presented here suggests that 160 K and 130 K are procollagen molecules that are associated with the plasma membrane. Isolation, immunological characterization, and preliminary data that suggest a possible interaction of these proteins with transferrin are also presented.

METHODS

Cell Culture

Cells were grown in Dulbecco-Vogt modified Eagle's medium containing 10% (vol/vol) calf serum (Colorado Serum Company) as previously described [8]. NRK-B C1 8, K-NRK C1 32, SV-NRK P8C12T7, and SV-NRK-B C1 2 were kindly supplied by Dr. R. Ting (Biotech Research, Inc.). Unless otherwise indicated subconfluent cells were used in this study.

It has been shown that desferrioxamine, a highly specific iron-chelating agent, selectively deprives iron from the cells [1, 7]. Thus to investigate the effects of iron deprivation on membrane protein patterns in normal and transformed cells, cultures were grown with or without desferrioxamine (325–396 μg/ml), and the membranes were analyzed after set incubation periods.

Cell Surface Labeling

Monolayer cultures were surface labeled as described by Hynes [9]. The growth medium was removed and the cells were washed three times with PBS (pH 7.2). The cells in a 100-mm dish were labeled in 2 ml PBS containing glucose (5 mM), lactoperoxidase (40 μg) (Calbiochem), glucose oxidase (0.2 U) (Calbiochem or Worthington), and 100 μCi carrier-free Na-^{125}I (New England Nuclear). After 10 min incubation at room temperature, labeling was terminated by addition of PBI (phosphate-buffered saline in which NaCl was replaced by NaI). The PBI contained 2 mM PMSF to inhibit proteases. The medium was removed and the cells were washed twice with PBS containing 2 mM PMSF. The cells were then scraped from the dish into Earle's BSS containing 10% sucrose, and the membranes were isolated as described below. In some experiments, the cells were labeled in suspension after removal from the dishes by scraping. All washings and fractionation buffers contained 2 mM PMSF.

Protease Treatment of the Cells

Subconfluent cells were treated as described in [10]. Collagenase treatment of the cells was carried out only in the presence of 2 mM N-ethylmaleimide and 1 mM PMSF. Purified collagenase and trypsin were obtained from Worthington Biochemical Corporation.

Metabolic Labeling of Cells

The cultures were metabolically labeled with radioactive precursors, [^{14}C]-glucosamine, [^{14}C]-galactose, [^{14}C]-glucose, [^{14}C]-mannose, and [^{14}C]-proline by culturing in complete DME medium. Pulse labeling of the cells with [^{35}S] methionine (644-1270 μCi/mmole) was performed as indicated elsewhere [7].

Preparation of Transferrin

Purified human (Boheringer) or bovine (Calbiochem) transferrin were stored at 4°C in the iron-free form. The transferrins were converted to the iron-saturated form and titrated [11] by addition of $Fe(C10_4)_2$ in the presence of 20 mM $NaHCO_3$ and 1 mM nitrilotriacetate (NTA). After iron saturation of transferrin, possible contaminating NTA was removed by Sephadex G-25 column chromatography. Transferrin peak (void volume) was lyophilized and purity of the preparation (greater than 98%) was determined by SDS electrophoresis.

Surface Labeling of Cells in the Presence of Transferrin

After 14 h of growth in the presence or absence of desferrioxamine (325–350 μg/ml), 90% iron-saturated differric transferrin in 200 μl iron-free DME medium (Custom made, Microbiological Associates) containing 0.1% bovine serum albumin (Chelex-100 treated, Bio Rad; Nutritional Biochemicals) was added to the cultures. Final transferrin concentration in growth media was 20 μM. The cells were incubated in the tissue culture incubator for 30 min. This incubation period was chosen because preliminary experiments showed maximal cellular binding of ^{125}I transferrin at this time (unpublished). A set of control cultures were identically treated, but transferrin was omitted from the preparation. Then the cells were surface labeled by the lactoperoxidase iodination procedure, and the plasma cell membranes were analyzed by SDS-PAGE.

Membrane Isolation and Analysis of Membrane Proteins by Polyacrylamide Gel Electrophoresis

The plasma membranes were isolated by the method of Brunette and Till [12] as modified by Stone et al [13]. SDS gel electrophoresis was performed following the basic procedure of Laemmli [14]. When radioactive proteins were analyzed, autoradiographs were obtained by exposing dried gels to Kodak RP Royal "X-Omat" film. The amount of protein or radioactivity in each band was estimated by measuring the areas under the peaks by a Digitizer (Elographic, Tennessee). Protein standard for molecular weight estimation were myosin (200 K), phosphorylase B (94 K), β-galactosidase (130 K), bovine serum albumin (68 K) and ovalbumin (43 K) (Bio Rad Laboratories).

Isolation of 160 K and 130 K from NRK Cells

The glycoproteins were isolated and partially purifed using plasma membranes from confluent NRK-B cells grown in 102 dishes 150 mm in diameter. After 72 h of growth in normal medium the cells were treated with 390 μg/ml of desferrioxamine for 11 h. Then the cells were collected and the plasma membranes were isolated. After boiling for 3 min and reduction with dithiothreitol (0.1 M), the preparations were electrophoresed on 5% to 12% exponential gradient preparative slab gels. Two narrow slices were cut near the edges of the fixed (50% trichloroacetic acid) gels and stained with Coommassie blue to locate the 160 K and 130 K glycoproteins. These glycoproteins were the major stained proteins in that molecular weight region. The stained strips were aligned with the fixed

gels, and the protein bands were cut from the gels. The gel was triturated at 0–4°C with a Dounce homogenizer, and the protein was eluted with three extractions at 37°C in 4 ml of 5 mM ammonium bicarbonate, 0.05% SDS, and 0.5 mM PMSF. This procedure yielded approximately 1,300 μg of 160 K and 1,200 μg of 130 K with a purity greater than 95% as estimated by reelectrophoresis and Coomassie blue staining. The protein preparations were lyophilized and resuspended in 1.5 ml PBS.

