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Isolation and Characterization of Normal Rat Kidney Cell Membrane Proteins with Affinity for Transferrin†

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ABSTRACT: Studies were performed to identify membrane receptors for transferrin in cultured normal rat kidney (NRK) cells. Cells were surface iodinated or metabolically labeled with radioactive glycoprotein precursors. Membrane receptors for transferrin were solubilized with the nonionic detergent Triton X-100. The soluble transferrin receptor has been purified ~1500-fold by affinity chromatography using transferrin coupled to Sepharose. Experiments demonstrated that the receptor can be adsorbed to a transferrin–Sepharose gel and be eluted specifically with transferrin. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the receptor preparations obtained by one cycle of affinity chromatography display, in addition to components of M_r lower than 20000, a major glycoprotein component of ~170000. Solubilized receptor preparations subjected to two cycles of affinity chromatography revealed a single polypeptide of ~20000 daltons. Further studies indicated that the 20000-dalton polypeptide is a degradation product of the 170000 glycoprotein. Immunological studies showed that antitransferrin antibodies specifically precipitate a transferrin–170000 complex and that a specific antibody against 170000 glycoprotein precipitates the same complex. These results suggest that the 170000 glycoprotein associates with transferrin in specific fashion and that this protein may be a subunit of the transferrin receptor of NRK cells.

Transferrin is a glycoprotein that is responsible for the delivery of iron to various cells (Aisen & Leibman, 1977). This glycoprotein is a factor required for growth and maintenance of some cultured mammalian cells (Bottenstein et al., 1979; Fernandez-Pol, 1978). As with other substances of related function, it appears that the first step of the interaction of transferrin with the cells is the association of the factor with a specific recognition site (receptor) on the surface of the cells (Aisen & Leibman, 1977; Hu & Aisen, 1978). The binding characteristics of this receptor from both red blood cell precursors and placenta have been extensively studied, and it has been shown that the transferrin receptors can be extracted from the cell surface by nonionic or ionic detergents without loss of its ligand binding activity (Aisen & Leibman, 1977; Sly et al., 1978; Hu & Aisen, 1978). More recently, cultured cells, including NRK[1] cells, have been found to possess transferrin receptors (Fernandez-Pol et al., 1979; Hemmaplardh & Morgan, 1974; Larrick & Cresswell, 1979; Hamilton et al., 1979). Little is known on the nature of the transferrin receptors in cultured NRK cells.

In this report we present the binding characteristics of a putative transferrin receptor extracted from NRK cells with detergents. Some properties of the soluble and partially purified receptor and preliminary identification of cell surface proteins interacting with transferrin are also presented.

Materials and Methods

Cell Culture. NRK-B cells (clone 8) were obtained from Dr. R. Ting (Biotech Research, Inc.). Cell stocks were cultured in 75-cm^2 Costar tissue culture flasks in DME containing 10% (v/v) calf serum (Colorado Serum Co., Denver, CO) as previously described (Fernandez-Pol et al., 1977). For experiments, cells were grown in 150-mm Falcon tissue culture

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[1] Abbreviations used: NRK, normal rat kidney; DME, Dulbecco–Vogt modified Eagle's medium; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol).

dishes containing 25 mL of DME and 10% calf serum. To deprive iron from the cells, cultures were grown in the presence of desferrioxamine (Fernandez-Pol, 1979).

Cell Surface and Metabolic Labeling of Cells. For surface labeling, cells were plated at 3×10^6/150-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 (400 μg/mL). After 6 h of growth in the presence or absence of desferrioxamine, monolayer cultures were surface labeled by the glucose oxidase–lactoperoxidase catalyzed iodination procedure as previously described (Fernandez-Pol, 1979; Hynes, 1973). The plasma membranes were isolated by the method of Brunette & Till (1971) as modified by Stone et al. (1974). All washings and fractionation buffers contained 2 mM PMSF.

For metabolic labeling, cells were plated at 3×10^6/150-mm dish. Twenty-four hours later the medium was removed, and new media containing [1-^{14}C]-D-glucosamine [1 μCi/mL; sp act. 57 mCi/mM (ICN, Irvine, CA)], [^{14}C]-L-proline [1 μCi/mL; sp act. 250 mCi/mM (ICN, Irvine, CA)], or [G-^{3}H]-L-amino acid mixture [10 μCi/mL (New England Nuclear, Boston, MA)] were added. After 24 h desferrioxamine was added to the cultures (final concentration 400 μg/mL), and cultures were incubated at 37 °C in the tissue culture incubator for 6 h. A set of control cultures was identically treated but without desferrioxamine. Then, the media were removed and the cultures were washed 3 times with PBS containing 2 mM PMSF. The cells were scraped from the dishes into Earle's BBS containing 10% sucrose, and the membrane were isolated as described above.

Preparation of Transferrin. Human (Boheringer Diagnostics, Sommerville, NJ) or rat (Cappel, Cochranville, PA) transferrins were converted to the 100% iron-saturated form and purified as previously indicated (Fernandez-Pol, 1979; Graham & Bates, 1976). The purity of the preparation determined by NaDodSO$_4$–polyacrylamide gel electrophoresis, was greater than 98%. Labeled transferrins were prepared by the lactoperoxidase procedure (Enzymobeads, BioRad, Richmond, CA). The procedures for purification of ^{125}I-labeled transferrin were performed as indicated elsewhere (Fernandez-Pol, 1979). Purity of the preparation was determined by NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography. Only one labeled transferrin band was observed (purity greater than 99%). The specific activity of the labeled transferrins was 150–200 μCi/μg. [^{125}I]Transferrin was 90–95% immunoprecipitable. The labeled transferrins were kept in aliquots at −20 °C until used.

Preparation of Soluble Transferrin Receptors. In order to characterize the transferrin receptor of NRK cells, we have utilized the nonionic detergent Triton X-100 as a membrane-solubilizing agent. A plasma membrane enriched fraction was prepared according to previously described methods. The membranes were solubilized (protein concentration 4–6 mg/mL) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.5% (v/v) Triton X-100 at 37 °C for 30 min; the incubation mixture was then centrifuged at 100000g for 1 h. The supernatant fraction, referred to as extract, contained the soluble receptors for the transferrin and other solubilized membrane proteins. Aliquots of the extract were stored at −20 °C until further use. We also investigated the solubilization of membrane proteins with NaDodSO$_4$, an ionic detergent previously utilized to solubilize transferrin receptors from reticulocytes (Garrett et al., 1973). The membranes were solubilized (protein concentration 1–2 mg/mL) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.5% (v/v) NaDodSO$_4$ at 37 °C for 30 min. Insoluble material was removed by centrifugation as described above, and the supernatant was utilized for affinity chromatography.

