Synthesis of the Transferrin Receptor by Cultures of Embryonic Chicken Spinal Neurons

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ABSTRACT   We have purified a glycoprotein from chicken sciatic nerves, sciatin, which has pronounced trophic effects on avian skeletal muscle cells in culture. Recent studies have shown that sciatin is identical to the iron-transport protein, transferrin, in terms of its physicochemical structure, immunological reactivity, and biological activity. To determine whether transferrin is synthesized and released by neuronal tissue, we incubated cultures of dissociated chicken spinal neurons in a medium free of L-leucine containing either L-^{3}H-amino acids or L-[^{14}C]leucine and immunoprecipitated transferrin with highly specific antibodies. The radiolabeled protein precipitated by rabbit heteroclonal, goat heteroclonal, or mouse monoclonal antitransferrin antibodies increased in specific activity in a linear manner for at least 30 min. Synthesis of this protein was abolished by the presence of puromycin (20 µg/ml) or cycloheximide (10^{-5} M). The disappearance of the radiolabeled protein from cells was linear with a half-life ($t_{1/2}$) of 8-10 h. When immunoprecipitates were separated by SDS gel electrophoresis, a prominent band corresponding to transferrin (M_r 84,000) was visualized by staining with Coomassie Blue. However, when such gels were fluorographed, no radioactivity was apparent in the transferrin region of the gel although a prominent radioactive band was visualized at an M_r of 56,000. The protein of M_r 56,000 was not simply a degradation product of transferrin because this particular protein band was not generated by incubating radiolabeled transferrin with unlabeled neuronal homogenates. The protein of M_r 56,000 was purified from embryonic chicken brain and spinal cord by immunoabsorption chromatography on mouse monoclonal antitransferrin IgG conjugated to Sepharose 4B followed by affinity chromatography on immobilized transferrin. The purified protein bound radioiodinated transferrin and was precipitated by rabbit anti-chicken transferrin-receptor antibodies. Furthermore, this receptor protein was found to be localized on the plasma membrane of dorsal root ganglion neurons by immunocytochemistry using the peroxidase–antiperoxidase technique, and by blocking experiments, which showed that antitransferrin receptor IgG could inhibit the binding of fluorescein-conjugated transferrin at 4°C to cultured neurons in vitro. From these data, we conclude that transferrin is not synthesized by cultures of chicken spinal cord neurons, but that the receptor for transferrin is synthesized by these cultures and is precipitated by antitransferrin antibodies as an antigen–receptor complex.

This laboratory has purified an acidic, myotrophic glycoprotein, sciatin, from the sciatic nerves of adult chickens (1). Sciatin has been shown to promote the morphological maturation and maintenance of chicken muscle cells in culture (2), to increase protein synthesis in postmitotic myotubes (3), to promote and maintain the aggregation of acetylcholine receptors into clusters (4), and to be an essential component for the initial differentiation of cultured chicken myogenic cells

in vitro (5). Furthermore, immunocytochemical studies showed that sciatin is localized in the perikarya of spinal neurons and the axoplasm of sciatic nerves in the chicken (6). Recent studies have shown that sciatin is identical to the iron-transport protein, transferrin, in terms of its physicochemical structure, immunological reactivity, and biological activity (7, 8).

In view of the numerous myotrophic effects of transferrin upon skeletal muscle, and the fact that the protein is so well localized in the cell bodies and processes of spinal neurons, we anticipated that this protein might represent a myotrophic substance synthesized and secreted by neurons. In fact, a recent report by Stamatos et al. (9) demonstrated that transferrin is indeed synthesized and released by embryonic chicken spinal cord neurons in vitro (see also reference 10). Thus, we decided to investigate further the synthesis and release of transferrin by cultured embryonic chicken neuronal tissue. In the present communication, we report the following: (a) a protein precipitable by antitransferrin antibodies is synthesized in neuronal cultures exposed to radioactive amino acids; (b) this labeled protein migrates at an M_r of 56,000 on SDS gels and is not transferrin or a degradation product of transferrin; (c) this 56,000-M_r protein appears to represent a receptor or binding protein for transferrin that is immunoprecipitated by antitransferrin antibodies as part of a transferrin/transferrin receptor complex; and (d) this 56,000-M_r protein is present on the surface of neurons as evidenced by its immunocytochemical localization with rabbit anti-chicken transferrin-receptor antibodies, and by blocking studies with antireceptor IgG that showed that pretreatment of cells with antireceptor IgG could inhibit the surface binding of fluorescein-conjugated transferrin to living neurons.