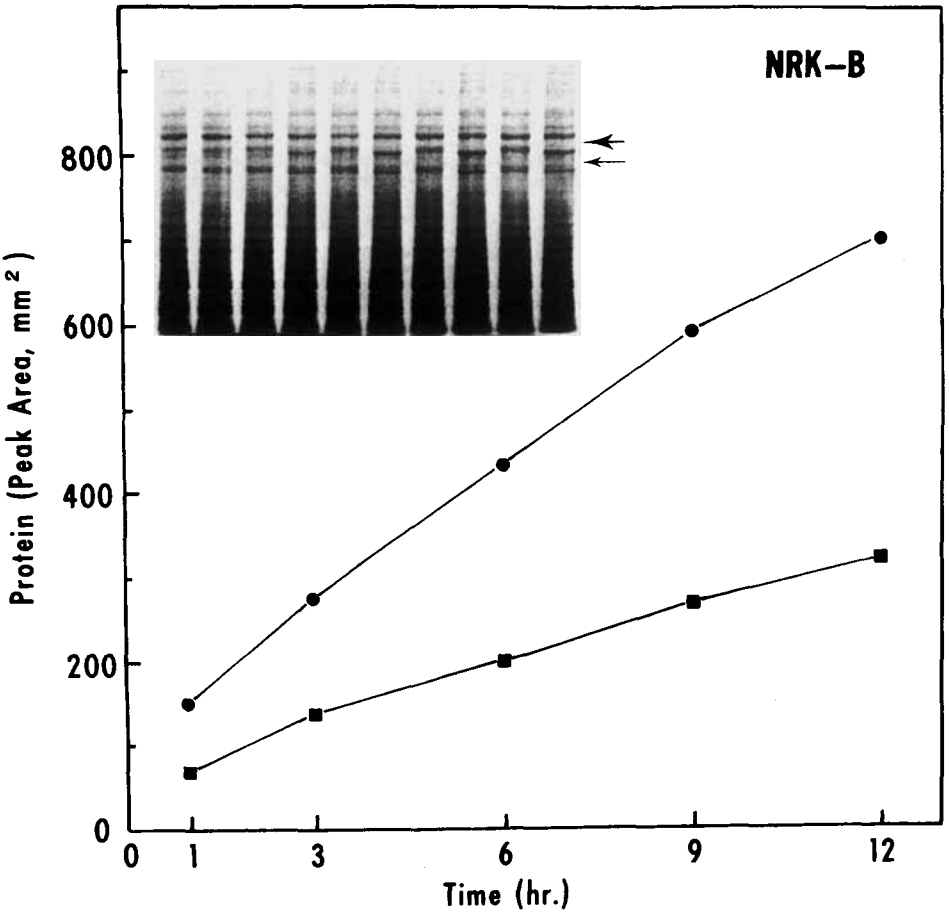


Fig. 1A. Selective effects of iron deprivation on plasma membrane profiles of NRK cells. Cells were plated at 1×10^6 per 100-mm dish. Forty-eight hours later, the medium was replaced. Dishes were divided into two sets: One set contained normal medium (control); the other set contained added desferrioxamine (350 μg/ml). At regular intervals after the initiation of the treatment, cells were collected and plasma membrane proteins were analyzed in exponential 5% to 12% acrylamide gels; polypeptide membranes that contained 100 μg of protein was applied to each lane. Protein bands were stained with Coomassie brilliant blue and were quantitated with an electronic planimeter and expressed as peak area. Inset: Duplicate lines correspond to control and desferrioxamine treated cells for each of the indicated times. Large and small arrows indicate 160 K and 130 K, respectively. Symbols: •, 160 K; ▪, 130 K.

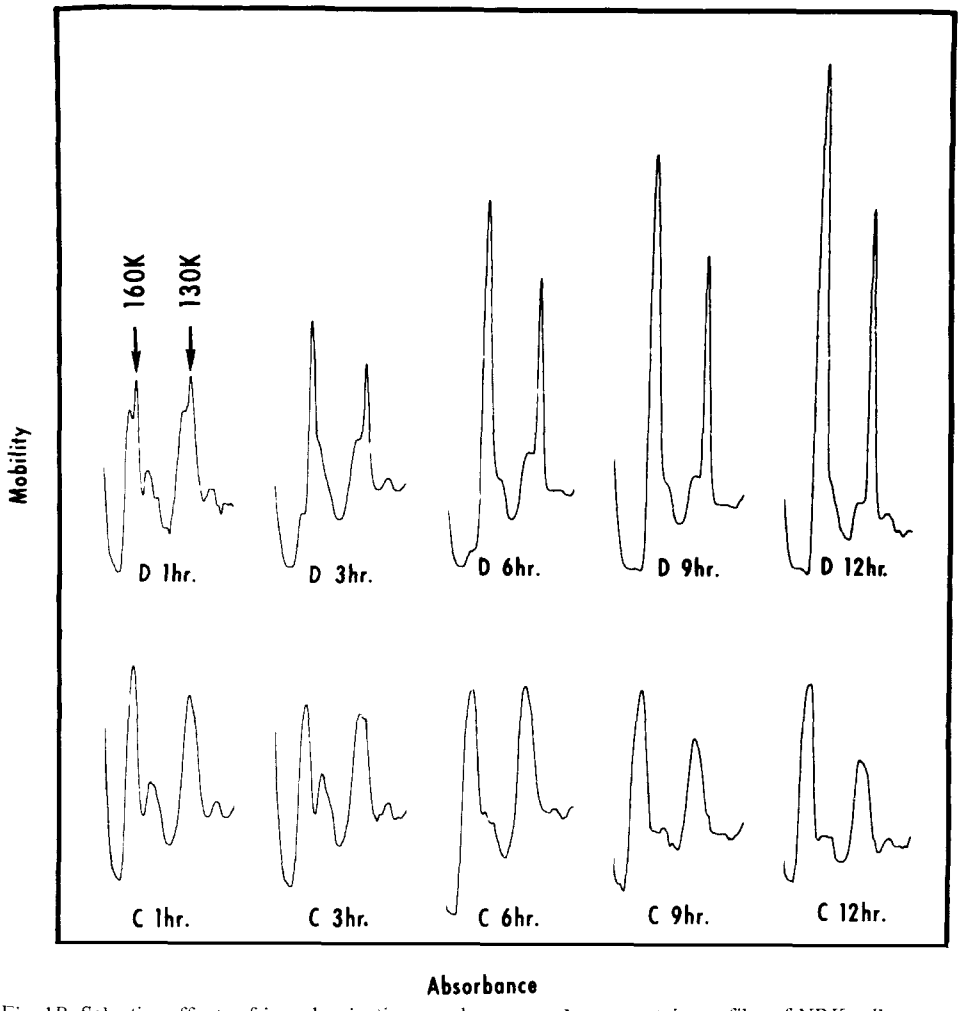


Fig. 1B. Selective effects of iron deprivation on plasma membrane protein profiles of NRK cells (continued). Densitometric scans of Coomassie blue stained gels of the regions of interest of control [C] and desferrioxamine treated cells [D] are shown.

Production of Anti 160 K and 130 K Antiserum

Rabbits were immunized three times at 2-week intervals intradermally at multiple sites with 80 μg of the protein preparations in 0.5 ml of PBS emulsified with 1 ml of Freund's complete (first injection) or incomplete (last two injections) adjuvant. Seven days after the last injection the rabbits were bled. Gamma globulins were obtained from the serum by three precipitations with 40% ammonium sulfate at 4°C. The pellet was dissolved in Ca^{++}, Mg^{++}-free PBS and was dialyzed extensively against the same buffer. Dialysis was stopped when dialysate was negative for sulfates [15].

Immunoreactivity in Polyacrylamide Gels

The membrane proteins were separated by SDS-PAGE as indicated above. The reactivity of the antiserum with membrane proteins was determined as described elsewhere [16].