Assay for Transferrin Receptor Activity. The presence of transferrin binding proteins was determined by the use of PEG to precipitate the transferrin-receptor complexes (Ecarot-Charrier et al., 1977) and antibodies against 170000 protein (Fernandez-Pol, 1979), to precipitate a transferrin–170000 protein complex (reasons for the utilization of this procedure are found under Results).

(*a*) *Precipitation of Transferrin-Receptor Complexes by Poly(ethylene glycol).* The presence of transferrin receptor binding activity in Triton X-100-solubilized membrane proteins was determined according to a procedure similar to that employed by Ecarot-Charrier et al. (1977). Aliquots (50–100 μL) of soluble protein were incubated with 5×10^4 cpm (0.1–1 ng) of [^{125}I]transferrin in 0.05 M Tris-HCl buffer with 0.1% Triton X-100 and 1 mg of bovine serum albumin per mL in a total reaction volume of 500 μL. Samples were incubated at 23 °C for 16 h. The [^{125}I]transferrin-receptor complex was precipitated by addition of 0.5 mL of 0.1% (w/v) rabbit globulin (fraction II) in ice-cold 0.1 M sodium phosphate buffer, pH 7.5, and 1 mL of ice-cold 25% (w/v) PEG 6000 (Fisher) dissolved in the same phosphate buffer. The final concentration of PGE was 12.5% (w/v). The samples were mixed and placed on ice for 15 min, and the transferrin–receptor complexes, precipitated by PEG, were centrifuged at 3000g at 4 °C for 30 min. The supernatant fractions were decanted and the pellets were counted. Under these conditions, 20–75% of the added labeled transferrin, depending on amount of receptors in the incubation mixture, was precipitated. Nonspecific binding (~15–20% of counts in pellet) was determined by carrying out parallel incubations in the presence of a 1000-fold excess of unlabeled transferrin; this represented nonspecific binding to proteins, tube, or nonspecific precipitation by PEG. Specific binding was calculated by the difference in binding observed between these two conditions.

(*b*) *Precipitation of Transferrin-Receptor Complexes by Anti-170000 Antibodies.* We have previously reported the isolation of an "iron-regulated" glycoprotein with an apparent M_r of ~170000 from plasma membranes of NRK cells and the generation of specific rabbit antibodies against this protein (Fernandez-Pol, 1979). Experiments described under Results showed that these antibodies precipitate solubilized 170000–transferrin receptor protein labeled with [^{125}I]transferrin in specific fashion. On the basis of these observations a convenient assay method which employed indirect immunoprecipitation to precipitate [^{125}I]transferrin-receptor complexes was utilized. The following procedure for immunoprecipitation of [^{125}I]transferrin-receptor complexes by antiserum against 170000 was used: aliquots (50–200 μL) of soluble protein were incubated with 5×10^4 cpm (0.1–1 ng) of [^{125}I]-transferrin in 0.05 M Tris-HCl buffer with 0.1% Triton X-100 and 1 mg of bovine serum albumin per mL in a total reaction volume of 100–250 μL. After 30 min at 37 °C, the rabbit antiserum to the 170000 protein (5 μL) was added to achieve a final dilution of 1:1000, and the incubation was continued for 18 h at 4 °C. Then goat antiserum to rabbit immunoglobulin G (IgG) (100 μL) was added. After 18 h at 4 °C, the resulting precipitate was separated from other proteins by centrifugation at 3000g at 4 °C for 30 min. The supernatant was removed, and the precipitates were washed 4 times in phosphate buffer, pH 7.5, containing 0.1% Triton X-100. Under these conditions, 10–85% of the added labeled trans-

ferrin, depending on the amount of receptors present, was precipitated. About 2–10% of the added [^{125}I]transferrin was precipitated in control tubes in which anti-170000 serum was omitted from the incubation mixture. The difference in precipitation observed under these two conditions was considered to represent specific precipitation. When solubilized receptor was omitted from the incubation mixture, ~5–10% of the added [^{125}I]transferrin precipitated.

The amount of [^{125}I]transferrin-labeled receptor precipitated by anti-170000 serum (1:1000 dilution) was compared with the amount of [^{125}I]transferrin-labeled receptor precipitated by PEG, 12.5% (v/v). The results obtained by both procedures were similar. The nonspecific binding observed with the PEG technique (20%) was higher than that with the anti-170000 antibodies (10%). Thus, the latter technique was preferentially utilized.

Affinity Chromatography. (*a*) *Preparation of Affinity Adsorbents.* Rat transferrin (10 mg) was conjugated to CNBr-activated Sepharose 4B (1 g dry weight) and was repetitively washed according to the instructions supplied by the manufacturer (Pharmacia, Piscataway, NJ). The efficiency of coupling was estimated by determining the amount of uncoupled transferrin by spectrophotometry. About 99% of the transferrin added was coupled under these conditions. Since the washing of the transferrin–Sepharose gel involved changes in pH, the possibility existed that the saturation of transferrin by iron was altered. To perform experiments under rigorous conditions, we saturated the transferrin coupled to Sepharose with iron by utilizing the procedure described in Van Snick et al. (1973). Rat apotransferrin, 10 mg, was also coupled to 1 g of CNBr-activated Sepharose in the same manner. Alternatively, rat apotransferrin was prepared by washing the transferrin–Sepharose gel with 0.1 M citric acid in order to release the iron bound to the protein (Van Snick et al., 1973) and then was equilibrated in an appropriate buffer. Human apotransferrin, human transferrin, lactoferrin (Calbiochem, LaJolla, CA) and bovine serum albumin (Sigma Chemical Co., St. Louis, MO) were coupled to CNBr-activated Sepharose by the same procedures.

(*b*) *Affinity Purification of Transferrin Receptors.* A receptor extract in $NaDodSO_4$ or Triton X-100 was incubated for 1 h at 23 °C in a column (0.8 × 3 cm) of transferrin-coupled Sepharose equilibrated with 0.05 M Tris-HCl buffer pH 7.6, containing 0.5 M NaCl with or without 0.1% Triton X-100. At least 20 bed volumes of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.5 M NaCl with or without 0.1% Triton X-100 was used to wash the column until no radioactivity and no receptor activity were eluted. The flow rate was ~15 mL/h. Fractions of ~1 mL were collected. The material bound to the Sepharose gel was eluted with 2 M KSCN in PBS, pH 7.4. Fractions of 1 mL were collected during elution. Normally, nonadsorbed (fractions 1–10) material and adsorbed (fractions 40–45) material were pooled, placed in separate dialysis tubings (Spectraphore 3000), and dialyzed against at least 200 volumes of borate buffer, Tris-HCl buffer, or 0.01 M acetic acid over a 72-h period, with three media changes at 4 °C. The samples were dialyzed against 0.01 M acetic acid or deionized H_2O, lyophilized, and stored at −20 °C until used. In experiments where more detailed studies were performed, fractions 1–20, 21, 31, and 40–45 were dialyzed in separate dialysis tubings. The high osmotic pressure generated by high salt concentrations in the dialysis tubings diluted the contents during dialysis. To correct for the change in volume for calculations of recovery, we noted the volume of each fraction after dialysis.