MATERIALS AND METHODS

Cell Culture: Spinal cord tissue from 10-d-old chicken embryos (Spafas, Inc., Norwich, CT) was dissociated in 0.2% trypsin and plated on collagen-coated Linbro plastic dishes (35 × 15 mm; FB-6-TC; Flow Laboratories, Inc., McLean, VA) at a density of 3 × 10⁶ cells/dish. Dorsal root ganglia tissue from 12-d-old chicken embryos was dissociated and plated in an identical fashion at a density of 2.0–5.0 × 10⁵ cells/dish (6). The standard culture medium consisted of 80% Dulbecco's modified Eagle's medium (DME[1]; high glucose; Gibco Laboratories, Grand Island, NY), 15% horse serum (North American Biologicals, Inc., Miami, FL; heat-inactivated and Millipore-filtered), and 5% chicken embryo extract (11). Cytosine arabinoside (10⁻⁵ M) was present in the medium for the first 3 d of culture to kill rapidly proliferating non-neuronal cells. Cultures were maintained at 36.5°C in a humidified atmosphere of 95% air/5% CO_2. The culture medium was changed every third day and no antibiotics were used.

Preparation of Monoclonal Antibodies: Monoclonal antibodies against chicken transferrin were produced using a modification of the procedure of Kenneth et al. (12). Balb/C female mice (8-wk-old; Jackson Laboratories, Bar Harbor, ME) were immunized by subcutaneous injection with 50–100 μg of purified transferrin on days 0, 7, and 14. The first injection was in the presence of complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and later injections were in the presence of incomplete Freund's adjuvant. On day 21, transferrin was injected intravenously and the spleens were removed on day 24. Spleen cells were harvested by grinding the organs between the frosted surfaces of microscopic slides, and these cells (10⁸) were fused with mouse myeloma cells (SP 2/0-Ag 14, 10⁸ cells) in the presence of 50% polyethylene glycol 1000 (J. T. Baker Chemical Co., Brick Town, NJ). Cells were suspended in DME containing 20% fetal bovine serum (Gibco Laboratories), distributed into Costar 96-well plates (Cambridge, MA) and incubated overnight at 37°C