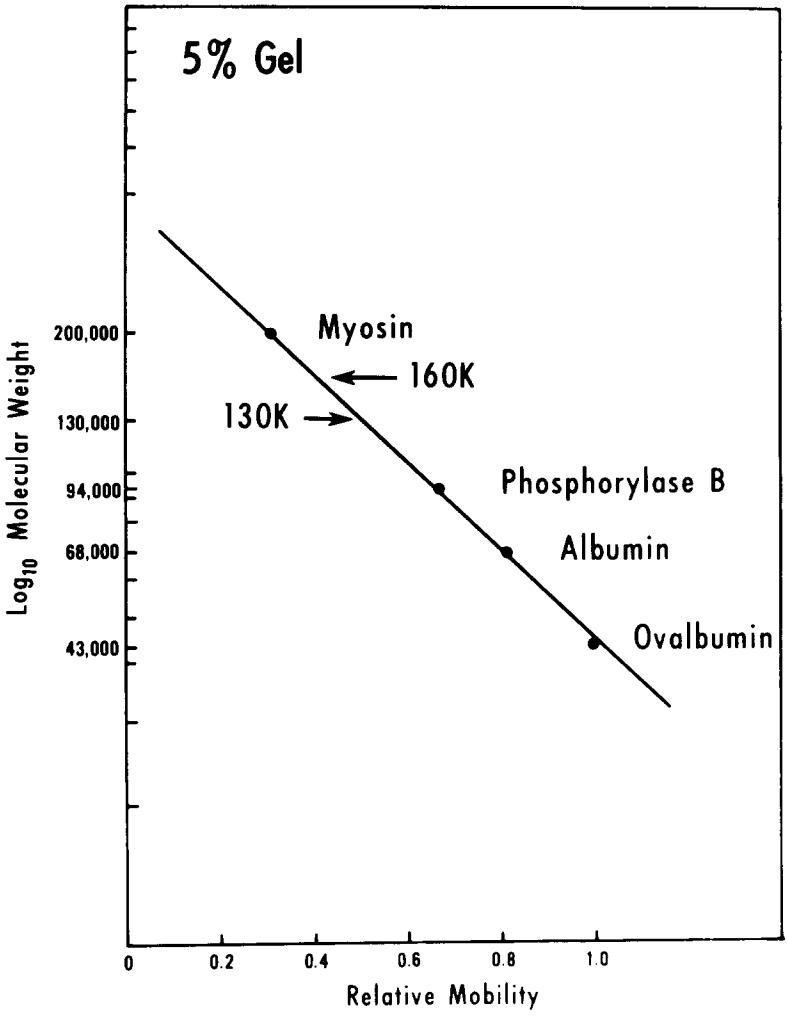


Fig. 1C. Selective effects of iron deprivation on plasma membrane protein profiles of NRK cells (continued). Molecular weight versus mobility plot of standard proteins separated in 5% SDS polyacrylamide gels. Positions of 160 K and 130 K are indicated.

Immunofluroscense Localization

NRK cells ($1-4 \times 10^4$ cells) were grown on coverslips (ethanol sterilized) in 35 mm culture dishes for 36 to 72 h. Then, new media with or without desferrioxamine (350 μg/ml) were added. After 3 h of incubation the cells were washed twice with PBS at 37°C and fixed in PBS containing 4% formaldehyde and 5% (wt vol) sucrose at room temperature for 30 min [17]. The cultures were then rinsed 4 times with PBS. A set of cultures was used without further treatment while another set was treated with acetone to permit intracellular staining [18]. After incubation of the cells with the antisera (1:50 dilution in 1 ml) for 60 min, the cells were rinsed with PBS and they were exposed to the IgG fraction of goat anti-rabbit IgG coupled to fluorescein (Miles Laboratories) for 60 min. After rinsing with PBS the cells were mounted in phosphate-buffered glycerol. Cells were examined with a Zeiss microscope equipped with fluorescence optics. For studies of antigen distribution, cultured cells were exposed to gamma globulin fractions (1 mg/ml) added to the culture medium. The cells were incubated for 1–3 h at 37°C, rinsed with PBS, fixed, and stained as described above.

Electron Microscopy

After incubation of the glycoproteins and/or transferrin in PBS containing 1 mM $CaCl_2$ and $MgCl_2$ at 37°C for 15 min, supernatant samples and pellets collected by centrifugation were prepared for electron microscopy as indicated elsewhere [19]. After negative staining with 1% uranyl acetate, the specimens were examined in a Philips 200 transmission electron microscope.

Other Procedures

Aliquots of the solubilized plasma membrane preparation were used for determination of protein [20], total radioactivity, and trichloroacetic acid-precipitable radioactivity. Metal-free water was used in the preparation of all transferrin solutions. Cells were counted with a Coulter counter. Protein was measured by the method of Lowry et al [21] or Bio Rad. Ouchterlony analyses were performed as indicated elsewhere [15]. Areas were estimated by a standard computer program developed at this laboratory. Unless otherwise noted, all the measurements were carried out in triplicate, and the results reported here were reproduced in four separate experiments each.

RESULTS

Relationship of the 163 K and 160 K Glycoproteins

We have evaluated this relationship by comparison of plasma membrane protein patterns of NRK cells grown in the presence or absence of desferrioxamine (Figs. 1A and B). The 5% to 12% exponential gel shown in Figure 1 demonstrates that the mobility of 163 K of NRK cells starved for iron is altered to a lower apparent molecular weight, 160 K. In the case of 132 K and 130 K a similar phenomenon was observed. Thus, when the cells were grown under conditions of iron deprivation, 163 K and 132 K ceased to be synthesized, and 160 K and 130 K were synthesized in their place. Further details of the relationship between iron depletion and kinetics of accumulation of 160 K and 130 K will be published elsewhere [7].

The 160 K and 130 K molecular weights used in the description of our data correspond to the lowest apparent molecular weight determined in a 5% gel (Fig. 1C) in cells treated with desferrioxamine for 24 h. The apparent molecular weight of the iron-regulated proteins after 6 h of treatment was about 170 K and 140 K, as determined in a 5% to 12% exponential acrylamide gel.

These glycoproteins were efficiently labeled with [^{14}C]-glucosamine and to a much lower specific activity with radioactive [^{14}C]-glucose, [^{14}C]-galactose, and [^{14}C]-mannose. They were also metabolically labeled with [^{35}S] methionine and [^{14}C]-proline. The amount of glycosylation particularly with [^{14}C]-galactose appeared to be dependent on the presence or absence of iron. Further details of these results are reported elsewhere [7].

Characterization of 160 K and 130 K Glycoproteins Induced by Iron Deprivation in NRK Cells

Cell surface proteins were iodinated by the lactoperoxidase-catalyzed iodination procedure which apparently labels cell surface proteins but not internal proteins [9]. Iodination of the surface of NRK cells subjected to iron deprivation labeled a protein of apparent molecular weight of 160 K daltons (Fig. 2). In control cells, 160 K was absent, and a relatively broad band of 163 K was found in its place. Thus, iron deprivation resulted in the expression of a cell surface protein, 160 K. No significant changes in

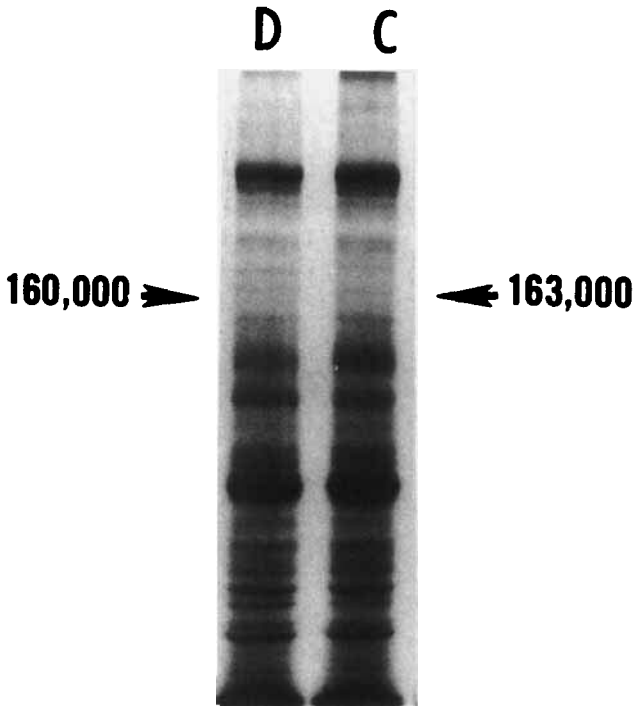


Fig. 2. Effect of iron starvation on surface proteins of plasma membrane of NRK cells. Cells were labeled in monolayer by the glucose oxidase lactoperoxidase-catalyzed iodination procedure [9]. The plasma membranes were isolated and analyzed in exponential 5% to 12% acrylamide gels. Plasma membrane samples applied to each lane contained equal quantities of cell membrane protein (100 μg). D. Iodination pattern of NRK cells grown in the presence of desferrioxamine. Cells were plated at 1×10^6 cells per 100-mm dish. Forty-eight hours later, new medium containing desferrioxamine (325 μg/ml) was added. Following 14 h of treatment, cells were iodinated. In this autoradiogram, a sharp protein band with a molecular weight of 160,000, is iodinated. C. Control, untreated cells, shows 163 K glycoprotein.

mobility were seen in other membrane-associated proteins of cells subjected to iron deprivation, though decreases in the labeling of some proteins were observed (Fig. 2). To ascertain whether 160 K was firmly bound to the cell membranes or loosely attached, the cells were incubated and washed extensively with PBS at 37°C. After this procedure, the ^{125}I-labeled 160 K was not decreased. Thus, 160 K appears to be firmly associated with the plasma membrane. The intensity of the iodinated band of 160 K daltons was enriched in plasma membrane preparations. The 160 K glycoprotein was iodinated in isolated membrane preparations. No evidence was found that the 130 K glycoprotein detected by Coomassie blue staining (Fig. 1) or [^{35}S] methionine labeling comigrates with a 130 K lactoperoxidase-labeled glycoprotein. The 130 K glycoprotein could not be significantly iodinated in isolated cell membranes. These data suggest that 130 K glycoprotein induced by iron deprivation is not accessible to iodinization. However, the possibility that iodination may have altered the mobility of 130 K detected by metabolic labeling to a protein of slightly lower molecular weight has not been ruled out.