In subsequent experiments in which the binding of soluble preparation to apotransferrin-, lactoferrin-, and albumin-coupled to Sepharose 4B was studied, the unadsorbed protein and the washing and the eluted materials were collected and processed as described above. In some affinity chromatography experiments an experimental procedure similar to that described by Light (1978) was utilized.

Immunoprecipitation. The precipitation of specific proteins contained in the 100000*g* supernates of Triton X-100-extracted radiolabeled proteins by antitransferrin or anti-170000 antibodies was studied. Incubations were performed in 10 × 75 mm glass tubes. To investigate the membrane components associated with transferrin, we added unlabeled transferrin (2 μg) to radiolabeled soluble membrane components (200 μL containing 20 μg of protein) and the mixture was incubated for 10 min at 37 °C. Then antibodies to transferrin (final dilution 1:2000) were added, and the sample was incubated for 30 min at 37 °C. After 16 h at 4 °C, precipitates were sedimented by centrifugation at 3000*g* for 30 min. The supernates were removed and the precipitates were washed 3 times with 0.02 M borate buffer, pH 8.0, containing 0.15 M NaCl and 1% Triton X-100 (v/v). To investigate the membrane components precipitated by anti-170000, we incubated solublized radiolabeled membrane components (200 μL containing 20 μg of protein) with 5 μL of anti-170000 serum (final dilution 1:1000), and the samples were incubated for 18 h at 4 °C. Indirect immunoprecipitation was performed as indicated in other sections of this paper. Precipitation studies determined that 1% Triton X-100 had no effect on the amount of transferrin or 170000 glycoprotein precipitated by antitransferrin or anti-170000 antibodies, respectively. These data are in accordance with results from other laborabories (Sullivan & Weintraub, 1978). Optimal antigen–antibody ratios for both antitransferrin and anti-170000 antibodies were determined in the presence of 1% Triton X-100. The immunoprecipitates were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Other Procedures. Monolayer cultures were treated with trypsin (Worthington Biochemical Co., Freehold, NJ) as described by Carter & Hakomori (1978). $NaDodSO_4$ gel electrophoresis was performed following the basic procedure of Laemmli (1970). Radioactive proteins were detected by autoradiography (Fernandez-Pol, 1979) or by fluorography (Laskey & Mills, 1975). Protein standards for molecular weight estimation were myosin (200000), β-galactosidase (130000), phosphorylase B (94000), bovine serum albumin (63000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (21000), lysozyme (14300) (Bio-Rad, Richmond, CA). Protein was determined by the Bio-Rad method or by the procedure of Lowry et al. (1951) with a minor modification (Peterson, 1977) if the solution contained detergents. Cells were counted with a Coulter counter. Unless otherwise noted, the results reported here were reproduced in at least four separate experiments each.

Results

Preparation of Detergent-Extracted Membrane Proteins. Triton X-100 at a concentration of 0.5% (v/v) solubilized 50–60% of the total protein in the membrane-enriched fraction in 30–45 min when the protein concentration was 4 mg/mL; above this protein concentration, the efficiency of solubilization with this detergent concentration was reduced. Under the conditions employed (1–2 mg of protein/mL; 37 °C; 30 min), 0.5% $NaDodSO_4$ solubilized over 80% of the cell surface bound ^{125}I radioactivity; the radioactivity remained associated to nondialyzable macromolecular structures. The effect of Na-

$DodSO_4$ concentration on the efficiency of solubilization has not been examined. Because Triton X-100 provided the mildest and most effective means of solubilizing transferrin receptors for both affinity chromatography and immunological studies, we utilized this detergent in most of our experiments.

Detection of Receptor Activity by Poly(ethylene glycol) or Anti-170 000 Precipitation. A simple and convenient assay for transferrin binding activity based on the differential precipitation of free and bound transferrin by PEG was utilized. It was found that a final concentration of 12.5% PEG is sufficient to precipitate the transferrin-receptor complexes. Nonspecific binding remained relatively constant at concentrations of PEG from 10 to 16%. About 8–10% of the nonspecific binding was due to nonspecific precipitation of [^{125}I]transferrin. Therefore, a final concentration of 12.5% PEG was used for separating bound and free [^{125}I]transferrin in assays for soluble receptors.

An interaction between 170 000 glycoprotein and transferrin was demonstrated by indirect immunoprecipitation. Triton X-100-solubilized receptor was labeled with [^{125}I]transferrin and incubated with rabbit antiserum to 170 000 glycoprotein. The [^{125}I]transferrin–170 000 antibody complex was then precipitated with an antiserum to rabbit immunoglobulin G (IgG). When a 1000-fold excess of native transferrin was added to the incubation mixture to prevent receptor labeling, the radioactivity in the precipitate was decreased to ~10% of the total radioactivity added to the incubation mixture. No [^{125}I]transferrin-labeled 170 000 glycoprotein was precipitated when anti-170 000 serum was replaced by preimmune serum or buffer alone or when solubilized receptor was omitted from the incubation mixture. These results excluded the possibility that the specific precipitiation of [^{125}I]transferrin was due to antibodies to transferrin rather than to the 170 000 glycoprotein. Results of these and other experiments indicated that anti-170 000 serum specificially precipitates a [^{125}I]-transferrin–170 000 complex, suggesting that 170 000 is a component of the transferrin receptor. These findings appeared to justify the detection of transferrin binding activity by the utilization of antibodies against 170 000 glycoprotein.

The 170 000 molecular weight used in the description of our present results corresponds to the highest apparent molecular weight determined in a 5–12% exponential acrylamide gel in cells treated with desferrioxamine for 6 h. The apparent molecular weight of this protein after 24 h of treatment was ~160 000, as determined in a 5% acrylamide gel (Fernandez-Pol, 1979).

Binding Characteristics of the Detergent-Extracted Receptor. Typical competitive binding experiments of the Triton-extracted transferrin receptor, in which aliquots of Triton extract (20 μg of protien in 200 μL) were incubated with 20 nM [^{125}I]transferrin alone and in the presence of increasing concentrations (10^{-10}–10^{-5} M) of unlabeled transferrin, were done. Approximately 80% of the [^{125}I]transferrin that was bound to the Triton extract in the absence of unlabeled transferrin could be prevented from binding by the presence of 1×10^{-6} M transferrin; this percentage represents the specific bindng to the receptor. The corresponding Scatchard (1949) binding analysis of the competitive binding data showed that the equilibrium binding constant, calculated from the slope, is $\sim 6 \times 10^{-9}$ M. This K_a was identical in receptor preparations of both NRK cells grown in normal media or in iron-deficient media.