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[1] *Abbreviations used in this paper:* APB, antiprotease buffer; DME, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; FITC, fluorescein isothiocyanate; PAP, peroxidase–antiperoxidase; TCA, trichloroacetic acid; Tf, transferrin; TfR, transferrin receptor.
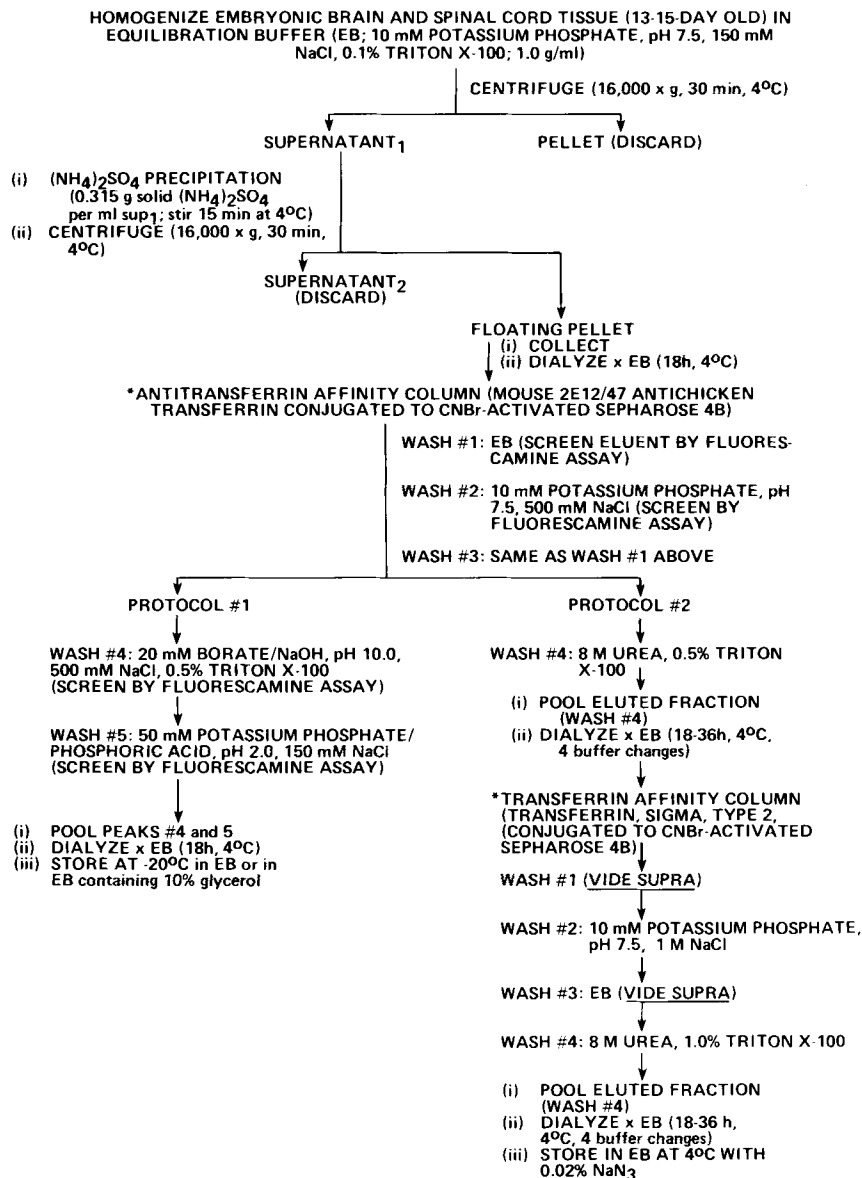
in a humidified atmosphere of 90% air/10% CO_2. The culture medium was replaced on days 1, 3, and 5 with HAT medium (DME containing 20% fetal bovine serum, 10% NCTC 135, 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine), and on days 7, 9, and 11 with HT medium (DME containing 20% fetal bovine serum, 10% NCTC 135, 100 μM hypoxanthine, and 16 μM thymidine). On days 12–15, visible clones were noted and the supernatants were tested for antibody to transferrin by an enzyme-linked immunosorbent assay established in this laboratory (Cha, C. Y., T. H. Oh, S. H. Shim and G. J. Markelonis, unpublished data). Positive clones were grown on 35-mm dishes and established as ascites tumors in Balb/C female mice which had been sensitized with Pristane (Aldrich Chemical Co., Metuchen, NJ). The ascites fluid from clone 2E12/47 had a titer of 1:250,000 against chicken transferrin and was therefore used in these experiments.

Immunocytochemical Procedure: Spinal cord cultures were rinsed with PBS and fixed in either absolute methanol for 30 min or 4% paraformaldehyde–0.1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2 for 1 h. Transferrin was visualized immunocytochemically on methanol-fixed cultures by the peroxidase–antiperoxidase (PAP) procedure of Sternberger (13) using rabbit anti-chicken transferrin serum (diluted 1:100 with PBS) as described previously (6). Preimmune rabbit serum was used as a control. The transferrin receptor was visualized on aldehyde-fixed cultures by the PAP procedure using rabbit anti-chicken transferrin receptor IgG (14) at a concentration of 100 μg/ml in PBS. This antibody was prepared against transferrin receptors purified from embryonic chicken erythrocytes. This IgG fraction was shown to agglutinate embryonic chicken reticulocytes, a process which could be inhibited by preincubation with the $F_{ab'}$ fragments of this IgG, to cause complement-mediated red cell lysis and to inhibit the uptake of [⁵⁵Fe]transferrin by these cells (14). Preimmune rabbit IgG was used as a control.