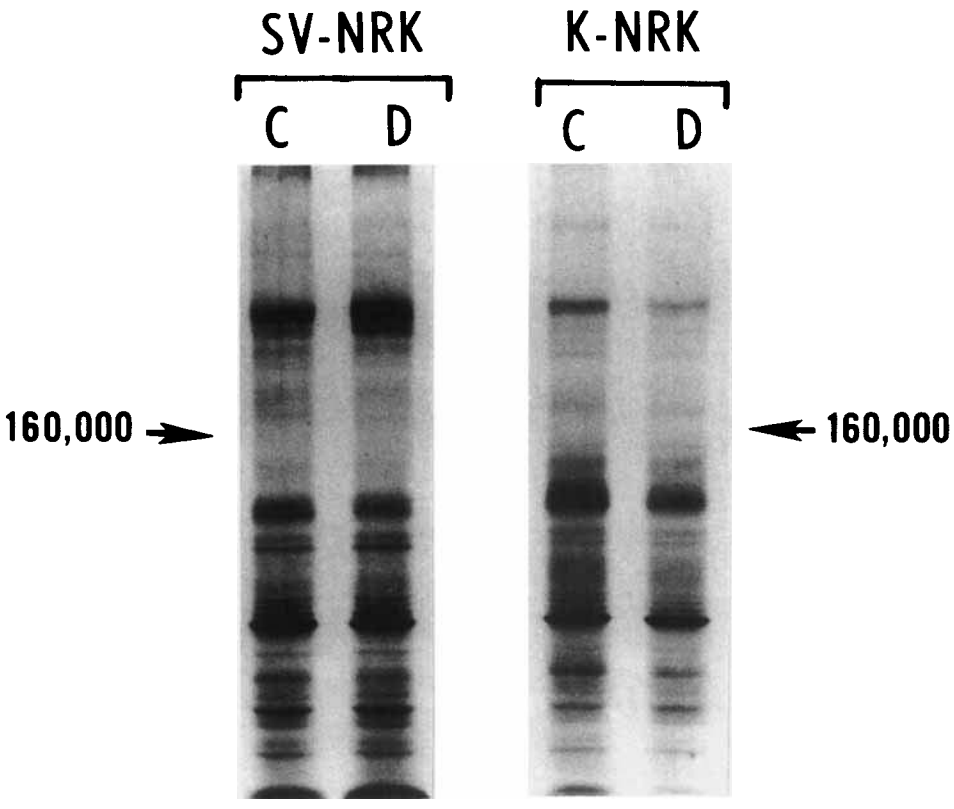


Fig. 3. Iodination of cell surface proteins of SV-NRK-B C12 and K-NRK cells subjected to iron deprivation. Cells were treated as indicated in Figure 2. C. Control, untreated cells; D. desferrioxamine treated cells.

Further experiments suggested that 160 K protein is exposed at the outer cell surface since trypsinization of the cells removed most of the label associated with 160 K. The 160 K glycoprotein, metabolically labeled with [^{35}S] methionine, was readily degraded by treatment with trypsin (1 μg/ml of trypsin at 37°C for 3 h). However, tryptic digestion with a decreased quantity of trypsin (0.1 μg/ml) did not remove all the label associated with 160 K. The 130 K glycoprotein could be partially removed by 1 μg/ml of trypsin. Treatment of the cell surface with highly purified collagenase (10 units/ml for 3 hr at 37°C) effectively removed 160 K and 130 K. The 160 K and 130 K bands co-migrated with authentic proline-labeled procollagen. These data suggest that 160 K and 130 K are related to the procollagen.

Surface Labeling of Transformed Cells Subjected to Iron Deprivation

We have examined transformed cell lines for differences in the distribution of cell surface proteins after 12 h or 16 h of iron deprivation. Figure 3 shows that the plasma membrane polypeptide patterns of K-NRK cells grown in the presence or absence of desferrioxamine were not significantly different, with the exception of one band of apparent molecular weight close to 160 K daltons. This band is weakly labeled in com-