Purification of Transferrin Receptors by Affinity Chromatography. The binding of the soluble fraction of NRK cell membranes metabolically labeled with glycoprotein precursors to transferrin–Sepharose was studied to determine if any of the labeled membrane components had affinity for transferrin. Aliquots of the soluble fraction of ^{3}H-labeled amino acids or [^{14}C]glucosamine-labeled cell membranes were incubated in columns of transferrin–Sepharose. After incubation and elution as described under Materials and Methods, a peak of retarded material, representing less than 5% of the total radioactivity added, was found. Additional experiments demonstrated that the retarded fraction could be much more efficiently labeled with [^{14}C]proline since between 25–30% of the total radioactivity added appeared in the retarded fraction. These results were repeatedly confirmed by using membranes from NRK cells grown in normal media or from NRK cells subjected to iron deprivation. Results of these experiments suggested that the transferrin-bound Sepharose recognizes specific proline-rich membrane glycoproteins.

Figure 1A shows a typical elution profile for the purification of transferrin-binding proteins from a soluble preparation obtained by treatment of [^{14}C]proline-labeled NRK cell membrane proteins with 0.5% $NaDodSO_4$. Of the total radioactivity, 50% was adsorbed, and of this, 10% was recovered from the adsorbent by 2 M KSCN. No further radioactivity was eluted by additional 2 M KSCN or with 8 M urea. Further experiments were performed to determine if other conditions would be more suitable for eluting labeled membrane components from transferrin–Sepharose. It was found that 0.1 M acetic acid dissociated 20–30% of the radioactivity from the transferrin–Sepharose column and also preserved the activity of the receptor. Acetic acid, pH 3.1, was a convenient eluant since with this dissociating agent, the material could be directly lyophilized. However, it was found that acetic acid removes iron from transferrin. Thus, to perform experiments under rigorous conditions, we preferred 2 M KSCN as the eluant, since this agent did not remove iron from transferrin and yielded a good recovery of radioactivity. It is not clear what factors contribute to the low recovery (20–30%) of the radioactivity eluted with 2 M KSCN or 0.1 M acetic acid from the affinity column. The low recovery was not due to the presence of $NaDodSO_4$ in the samples because similar results were obtained with samples solubilized with Triton X-100. It was also found that the presence of 0.5% Triton X-100 in the elution buffer increased the yield of radioactivity by 10%. The receptor activity was found to be labile when exposed to 2.5 M $MgCl_2$; $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the adsorbed material eluted with 2.5 M $MgCl_2$ revealed radioactive polypeptides of M_r lower than 15 000. Other salts have not been tested.

Figure 1B shows a typical elution profile of a $NaDodSO_4$-solubilized receptor preparation from NRK cells subjected to iron deprivation that has been chromatographed successively through two transferrin–Sepharose gel steps. The first fractionation of the soluble receptor preparation on the transferrin gel is shown in Figure 1A. Subsequent chromatography of the material eluted with 2 M KSCN from the first column showed that this material rebinds specifically to the second column and yields a recovery of 15% of the initial material. To determine the protein and radioactivity profile of the eluted material, we performed $NaDodSO_4$–polyacrylamide gel electrophoretic analysis. Figure 2, lane d, shows that one major radioactive band of 20 000 daltons was observed in the material subjected to two cycles of affinity chromatography. Minor bands observed in the preparation demonstrated heterogeneity. Figure 2, lane f, shows that only one distinct band giving Coomassie blue stain was identified in the gel with M_r identical with that of radioactive 20 000 protein. A parallel two-cycle

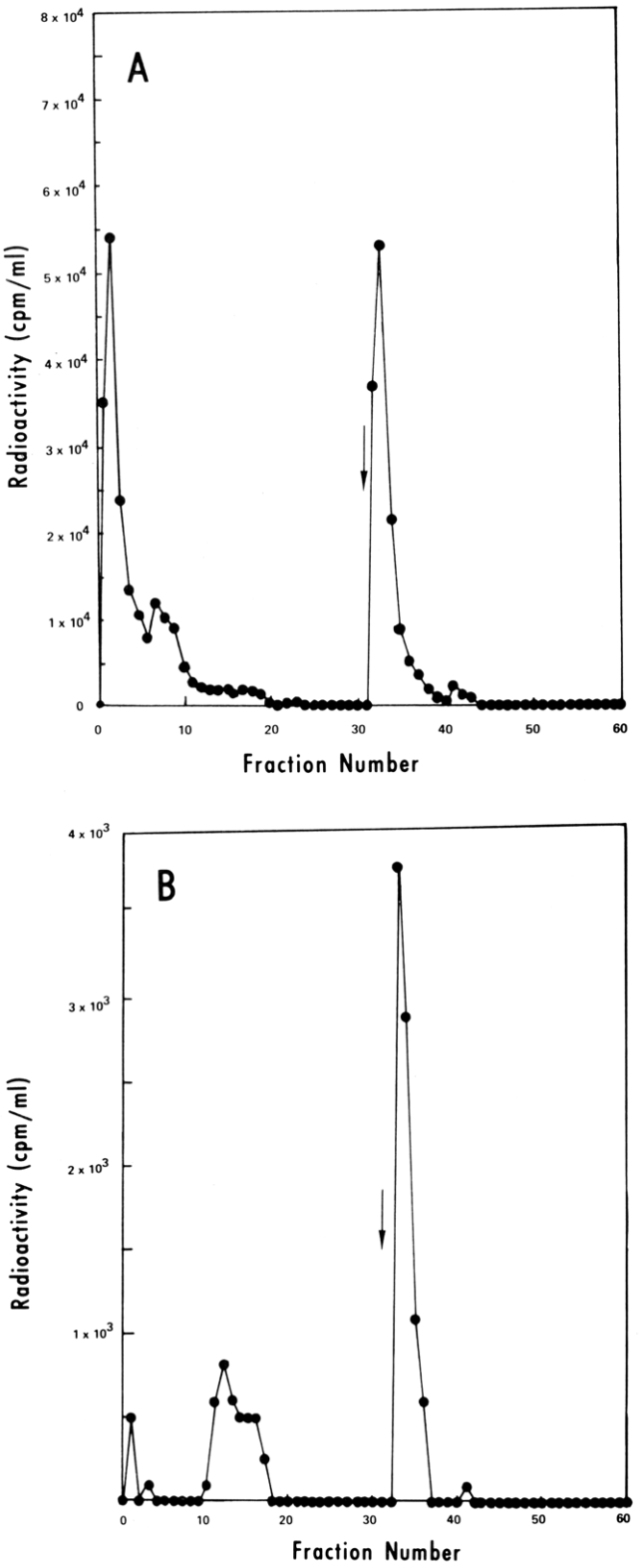

FIGURE 1: Affinity chromatography of $NaDodSO_4$-solubilized [^{14}C]proline-labeled NRK cell membranes on rat transferrin–Sepharose columns. (A) The column (0.8 × 3 cm) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.6, containing 0.5 M NaCl. Approximately 1.5 × 10^5 cpm of solubilized material was applied in 0.45 mL of the same buffer and was incubated for 1 h at 23 °C. The nonbound material was washed from the column with 0.05 M Tris-HCl buffer, pH 7.6, containing 0.5 M NaCl. After 30 mL of buffer had passed through the column, the adsorbed fraction was eluted with 2 M KSCN in PBS, pH 7.4 (arrow). The flow rate was regulated at ~20 mL/h. Approximately 1 mL of eluate was collected in each tube. (B) Adsorbed material obtained from the first column (A) was pooled, lyophilized, and chromatographed as described above.