Isotopic Labeling of Cultures: Newly synthesized proteins in 7-d-old cultures were routinely labeled in the presence of 10 μCi L-³H-amino acids (New England Nuclear, Boston, MA; NET-250; approximate specific activity, 7.7 Ci/mmol) in L-leucine-free DME (custom product; KC Biologicals, Inc., Lenexa, KS) or, on some occasions, in 10 μCi L-[¹⁴C]leucine (New England Nuclear; 40 Ci/mmol) in the same medium. To inhibit protein synthesis, puromycin (20 μg/ml [15]) or cycloheximide (10⁻⁵ M [16]) was added 4 h or 1 h, respectively, before addition of isotope. The labeling reaction was stopped by washing cultures thrice with 1.0-ml portions of an antiprotease buffer (APB) consisting of 10 mM sodium phosphate, pH 7.2, 5 mM L-leucine, 10 μg/ml leupeptin, 0.5 trypsin-inhibitor U/ml aprotinin, and 1 mM EGTA. On certain occasions, APB was further supplemented with 5 mM phenylmethylsulfonyl fluoride, but this protease inhibitor had no recognizable effect on the results obtained with routine APB. The cells were then scraped from the dish and disrupted in 1.0 ml of APB in a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The supernatant obtained after centrifuging at 1,000 g for 20 min was utilized in immunoprecipitation experiments.

Immunoprecipitation: The post-1,000 g supernatant obtained above was mixed with an equal volume of either rabbit heteroclonal anti-chicken transferrin serum (final titer, 1:4 [6]), goat heteroclonal anti-chicken transferrin serum (final titer, 1:4) or mouse monoclonal anti-chicken transferrin antibody (2E12/47 ascites fluid; final titer, 1:10,000) in PBS. Control experiments showed that these antibody dilutions all gave quantitative immunoprecipitation of radiolabeled transferrin. After incubation overnight at 4°C, the immune complexes present were precipitated by centrifuging at 1,000 g for 20 min. For quantitative experiments, the pelleted antigen–antibody complexes were precipitated with 1.0 ml of 10% trichloroacetic acid (TCA), collected by centrifuging at 1,000 g, dissolved in 1.0 N NaOH and counted in 10 ml of Aquasol (New England Nuclear) acidified with an appropriate quantity of 10% TCA to clarify the sample.

Electrophoresis and Fluorography: For electrophoresis experiments, immunoprecipitates labeled with L-[¹⁴C]leucine were dissolved in a buffer consisting of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2% SDS, 5% 2-mercaptoethanol, 4 M urea, and 25% sucrose at 100°C for 10 min. The immunoprecipitates were separated on 12.6% SDS gel electrophoresis, and the gel proteins were stained with Coomassie Blue and destained as previously described (17; also see reference 2). The dehydrated gels were rehydrated with 1% glycerol in 10% acetic acid for 2 h and dried using a Bio-Rad gel dryer (Bio-Rad Laboratories, Richmond, CA). The dried gels were placed in contact with Kodak XAR-5 film in a Kodak X-Omatic cassette (Eastman Kodak Co., Rochester, NY), and the fluorographic film was exposed for 14 d at 4°C. The exposed film was developed in Kodak GBX developer by the standard method.