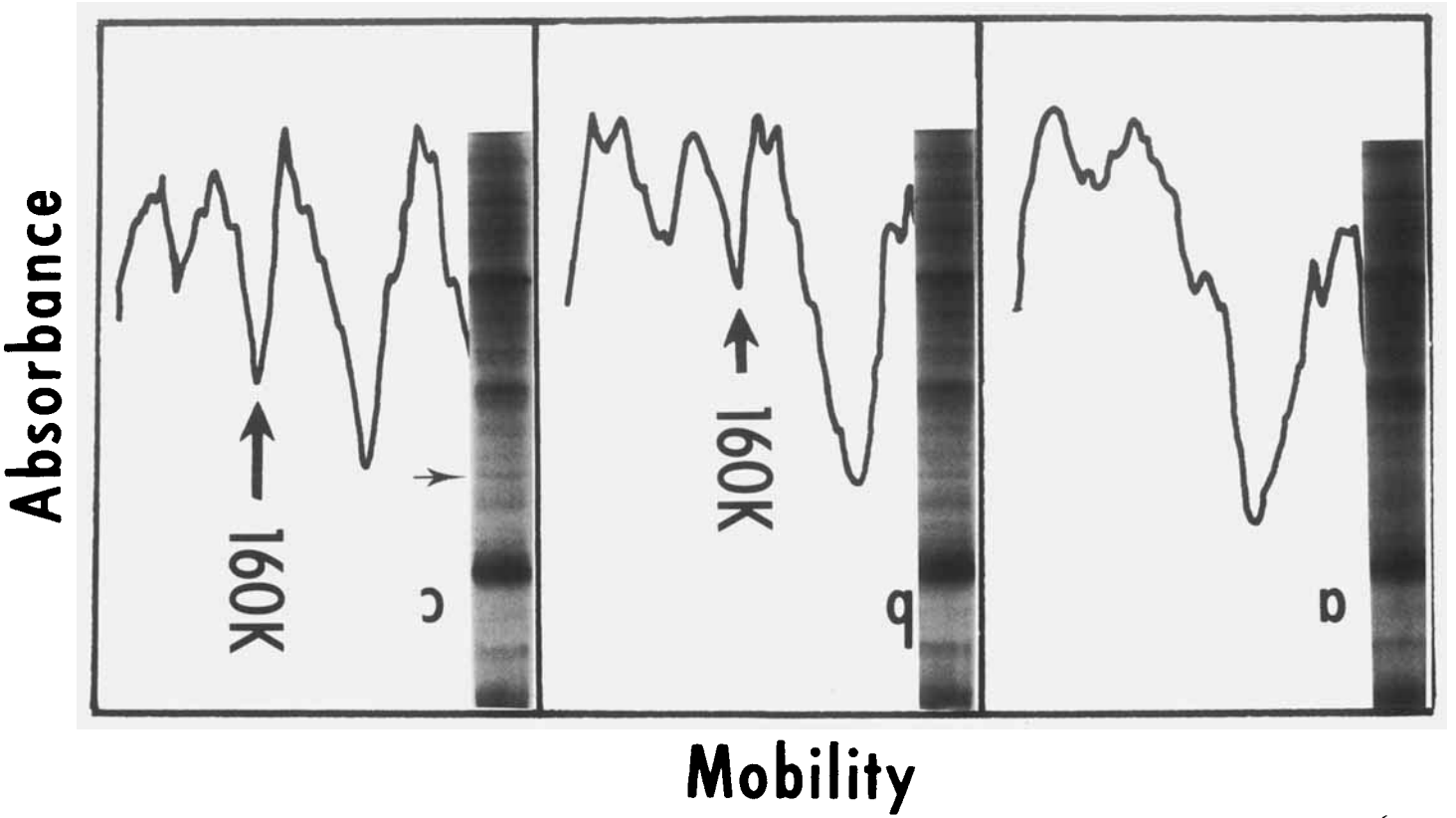


Fig. 4. Iodination of cell surface proteins of NRK cells subjected to iron deprivation after exposure to added transferrin. Cells were plated at 1×10^6 per 100-mm dish. Forty-eight hours later, the medium was removed and new medium with or without desferrioxamine (325 μg/ml) was added. After 14 h, transferrin was added to the cultures (final concentration, 20 μM) and cultures were incubated at 37°C for 30 min. A set of control cultures were identically treated but without transferrin. Then the cells were iodinated by the lactoperoxidase procedure. Plasma membranes were prepared from 5 to 8 dishes for each condition, normalized for equal protein (100 μg), and analyzed by SDS electrophoresis in 5% to 12% exponential acrylamide gels. Autoradiograms of (a) control, untreated cells, (b) desferrioxamine-treated cells plus 20 μM transferrin, (c) desferrioxamine treated cells, no additions. Note that the labeled protein at 160,000 mol wt is present in both (b) and (c) preparations but the relative activity is different. Prior exposure of the cells to transferrin decreases the specific activity of the 160 K species. Densitometric scans of autoradiogram of the regions of interest are shown.

parison to 160 K in NRK cells (Fig. 2). Thus, 160 K is a surface protein in K-NRK cells but apparently is less accessible to iodination, or the quantities on the cell surface are reduced in comparison to the untransformed counterpart. As shown in Figure 3, when SV-NRK-B C12 cell cultures were grown in the presence of desferrioxamine, 160 K showed weak labeling. In another clone of SV-NRK cells, 160 K could not be detected (not shown). The difference between these two clones in membrane polypeptide distribution and response to iron deprivation indicates clonal variation. These findings are in accordance with results obtained by metabolic labeling of transformed cells [7]. 130 K could not be detected in any transformed cell line. The data suggest that 160 K and 163 K are surface proteins whose surface expression is modified by iron and is altered by viral oncogenic transformation.

Possible Interaction of 160 K with Transferrin in Cultured NRK Cells

Intact NRK cells, grown in the presence or absence of desferrioxamine, were loaded with exogenous differic transferrin prior to the lactoperioxidase iodination procedure. In the autoradiographs, 163 K and 160 K were evident in control and treated cells, respectively (Fig. 4). Note that the labeled 160 K band is very sharp and easily seen in the autoradiographs of cells unexposed to exogenously added transferrin (Fig. 4c). In contrast, this component is significantly reduced in cells preloaded with transferrin (Fig. 4b). Although in our hands, lactoperoxidase iodination is a semiquantitative procedure, we decided to estimate the loss of label in the surface polypeptide bands of cells exposed to transferrin prior to iodination. As can be observed in Figure 4b, a significant decrease of about 55% in the iodination of the 160 K band after prior incubation with transferrin was detected.

TABLE I. Estimated Inhibition of Lactoperoxidase Iodination of 160 K and 163 K Proteins by Transferrin in NRK Cells

	% Inhibition	
Protein	Human transferrin	Bovine transferrin
160 K (induced)	62	79
163 K (control)	45	73

Cells were plated at 1×10^6 per 100-mm dish. Forty-eight hours later, the medium was removed and new medium with or without desferrioxamine (325 μg/ml) was added. After 14 h, transferrin was added to the cultures (final concentration, 20 μM) and cultures were incubated at 37°C for 30 min. A set of control cultures was identically treated but without transferrin. Then the cells were iodinated by the lactoperoxidase procedure. Plasma membranes were prepared from 5 to 8 dishes for each condition, normalized for equal protein, and analyzed by SDS electrophoresis in 5% to 12% acrylamide gradient. Autoradiograph bands were quantitated and expressed as peak area. The percent inhibition was calculated by dividing the decrease in area of iron-regulated proteins in transferrin-treated cultures by the area of iron-regulated proteins in cells unexposed to transferrin. The data are expressed as an average of three experiments and, for bovine transferrin, as an average of two experiments. For details, see text.

The loss of radioactivity, however, was never complete. No other major differences were noted. Experiments with bovine transferrin showed that this protein also partially prevented the labeling of 163 K and 160 K of NRK cells (Table I). Thus, human or bovine transferrin, with certain selectivity, partially inhibits the iodination of these bands. The experiments described above were also performed with K-NRK and SV-NRK cells to ascertain whether differences in the pattern of 160 K labeling or other membrane proteins would be found in these cells. There was a significant difference in the labeling of 160 K in K-NRK cells with or without prior incubation with transferrin in three separate experiments (65% inhibition). Other membrane proteins remained unchanged. No significant differences were observed between protein bands of SV-NRK cells exposed or unexposed to transferrin prior to iodination. No significant changes in other membrane proteins were found when the membrane proteins of transformed cells were analyzed in exponential 7% to 16% gels.

Isolation of 160 K and 130 K Glycoproteins from NRK Cells

The partial purification procedure of 160 K and 130 K glycoproteins involved plasma membrane preparation followed by preparative SDS-PAGE. We estimated by Coomassie blue staining that 160 K and 130 K represent about 3% and 1%, respectively, of total proteins of the membrane preparation. One major band was present upon re-electrophoresis on analytical SDS-PAGE in each of the cases. The isolated glycoproteins migrated within the same molecular weight as the original proteins and showed minimal ($< 2\%$) or undetectable contamination by other cell proteins of different molecular weights, as determined by Coomassie blue staining of SDS-PAGE. The isolated 160 K and 130 K proteins apparently correspond to proteins originally identified by metabolic labeling, since the 160 K and 130 K membrane proteins detected by Coomassie blue staining and the 160 K and 130 K proteins metabolically labeled comigrate [7]. Thus, because the 160 K and 130 K proteins are well separated by SDS-PAGE from other proteins, the isolation procedure apparently afforded a relatively homogenous preparation of the glycoproteins with minimal contamination by other proteins of different molecular weight. These preparations appeared to be of sufficent purity to justify further physical-chemical and immunological characterization.

Possible Interaction of 160 K With Transferrin as Revealed by Electron Microscopy

An interaction of possible biological relevance between 160 K and transferrin was suggested by the lactoperoxidase study. These observations prompted us to investigate the possible interaction of 160 K, 130 K, and transferrin under a variety of experimental conditions by transmission electron microscopy after negative staining. When 160 K (0.03 μg/ul), 130 K (0.03 μg/ul), or transferrin (0.1 μg/ul) (< 5 or 90% iron-saturated) were incubated at 37°C in PBS containing 1 mM Ca^{++} and Mg^{++}, a random distribution of protein particles was observed (not shown). In a few instances molecular aggregates were seen in 160 K preparations, which did not show a tendency to be organized. When 160 K was incubated with 5% iron-saturated transferrin striated aggregates were visible in the preparations (Fig. 5a). The incubation of 160 K with 90% iron-saturated transferrin was found to produce extensive aggregates showing striations, but apparently to a lower extent (not shown). The incubation of 160 K, 130 K, and transferrin resulted in the formation of globular protein aggregates that appear to coprecipitate (or copolymerize) with filaments in certain organized fashion (Fig. 5b). In none of the control conditions, which consisted in all other possible combinations of 160 K, 130 K, and transferrin were these structures observed.