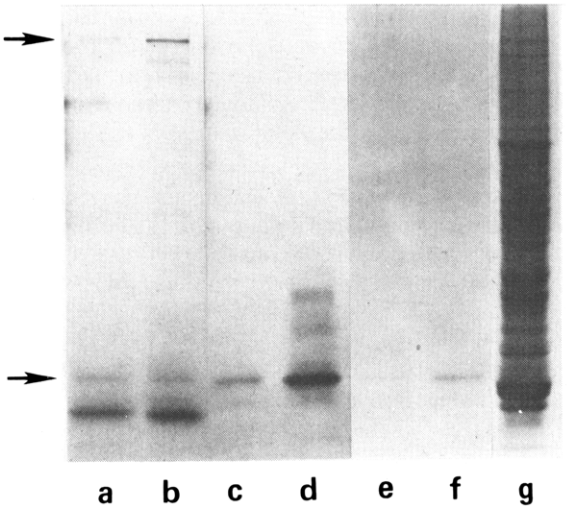

FIGURE 2: Radioactivity and protein patterns from $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of nonadsorbed and adsorbed fractions obtained by affinity chromatography of NRK cell membrane proteins. (a and b) ^{125}I-Labeled NRK cell membrane proteins solubilized with 0.5% Triton X-100 and subjected to one cycle of affinity chromatography in a rat transferrin–Sepharose column. (c and d) [^{14}C]Proline-labeled NRK cell membrane proteins solubilized with 0.5% $NaDodSO_4$ and subjected to two cycles of affinity chromatography in a rat transferrin–Sepharose column. (e and f) Corresponding Coomassie blue stained gel of lanes c and d, respectively. (a–f) Bound material; (g) protein stain of nonbound material obtained in the first cycle of affinity chromatography; (a, c, and e) cells cultured in normal medium; (b, d, f, and g) cells cultured in the presence of desferrioxamine. Conditions of sample preparation, electrophoresis, and fluorography described under Materials and Methods. The samples were analyzed in exponential 7–16% acrylamide gels. The positions of 170 000 (top arrow) and 20 000 (bottom arrow) proteins are indicated. Proteins used a standard (not shown) were as follows: myosin, M_r 200 000; β-galactosidase, M_r 130 000; phosphorylase B, M_r 94 000; albumin, M_r 68 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 000.

affinity chromatography experiment was performed with membranes from NRK cells grown in normal media. Figure 2, lanes c and e, shows that in this case only one major band of ~20 000 daltons was detected by autoradiography or protein stain. Similar results were obtained when [^{14}C]proline-labeled membrane protein was solubilized with Triton X-100.

The binding of ^{125}I-labeled membrane proteins to transferrin–Sepharose was studied to determine if one or more external cell surface proteins had affinity for transferrin. A soluble preparation of ^{125}I-labeled membrane proteins obtained by extraction of purified membrane proteins with 1% Triton X-100 was chromatographed on a transferrin–Sepharose column. Following washing of the column with 0.05 M Tris-HCl, pH 7.6, containing 0.5 M NaCl and 0.5% Triton X-100, the bound fraction was dissocated with 2 M KSCN. Both bound and unbound fractions were individually pooled, dialyzed, and prepared for $NaDodSO_4$–polyacrylamide gel electrophoretic analysis. Figure 2, lane a, shows that the adsorbed fraction of NRK cells grown in normal medium had components of about 170 000, 20 000 and 10 000 daltons as determined in a 7–16% exponential acrylamide gel. Figure 2, lane b, shows that a similar pattern was observed in NRK cells subjected to iron deprivation with the exception of one additional band of 140 000 daltons. By comparison of the pattern obtained with cells labeled with ^{125}I (Figure 2, lanes a and b) and labeled with [^{14}C]proline (Figure 2, lanes c and d), a protein of 20 000 daltons from cells grown in normal media or in iron-deficient media could be identified as common constituents of the specifically eluted materials. The comigration of those proteins labeled with two different isotopes

Table 1: Summary of Purification of Transferrin Receptors[a]

sample	total yield of protein (mg)	[^{125}I]transferrin binding capacity (pmol of transferrin/mg of protein)	purifn (x-fold)	total [^{125}I]transferrin binding capacity recovered	
				pmol	%
homogenate	42.20	1.24	1	52	100
crude membrane fraction in Triton X-100	1.02	20.73	16.7	21	40
Triton X-100 extract after ultracentrifugation	0.6105	35.09	28.3	21	40
partially purified receptor (one cycle of affinity gel)					
A	0.009	1502	1211	13	25
B	(0.007)	(1460)	(1352)	(10)	(23)

[a] Data represent summary of purification of the transferrin receptor of NRK cells. 1.5 g of cells (net weight) was used as the starting material to obtain preparation A and 1.8 g was used to obtain preparation B (in parentheses). Material obtained from 6 mL of the 1% Triton X-100-solubilized preparation was chromatographed as described in Figure 1. Detection of transferrin receptor activity was performed by utilizing anti-170 000 antibodies as described under Materials and Methods. Preparation A was obtained from cells cultured in normal media and preparation B was obtained from cells cultured in the presence of desferrioxamine.

suggests identity. These results indicated that transferrin-bound Sepharose recognizes specific cell surface proteins.

Figure 3A shows a typical elution profile when a soluble preparation of the transferrin receptor, obtained by treatment of purified cell membranes with 1% Triton X-100, was chromatographed on the transferrin–Sepharose gel. While ~90% of the total receptor activity was specifically bound, more than 90% of the total protein was unretarded by the column. Following washing of the column with 0.05 M Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.5% Triton X-100, more than 45% of the adsorbed receptor activity was eluted by addition of 2 M KSCN to the column. Figure 3B shows that similar results were obtained in a parallel experiment performed with solubilized membranes from NRK cells subjected to the iron deprivation. Subsequent experiments showed that the material eluted from the first column rebinds specifically to a second column and yields a preparation containing receptor activity. These experiments demonstrated that the receptors adsorbed by the transferrin–Sepharose gel retain receptor activity after elution from the column.