Transferrin Radiolabeling: Transferrin (Tf) was purified from either chicken serum or chicken sciatic nerves by affinity chromatography on concanavalin A-agarose followed by ion exchange on DEAE cellulose as previously described (7), or was obtained commerically (Sigma Chemical Co., St. Louis, MO). The protein was labeled by reductive methylation as described by Tack et al. (18). The protein (3 nmol) was methylated in the presence of 1 μmol

HOMOGENIZE EMBRYONIC BRAIN AND SPINAL CORD TISSUE (13-15-DAY OLD) IN
EQUILIBRATION BUFFER (EB; 10 mM POTASSIUM PHOSPHATE, pH 7.5, 150 mM
NaCl, 0.1% TRITON X-100; 1.0 g/ml)

CENTRIFUGE (16,000 x g, 30 min, 4°C)

SUPERNATANT₁ PELLET (DISCARD)

(i) (NH₄)₂SO₄ PRECIPITATION
    (0.315 g solid (NH₄)₂SO₄
    per ml sup₁; stir 15 min at 4°C)
(ii) CENTRIFUGE (16,000 x g, 30 min,
    4°C)

SUPERNATANT₂
(DISCARD)

FLOATING PELLET
    (i) COLLECT
    (ii) DIALYZE x EB (18h, 4°C)

*ANTITRANSFERRIN AFFINITY COLUMN (MOUSE 2E12/47 ANTICHICKEN
TRANSFERRIN CONJUGATED TO CNBr-ACTIVATED SEPHAROSE 4B)

WASH #1: EB (SCREEN ELUENT BY FLUORES-
CAMINE ASSAY)

WASH #2: 10 mM POTASSIUM PHOSPHATE, pH
7.5, 500 mM NaCl (SCREEN BY
FLUORESCAMINE ASSAY)

WASH #3: SAME AS WASH #1 ABOVE

PROTOCOL #1

WASH #4: 20 mM BORATE/NaOH, pH 10.0,
500 mM NaCl, 0.5% TRITON X-100
(SCREEN BY FLUORESCAMINE ASSAY)

WASH #5: 50 mM POTASSIUM PHOSPHATE/
PHOSPHORIC ACID, pH 2.0, 150 mM NaCl
(SCREEN BY FLUORESCAMINE ASSAY)

(i) POOL PEAKS #4 and 5
(ii) DIALYZE x EB (18h, 4°C)
(iii) STORE AT -20°C in EB or in
EB containing 10% glycerol

PROTOCOL #2

WASH #4: 8 M UREA, 0.5% TRITON
X-100

(i) POOL ELUTED FRACTION
(WASH #4)
(ii) DIALYZE x EB (18-36h, 4°C,
4 buffer changes)

*TRANSFERRIN AFFINITY COLUMN
(TRANSFERRIN, SIGMA, TYPE 2,
(CONJUGATED TO CNBr-ACTIVATED
SEPHAROSE 4B)

WASH #1 (VIDE SUPRA)

WASH #2: 10 mM POTASSIUM PHOSPHATE,
pH 7.5, 1 M NaCl

WASH #3: EB (VIDE SUPRA)

WASH #4: 8 M UREA, 1.0% TRITON X-100

(i) POOL ELUTED FRACTION
(WASH #4)
(ii) DIALYZE x EB (18-36 h,
4°C, 4 buffer changes)
(iii) STORE IN EB AT 4°C WITH
0.02% NaN₃

FIGURE 1 Purification protocols for the transferrin receptor.

$H^{14}CHO$ (New England Nuclear; 55 mCi/mmol) and 0.3 μmol NaBH₄ for 10 min at 0°C in 0.2 M sodium borate buffer, pH 9.0 (final reaction volume, 300 μl). The ^{14}C-methylated protein was then desalted on a column of superfine Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) and dialyzed extensively against Hanks' balanced salt solution. The ^{14}C-methylated protein had an average specific activity of 8.0 × 10⁶ dpm/mg, migrated as a single band on SDS gel electrophoresis and was quantitatively precipitated by goat antitransferrin antibodies (95%). Transferrin (75 μg) was radioiodinated in the presence of 1.0 mCi $Na^{125}I$ (carrier-free; New England Nuclear) to a specific activity of 4.4 μCi/μg using the chloramine-T method of Hunter and Greenwood (19). Free $Na^{125}I$ was removed from the iodinated protein by gel filtration on superfine Sephacryl S-200 using 10% heat-inactivated horse serum in PBS as the eluent. The labeled protein was stored as aliquots at −20°C. The iodinated protein migrated as a single band on SDS gel electrophoresis and was quantitatively precipitated by goat antitransferrin antibodies (80%).