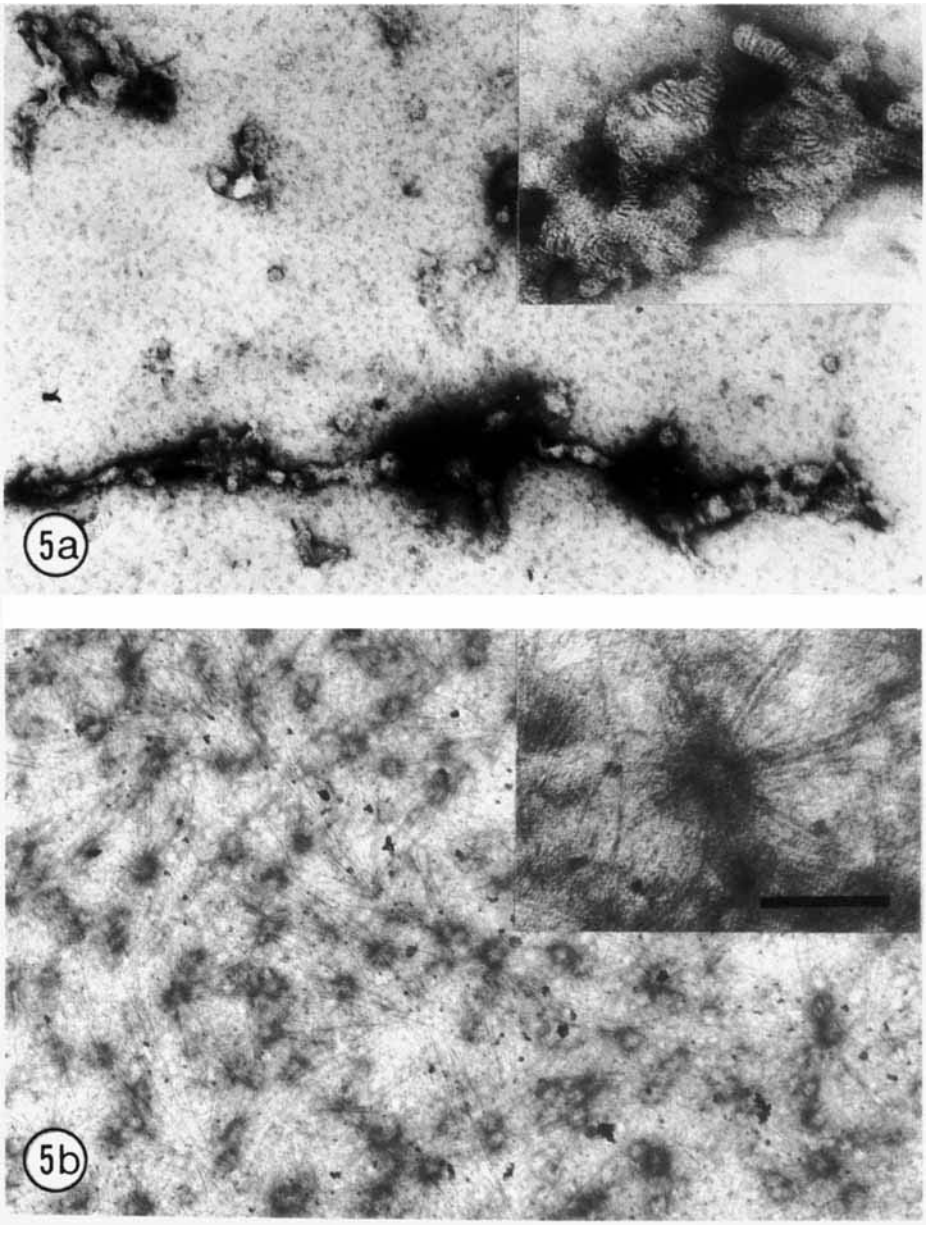


Fig. 5. Electron micrographs of 160 K, 130 K, and transferrin. The 160 K glycoprotein from NRK cells can assume different supramolecular structures depending on the presence or absence of transferrin and/or 130 K glycoprotein. The proteins were incubated in PBS (pH 7.4) containing 1 mM $CaCl_2$ and $MgCl_2$ at 37°C for 30 min. The supernatant of the reaction was applied to carbon-coated specimen grids and negatively stained with 1% uranyl acetate as described by Huxley [19]. Preparations were observed in a Philips 200 transmission electron microscope. (a) Irregulary striated aggregates are the most prevalent structure observed in the preparations of 160 K and 5% iron-saturated transferrin (magnification, × 54,538). Inset: Corresponds to right lower field (magnification, × 176,800). (b) Reticular networks and protein aggregates are observed in preparations of 160 K, 130 K, and 5% iron-saturated transferrin (magnification, × 55,566). Inset: Shows detail of a structure in close association with filaments (magnification, × 176,800), bar measures 1,000 Å.