Table I summarizes the data of the purification of receptor from NRK cells grown in normal media (preparation A). Values in parentheses represent data obtained from a preparation of receptors purified from cells grown in iron-deficient media (preparation B). By combining membrane separation, detergent extraction, and affinity chromatography, we purified the transferrin receptor 1200–1400-fold. The 40–50-fold increase in specific activity over that of its starting material shows the effectiveness of the affinity chromatography step. The final yield, determined by comparing the total capacity to bind transferrin, was ~25%.

Properties of Partially Purified Soluble Receptors. Experiments were performed to determine whether the interaction of soluble receptors with immobilized transferrin was on the basis of biospecific affinity interactions. First, the nonderivatized support did not have the ability of retaining solubilized membrane proteins specifically. Second, when bovine serum albumin coupled Sepharose gel was used as the adsorbent, no receptor activity was retained. Third, the presence of bovine serum albumin in the buffer system of columns of transferrin–Sepharose did not lead to an increase in yield of radioactivity or receptor activity. Fourth, when lactoferrin–Sepharose was utilized as the ligand, no receptor activity was retained and close to 98% of the [^{14}C]proline-labeled material applied to the column was recovered. Fifth, by preincubating the soluble preparations with transferrin, it was possible to demonstrate that transferrin partially blocked the retention of receptors by apotransferrin–Sepharose columns. Finally,

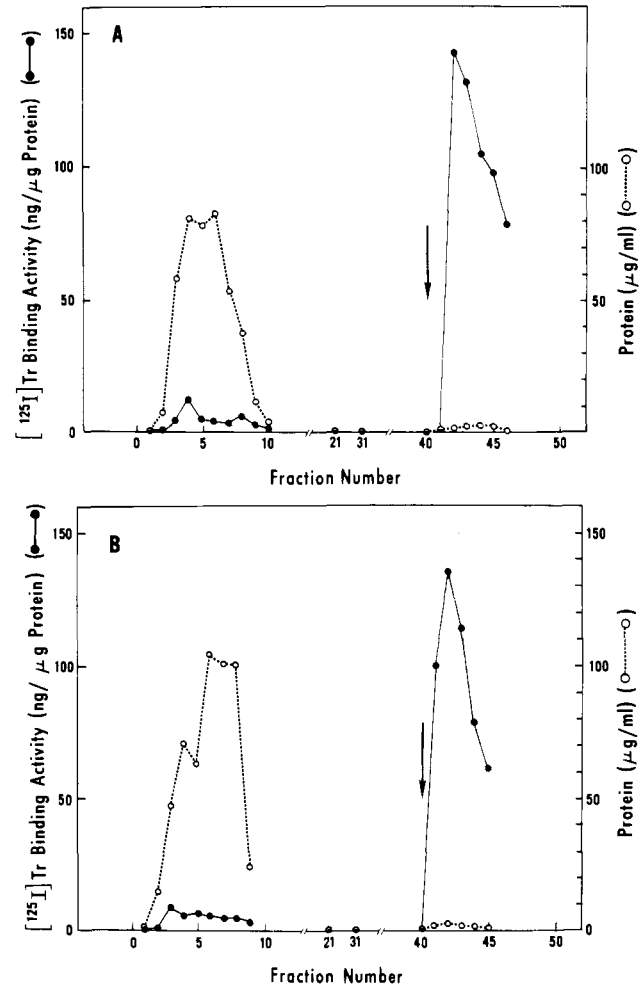

FIGURE 3: Chromatography of solubilized NRK cell membranes on Sepharose 4B–rat transferrin. In the experiments shown 6 mL of 1% Triton-solubilized preparation [(A) 600 μg/mL; (B) 560 μg/mL] were chromatographed on a 5-mL column of Sepharose 4B–rat transferrin gel equilibrated with 0.5% Triton X-100, 0.5 M NaCl, and 0.05 M Tris-HCl, pH 7.4. After loading the soluble preparation at 23 °C at a rate of 8–10 mL/h, we washed the column at a rate of 20 mL/h with equilbration buffer. Receptor activity was eluted at room temperature with 2 M KSCN in PBS, pH 7.4. Eluted fractions (1 mL) were prepared for determination of receptor activity by the immunoprecipitation procedure as described under Materials and Methods. The highest concentration of protein in the eluted fractions was less than 2.5 μg/mL. (A) Membranes obtained from control cells; (B) membranes obtained from cells treated with desferrioxamine.

Figure 4 shows the results of a typical experiment in which the peak of unbound radioactivity was followed by a smaller

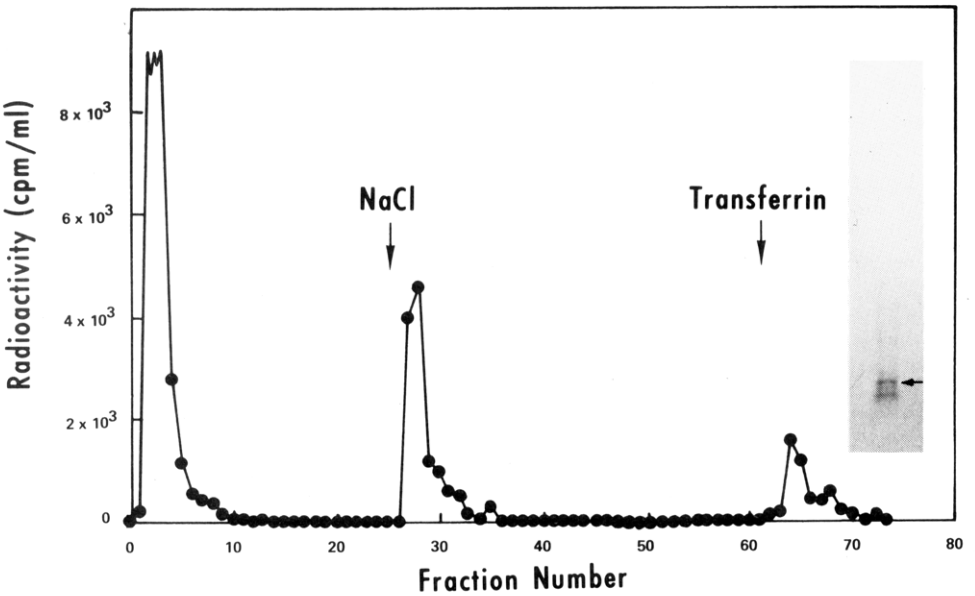

FIGURE 4: Affinity chromatography of Triton X-100-solubilized [^{14}C]proline-labeled NRK cell membranes on a Sepharose 4B–rat transferrin column. In the experiment shown, 0.5 mL of 1% Triton X-100-solubilized preparation (1.5×10^5 cpm/mL) was chromatographed on a 2-mL column of Sepharose 4B–rat transferrin gel equilibrated with 54 mM borate buffer, pH 7.4, containing 0.5% Triton X-100. After loading the soluble preparation at 23 °C and washing with equilibration buffer, we eluted the column with 0.3 M NaCl in borate buffer, pH 7.4, containing 0.5% Triton X-100. Receptor activity was biospecifically eluted at room temperature after 16 h of incubation with 5 mg/mL human transferrin solution in borate buffer, pH 7.4, containing 0.5% Triton X-100 and 0.3 M NaCl. Inset: Autoradiograph of the eluted fraction after $NaDodSO_4$–polyacrylamide gel electrophoresis on a 7–16% exponential gradient gel. The position of 20 000 is indicated (arrow).

peak of nonspecifically bound material eluted with 54 mM borate buffer, pH 7.4, containing 0.5% Triton X-100 and 0.3 M NaCl. The third peak was eluted with a 5 mg/mL human transferrin solution. This demonstrated that the elution of specifically bound material from a transferrin–Sepharose column could be obtained by incubating the column with transferrin. Figure 4, inset, shows that one major radioactive band of 20 000 daltons was observed in the material eluted from the column. Minor bands observed in the preparation demonstrated heterogeneity. Taken together, these results indicate that the solubilized transferrin receptors are specifically adsorbed by the transferrin–Sepharose gels.

No significant differences were found in affinity experiments performed with human or rat transferrin–Sepharose columns. It is worth noting that our preparations are quite stable. The solubilized receptor activity was stable for at least 6 months at −20 °C. The most purified preparations were also quite stable to freeze–thawing, lyophilization, or dialysis.

Immunoprecipitation of Membrane Components Associated with Transferrin. To determine whether any of the membrane proteins interact with transferrin, we have compared immunoprecipitates formed by incubating extracts of ^{125}I-labeled membranes with either antitransferrin or anti-170 000 antibodies. When we incubated an ^{125}I-labeled membrane extract, containing added transferrin, with antiserum against transferrin and analyzed the resulting immunoprecipitate on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5a,b), we detected a major protein band of ~170 000 daltons. This protein comigrates with a protein of 170 000 dalton found by affinity chromatography (Figure 2, lanes a and b). When we incubated an ^{125}I-labeled membranes extract with antibodies against the "iron-regulated" 170 000 glycoprotein, we detected a major protein band of M_r 170 000 (Figure 5c,d) that comigrates with the protein of M_r 170 000 found in membranes immunoprecipitated by antitransferrin. In membranes extracted from NRK cells exposed to trypsin, antitransferrin or anti-170 000 antibodies did not precipitate the 170 000 protein. This protein was not immunoprecipitated when a preimmune serum was used. If the protein precipitated with antitransferrin

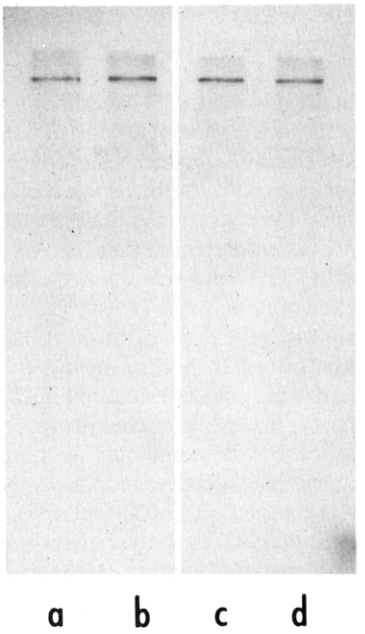

FIGURE 5: Immunoprecipitation of iodinated membrane proteins by antitransferrin (a and b) or anti-170 000 (c and d) antibodies. Autoradiographs after $NaDodSO_4$–polyacrylamide gel electrophoresis on a 7–16% exponential gradient gel. Lanes a and c: cell membranes obtained from control cultures. Lanes b and d: cell membranes obtained from desferrioxamine-treated cultures. Conditions for iodination of cells, solubilization of membrane proteins, immunoprecipitation, and electrophoresis are described in the text and in the legend to Figure 2.

antibodies is identical with that precipitated by anti-170 000 antibodies, then our data suggests that the iron-regulated 170 000 protein interacts with transferrin and a transferrin–170 000 complex is precipitated by antitransferrin antibodies. Evidence that this may be the case was provided by the fact that anti-170 000 antibodies specifically precipitated [^{125}I]-transferrin. Results of additional experiments showed that material subjected to two cycles of affinity chromatography (Figure 2c,d) contained a 20 000 [^{14}C]proline-labeled protein

which could be specifically immunoprecipitated by anti-170 000 antibodies.

Discussion

In the present study we have identified by affinity chromatography and immunological techniques one NRK cell membrane-associated polypeptide of subunit M_r 170 000 that appears to bind specifically to transferrin. This protein is localized on the external surface of the cell as indicated by previous (Fernandez-Pol, 1979) and present findings that the protein can be iodinated by the [^{125}I]lactoperoxidase procedure, that it is sensitive to trypsinization, and that antibodies against 170 000 localized on the cell surface. Additonally, previous results showed that the production of 170 000 can be experimentally regulated by intracellular iron concentrations (Fernandez-Pol, 1979, 1980). Preliminary data suggesting a possible interaction of 170 000 with transferrin have also been presented (Fernandez-Pol, 1979). The present studies provide further information on transferrin and 170 000 binding and on the solubilized transferrin receptor as it may exist in the plasma membrane of NRK cells. The most important conclusion that can be drawn from the present data is that 170 000 is a membrane-associated protein which appears to interact with transferrin in specific fashion, suggesting that it is a functional or structural subunit of the transferrin-receptor complex.

Data presented on the solubilization of the receptors revealed a number of interesting points. The most important conclusion that can be drawn from the data is that the transferrin receptor is an intrinsic external membrane protein. Since solubilized membrane proteins of cells exposed to trypsin do not bind transferrin, transferrin receptors must be located on the external surface of the cell membrane and they must have protein moieties as essential components. That the transferrin receptor is embedded in the membrane bilayer is indicated by the requirement of detergent to solubilize the protein. In our studies we have noted that in the absence of detergent, the extracted receptor protein tends to aggregate and precipitate, a property typical of an intrinsic membrane protein.