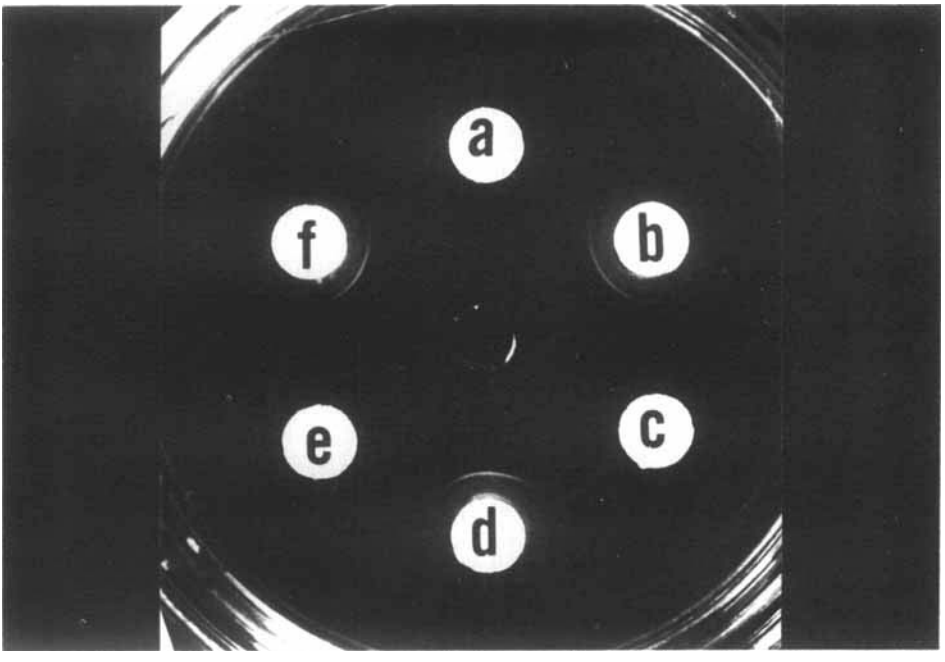
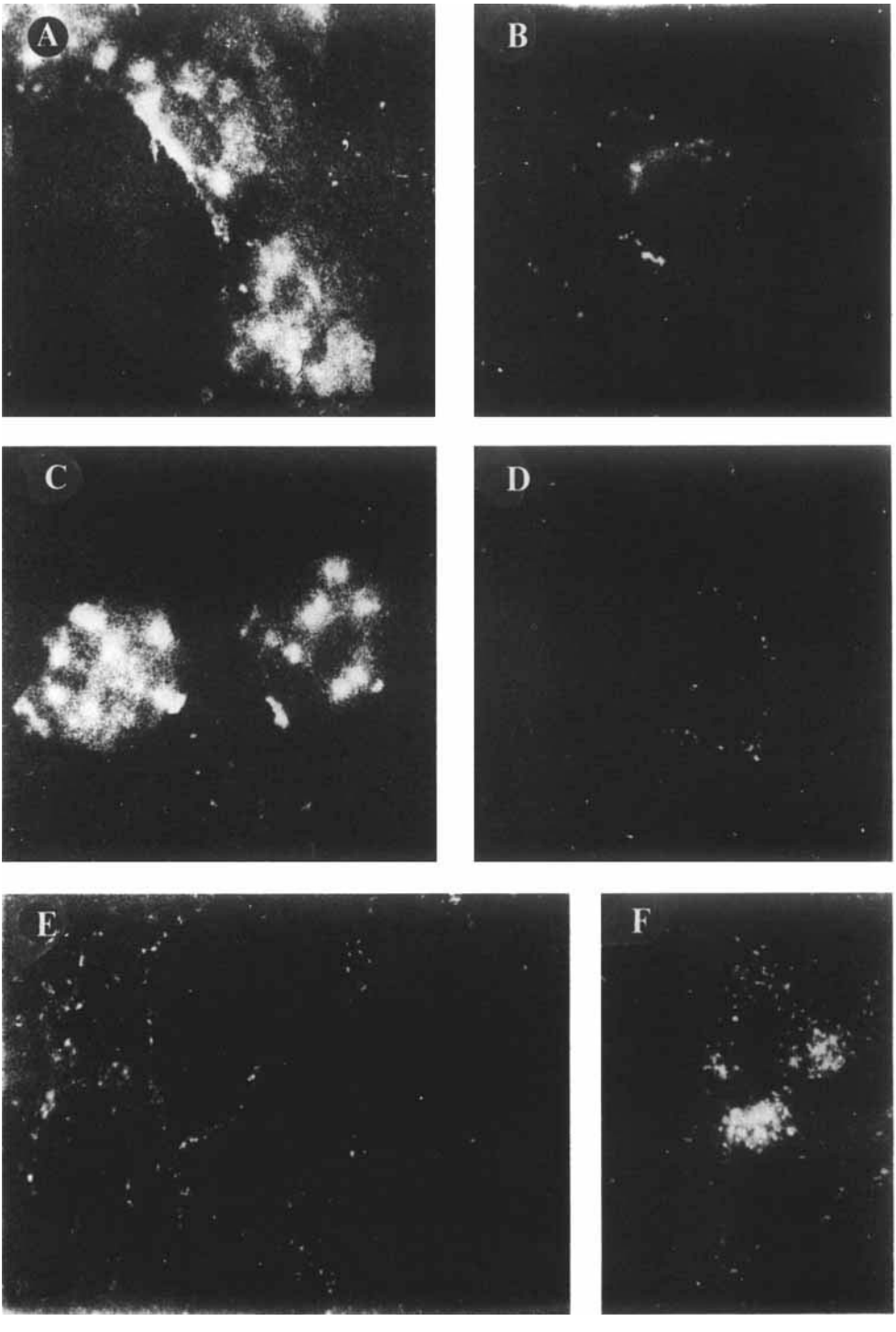


Fig. 6. Immunodiffusion of isolated 130 K glycoprotein in center well against preimmune (diluted 1:12) (wells a, c, and e) and immune (diluted 1:1,000) (wells b, d, and f) from a rabbit injected three times with 130 K protein as described in Methods.

Immunological Specificity of the Antiserum to the 160 K Glycoprotein

The antiserum to the 160 K glycoprotein reacted with 160 K, producing a single sharp precipitin band as determined by Ouchterlony analysis. The anti-160 K antibody did not react with 130 K or with a plasma membrane preparation obtained identically as described above but from which 160 K and 130 K were omitted. The reaction of 160 K with anti-160 K antisera apparently was specific since preimmune serum did not significantly react with 160 K. Additional evidence of specificity of anti-160 K antisera was provided by the method of Olden and Yamada [16]. With this procedure, the antiserum to the 160 K glycoprotein reacted only with one protein in an SDS-PAGE of total membrane proteins, as estimated by a significant densitometric increase (50% over controls), which comigrates with the 160 K glycoprotein. The reaction apparently was specific, since nonimmune sera did not react with any proteins in control gels. Anti-130 K

Fig. 7. A. Immunological localization of 160 K in NRK cells. Cells were plated on coverslips at 2×10^4 per 35-mm dish. Forty-eight hours later, the medium was replaced. Cells were grown for 3 h in the presence of 350 μg/ml desferrioxamine. The cells were then fixed in formaldehyde and stained by indirect immunofluorescence with anti-160 K antibody (diluted 1:50) as described in Methods. Cells photographed with the microscope focused on lower surfaces. (B, C, D, E). Antibody induced redistribution of 160 K in NRK cells. Density of plating was 1×10^4 (B, C), 4×10^4 (D), and 2×10^4 (E, F) cells per 35-mm dish. Forty-eight hours later the medium was replaced and the cells were cultured in the presence of 350 μg/ml desferrioxamine for 3 h. The cells were then cultures for 1 h (B), 2 h (C, D, E), or 3 h (F) with 1 mg/ml anti-160 K globulin, then fixed and stained. (B, D, E): NRK cells photographed with the microscope focused on mid-portions of the cells; (C, F): Microscope focused on upper surfaces of cells (fluorescence micrographs; magnification, × 500).



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reacted with 130 K and cross-reacted with 160 K, indicating that 130 K and 160 K glycoproteins share common antigenic determinants. The reaction of 130 K with anti-130 K antibodies appeared to be specific, since preimmune serum did not significantly react with 130 K (Fig. 6). The 130 K antiserum did not significantly react with other proteins of the membrane preparations. The antiserum to 130 K is therefore not entirely specific, whereas the anti-160 K is directed against the 160 K cell surface glycoprotein.

Immunofluorescence Localization and Antigen Redistribution

NRK cells were fixed, exposed to antibody against 160 K, and stained using indirect immunofluorescence. Figure 7A shows that 160 K was found on the cell surface of subconfluent NRK cells in a diffuse pattern. Focal points of increased staining appeared to correspond to cell attachment areas and blebs. By cell density analysis we were able to demonstrate spatial transitions in the nature of 160 K antigen since confluent cells showed staining located preferentially in the intercellular spaces (not shown). Immunofluorescence data showed surface staining in both control and treated cells, indicating either that 160 K is present in controls or, more likely, that anti-160 K cross-reacts with 163 K. When the interior of NRK cells was rendered accessible to the antibody by altering the plasma membrane with acetone, the antibody stained both the cell surface and the perinuclear cytoplasm of subconfluent cells (not shown). In confluent cells, however, the cell surface but not the cytoplasm was stained. The 160 K glycoprotein of NRK cells could be redistributed by exposure of cultured cells to the anti-160 K antibody (Fig. 7B). In subconfluent cultures of NRK cells rearrangements of surface antigens occurred after incubation for 1–3 h in the presence of anti-160K added to the culture medium (Fig. 7C). The 160 K antigen concentration and spatial distribution appeared to be dependent upon cell density, since confluent cells showed significant staining and antigen redistribution in peripheral areas of the cell (Figs. 7D and E). The apparent intensity or frequency of staining of the cell surfaces of iron-deprived and control cells was compared. The cell surface fluorescence of the desferrioxamine-treated cells appeared to be increased in comparison to untreated cells (not shown). The difference in the staining of the two types of conditions was consistent and evident both in dorsal and equatorial sections of the cell surface. Areas of concentration of antibody were seen in about 30% of the cells after 3 h of incubation with anti-160 K antiserum (Fig. 7F). Redistribution of antigens with nonimmune rabbit gamma globulins was negative. These antibody-induced redistributions indicate that the 160 K glycoprotein is exposed at the cell surface and is mobile in the plane of the membrane. Similar results were obtained with antiserum against 130 K (data not shown). Since the anti-130 K antiserum cross-reacts with 160 K glycoprotein, these results with 130 K are difficult to interpret. Thus, further work will be necessary to define more precisely the nature of the association of 130 K to cell membranes. Results of these experiments suggest that the 160 K or a related antigen is present in controls, that antigen concentration is related to cell density, and that iron depletion appeared to significantly alter the antigen abundance.

DISCUSSION

In the present study we have shown that cultured cells express different surface protein patterns as a result of iron deprivation. Under such conditions an exterior plasma membrane-associated protein, 160 K of NRK cells, was detected in small concentrations or not at all on virus-transformed derivatives. Additionally, the data suggest that the expression of 160 K to the cell exterior may be experimentally regulated by intracellular

iron concentrations. Because lactoperoxidase-catalyzed iodination involves the possibility of cell penetration by ^{125}I [22], the possibility arose that the results observed with 160 K might be artifactual. That this was not the case was suggested by the facts that antibodies against 160 K localized on the cell surface, that the protein is enriched in membrane preparations, and that it is sensitive to trypsinization. The 130 K polypeptide was apparently not accessible to iodination in both untransformed and transformed cells. This suggests that it is either not located at the surface or contains no iodinizable groups, or its configuration is such that the residues are not available to iodination. Experiments with virus-transformed cells suggested that changes in iodinization of 160 K manifested by absent or weak iodination may be related to transformation. The loss or decrease concentrations of a number of membrane proteins have been noted before and may be a direct result of the expression of viral genetic information [23].

Treatment of the cell surface with purified collagenase removed 160 K and 130 K, suggesting that these molecules are related to procollagen. Experimental work has established a critical role for iron in the synthesis of collagen [6, 24]. The biosynthesis of collagen occurs in a series of sequential steps [24–26]. Iron has been shown to be required for the hydroxylation of both proline and lysine in procollagen. It has been shown that in connective tissue treated with an iron chelator such as 2,2′-dipyridyl, proline and lysine are incorporated into procollagen but there is no synthesis of hydroxyproline or hydroxylysine [24, 26]. It has been recently demonstrated that fibroblast cultures treated with desferrioxamine show inhibition of DNA synthesis and reduced collagen formation, with an apparent increase in the amounts of collagen produced with reduced doses of the agent [27]. The present findings support the view that desferrioxamine effects on membrane-associated proteins involve collagen synthesis and inhibition of proline and lysine hydroxylase activity. This in turn would result in the accumulation in the cell and also on the cell membrane of under-hydroxylated collagen precursors. Furthermore, the protein would be under-glycosylated since the incorporation of galactose and glucose, which attach to the hydroxylysine residues, would be prevented [24]. Thus, the present data and other results [7] suggest that the under-glycosylated 160 K and 130 K seen in our preparations are under-hydroxylated procollagen molecules. It has been shown that procollagen is retained by the cells in the endoplasmic reticulum when treated with 2,2′-dipyridyl [28]. This suggests that procollagen should not be susceptible to iodination unless a fraction of the molecules are associated with the cell membrane and are exposed to the extracellular space. Under our conditions, it appears that 160 K is exposed to the extracellular space. Our data are in accordance with current ideas about cell surface architecture of collagen [29] and also with the evidence reported that collagen is membrane bound and that it is patched and capped by anti-collagen sera in human fibroblasts [30]. Thus, the data suggest that 160 K is related to procollagen, is associated with the cell membrane, and is exposed to the external environment.

The significance and mechanisms of the decreased or absent 160 K on the surfaces of transformed cells as determined by surface iodination remain to be determined. Several possible explanations exist for this finding: It could be due to reduced synthesis, increased degradation, or enhanced release of the glycoprotein. Recent work has shown decreased cellular messenger RNA coding for various high molecular weight proteins in transformed cells [31]. The results presented in a subsequent paper [7] allow one to consider the first model since the proteins appear in the surface of transformed cells at reduced rates, in comparison to normal cells, as demonstrated by metabolic labeling. Since 160 K and 130 K are most likely related to procollagen, our data are in accordance with the observa-

tion that collagen synthesis is altered in cultured fibroblasts after transformation by some oncogenic viruses [32, 33], chemicals [32], and the potent tumor promoter phorbol 12-myristate 13-acetate [34]. Thus, the decreased accumulation of 160 K and 130 K may be due to the inability of virus-transformed NRK cells to synthesize sufficient quantities of these proteins. The present findings also imply that the concentration of these proteins in or on the plasma membrane may be of certain importance to growth arrest [7] and morphological changes [7, 27] induced by iron deprivation. Thus, our data provide evidence of biochemical concomitants of morphological and growth changes which are altered by transformation.

A point of particular interest is the function of 160 K and 130 K associated with the cell membranes and the apparent inhibition of iodination of 160 K glycoprotein by transferrin. They may simply have the well-known structural role as precursors of collagen [26]. However, other possibilities may also be considered. One obvious possible role for 160 K or a subset of this protein is that it is a component of the transferrin receptor, because it is a cell surface protein, and because one might expect that the receptor complex for transferrin is regulated by iron availability. That the transferrin receptor in some cells may be susceptible of detection by lactoperoxidase iodination appears to be likely for several reasons discussed elsewhere [35]. Furthermore, Witt and Woodworth have recently shown that the iodination of a putative 190 K transferrin receptor of reticulocyte is partially inhibited by its association with transferrin, presumably because the interaction of transferrin and its receptor shields some of the tyrosine residues from attack by the lactoperoxidase molecule [35]. Using this approach it was possible to demonstrate a differential decrease in the iodination of the 160 K protein in the membranes of intact NRK cells that have been preloaded with differic transferrin in those unexposed to transferrin. However, an alternative explanation for these findings is that transferrin interacts with 160 K and the complex transferrin-160 K is internalized [36], with a subsequent reduction in the number of molecules of 160 K available for iodination. Electron microscopy evidence provided further support for the idea that transferrin, 160 K, and 130 K interact in certain fashion. This finding may not be suprising since precursors of collagen or collagen itself have been shown to interact with glycoproteins [37], to produce specific mono- or polymorphic arrangements [37, 38], and to bind Fe^{3+} [39]. Furthermore, it is known that collagen molecules may be precipitated in a variety of different polymorphic forms; which polymorph occurs is determined mainly by the conditions of precipitation [38]. Taken together, these few data and published reports indicate in vivo and in vitro interactions that may or may not be entirely specific but that may be of possible physiological relevance. The molecular events described in this paper may represent an early step in the transferrin-cell interaction, which is followed by binding of transferrin to the receptor. Further extensive study is necessary to confirm the speculation concerning specificity and physiological implications of this interaction.

In conclusion, the present findings show that, upon iron starvation, normal and some transformed cells display a new cell surface glycoprotein, related to procollagen, which can be shown to interact with transferrin. Our results on the latter point are only suggestive, and only when the proteins are isolated in pure form can definite knowledge of these interactions be obtained. Finally, the results presented in this paper yield additional insight into interactions between surface proteins, growth media proteins, and iron, which could facilitate the detailed understanding of factors controlling cell shape and growth in vitro.

NOTE ADDED IN PROOF

Specific antibodies against procollagen propeptides, generously provided by Dr. Kao, showed similar staining characteristics as the anti-160 K antibodies.

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