The high affinity and great specificity of ligand–receptor interactions made affinity chromatography an attractive method for purification of transferrin receptors. Although it is difficult to exclude electrostatic interactions, as opposed to biospecific ones, and harsh nonbiospecific conditions have been necessary to elute bound receptor protein, the results of a number of experiments suggested specificity. Indeed, the interaction of soluble receptors with the immobilized ligand appears to be solely on the basis of biospecific affinity interactions. By incubating the soluble preparations in a transferrin–Sepharose column and eluting with transferrin, it was possible to demonstrate that retention of transferrin receptors in transferrin–Sepharose columns has the characteristics expected for those of a transferrin-receptor process. By utilizing other ligands bound to Sepharose, such as albumin and lactoferrin, it was possible to demonstrate that retention of receptor by these gels does not occur with these substances. One unexpected finding was the binding of 170 000 and transferrin receptor activity to apotransferrin–Sepharose, since it might be expected that apotransferrin will not bind transferrin receptors. Yet, we do not have an estimate on the relative affinity of transferrin and apotransferrin to the receptors or to the 170 000 protein. Therefore, it may be that there is no difference between the binding of transferrin and apotransferrin to the receptor, that the selectivity of the immobilized ligand has been reduced considerably, or both. Thus, the findings reported here appear to be closely representative of true affinity chromatography as evidenced by the specificity of adsorption and elution.

On subjecting the receptor partially purified by affinity chromatography to $NaDodSO_4$–polyacrylamide gel electrophoretic analysis, more than one band of radioactivity was observed. It is not clear at present which band, if any, corresponds to the transferrin receptor activity. The most important conclusion that can be drawn from the present data is that transferrin receptor preparations subjected to one cycle of affinity chromatography contained major components of M_r 170 000, 20 000 and 10 000. In the final product obtained by two cycles of affinity chromatography only one major protein of 20 000 was found. Since antibodies against 170 000 protein specifically precipitated the 20 000 protein, this polypeptide appears to be a degradation product of 170 000. A less attractive possiblity is that the 170 000 protein is composed of a combination of 20 000 subunits. These results are consistent with the possibility that the 170 000 protein is a component of the transferrin-receptor complex.

That the 170 000 protein is part of the ligand for transferrin in the preparations and not an impurity of the preparation is suggested by several lines of evidence. One ^{125}I-labeled membrane protein of M_r 170 000 appears to be closely linked to transferrin, since it is precipitated by antitransferrin antibodies. This protein comigrates with 170 000 protein precipitated by anti-170 000 antibodies, and these antibodies can precipitate transferrin. These results indicate that transferrin binds to 170 000 and the complex transferrin–170 000 is precipitated. These studies are in accordance with affinity chromatography studies which showed that the ^{125}I-labeled NRK membrane proteins from transferrin–Sepharose affinity columns also contain a 170 000 glycoprotein with a mobility identical with that of 170 000 obtained by immunoprecipitaton. Futhermore, the lack of ability of antitransferrin or anti-170 000 serum to specifically precipitate 170 000 membane protein from solubilized membranes of NRK cells subjected to trypsinization parallels the ability of the solubilized membrane preparations to bind specifically transferrin. Thus, the results of immunoprecipitation studies further support the concept that 170 000 and transferrin interact in a specific fashion.

Review of the current literature indicates that transferrin receptors have been apparently identified in reticulocytes (Aisen & Leibman, 1977; Ecarot-Charrier et al., 1977; Light, 1978; Sly et al., 1978; Sullivan & Weintraub, 1978; Witt & Woodworth, 1978; Hu & Aisen, 1978), placenta (Seligman et al., 1979), and other tissues (Bottenstein et al., 1979; Fernandez-Pol et al., 1979; Hemmaplardh & Morgan, 1974; Larrick & Cresswell, 1979; Hamilton et al., 1979). Of the membrane receptors for transferrin, few have been as intensively studied over the past several years as the transferrin receptors of reticulocytes. It has been estimated that as much as 6% of the total membrane protein of a reticulocyte bearing 125 000 transferrin receptors of M_r 175 000 could consist of the specific transferrin receptor (Hu & Aisen, 1978). Aisen et al. analzyed transferrin-receptor complexes from reticulocytes and suggested a subunit composition for the receptor of 95 000 and 165 000 (Aisen & Leibman, 1977; Hu & Aisen, 1978). Witt & Woodworth (1978) found a 190 000 dalton membrane protein in rabbit reticulocytes and suggested that this protein is the primary transferrin receptor. Sullivan & Weintraub (1978) used antibodies to rat transferrin to precipitate transferrin–receptor complexes from ^{125}I-labeled reticulocytes and reported the identification of transferrin-

binding proteins of M_r 95000 and 145000. In contrast, Light (1978) found that a cell surface glycoprotein of M_r 18000 of the rabbit bone marrow erythroid cells may be the transferrin receptor. Results from another laboratory further show that little agreement has prevailed on the molecular characterization of the transferrin receptor of erythroid cells (Sly et al., 1978). Seligman et al. (1979) identified a placental transferrin receptor of M_r 150000 which dissociated into 90000, 60000, and 30000 components. Finally, Hamilton et al. (1979) identified a single polypeptide of 90000 daltons as a subunit of a putative transferrin receptor of human cultured cells. As further information about transferrin receptors become available, the similarities and differences between individual cells should be clearer. The possible relevance and connection of these studies with the studies presented here remains to be determined.

We have previously suggested a possible role of 170000 of a subset of this protein as a component of the transferrin receptor because it is a membrane protein exposed to the external environment and because one might expect that the receptor complex for transferrin is regulated by iron availability (Fernandez-Pol, 1979). The fact that the M_r of 170000 can be experimentally regulated by iron availability (Fernandez-Pol, 1980) may have physiological significance in regulating the binding of transferrin to the cells. However, the possibility exists that the 170000 protein in a structural component of the transferrin receptor rather than the protein responsible for specific binding of transferrin. Our previous data suggest that 170000, a protein mobile in the plane of the membrane, interacts with transferrin and the complex transferrin–170000 is internalized (Fernandez-Pol, 1979). The process of receptor-mediated internalization is recognized as an important and general mechanism by which animal cells take up regulatory proteins from the extracellular fluid (Goldstein et al., 1979). Internalization of transferrin appears to be essential for iron delivery to the cells (Sullivan & Weintraub, 1978). Thus, the mechanism by which transferrin is internalized may involve the binding of transferrin to 170000, subsequent internalization of the complex, and delivery of Fe to specific intracellular receptor sites (e.g., mitochondria). Further studies will be necessary to confirm these speculations.

In conclusion, the present findings show that solubilized NRK cell membrane proteins contain two major proteins of subunit M_r of 170000 and 20000, the latter presumably a degradation product of 170000, that have affinity for transferrin. The present study, however, does not demonstrate whether or not the 170000 or the 20000 proteins are directly responsible for transferrin receptor binding activity in solubilized receptor preparations. Furthermore, the possibility exists that structures other than receptors can potentially bind transferrin and that many nonreceptor materials may bind transferrin in a manner similar to true receptor binding. Although transferrin binding activity is not found in the absence of the 170000 or 20000 proteins, their presence in our preparations may be a fortuitous coincidence of events. Finally, the results presented in this paper, considered in conjunction with previous reports, provide biochemical insight into interactions between transferrin and specific membrane proteins which may facilitate studies on the biochemical, physiological, and immunological properties of the transferrin receptors in mammalian cells.

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