Purification of Transferrin Receptors from Chicken Neural
Tissues: Brain and spinal cord tissues were harvested from 13–15-d-old chicken embryos, washed in Hanks', and blotted dry. The tissues were homogenized in 10 mM potassium phosphate buffer, pH 7.5 containing 150 mM NaCl and 0.1% Triton X-100 (1.0 g of tissue/ml) using a Polytron homogenizer (20). Preliminary studies showed that Triton X-100 or SDS was required for complete solubilization of the 56,000-M_r protein from embryonic neural tissue. The homogenate was centrifuged at 16,000 g for 30 min at 4°C and the supernatant was collected. The transferrin receptor (TfR) was purified by the

protocols shown in Fig. 1, which are based on the procedure originally described by Seligman et al. (20). Mouse monoclonal anti-chicken transferrin IgG was purified from ascites fluid (2E12/47) by DEAE Affi-Gel Blue column chromatography (Bio-Rad product bulletin no. 1062, Richmond, CA) and conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) at a ratio of 10 mg of IgG/g Sepharose by a standard procedure (21). Transferrin (Sigma Chemical Co., conalbumin, type II) was conjugated to cyanogen bromide-activated Sepharose 4B in an identical manner. Where specified, column fractions were screened by the fluorescamine assay (22) using a Farrand spectrophotofluorometer (Farrand Optical Co., Inc., Valhalla, NY). Column peaks were pooled and the protein composition of each peak was determined by SDS gel electrophoresis. Immunoreactivity of proteins against antitransferrin antibodies was tested by a double-immunodiffusion assay in agar as previously described (6).

Transferrin Receptor (TfR) Binding to ^{125}I-Transferrin (^{125}I-Tf): Biologically active TfRs were purified using protocol 2 as outlined in Fig. 1. Even though these receptors were purified by affinity chromatography on immobilized Tf, we further tested their ability to bind ^{125}I-Tf by using a gel filtration procedure. Purified TfRs were mixed with ^{125}I-Tf (81 ng; 4.6 μCi/μg) in equilibration buffer and the mixture was stored overnight at 4°C. The resulting ^{125}I-Tf/TfR complexes were separated from free ^{125}I-Tf on a calibrated column (1 × 50 cm) of superfine Sephadex G-200 at a flow rate of 2.0 ml/h using equilibration buffer as the eluent. Fractions of 0.35 ml were collected and counted in a γ-counter. Calibration proteins were chromatographed under

identical conditions, and the fluorescamine assay (22) was used to construct an elution profile. The approximate molecular weight of the Tf/TfR complex was ascertained by comparing its relative elution volume with those obtained for the calibration proteins.

Binding of Fluorescein Isothiocyanate Transferrin to Neuronal Cultures: Transferrin (15 mg; Sigma Chemical Co.) was reacted in the dark with 100 μg of fluorescein isothiocyanate (FITC; Sigma Chemical Co.) for 1 h at room temperature in 1.0 M NaHCO₃, pH 9.5 (23). The FITC-conjugated protein was then purified by gel filtration on a 1.0 × 30-cm column of superfine Sephacryl S-200 using PBS as the eluent. The protein peak was localized by screening column fractions for absorbance at 280 nm in a spectrophotometer, and by screening for fluorescence in a spectrophotofluorometer (excitation, 495; emission, 520). The pooled peak was dialyzed overnight in the dark at 4°C against PBS and had a fluorescence yield of 1.94 fluorescence U/μg of protein. For binding experiments, dorsal root ganglion (DRG) cultures grown on glass coverslips as described above were incubated in the presence of 29–86 μg of FITC-Tf at 4°C for 2 h after a 2-h preincubation in 1% preimmune serum. For internalization studies, receptors were saturated with 29–86 μg of FITC-Tf for 30 min at 4°C and then moved to a 37°C incubator for 90 min. TfR blockade was accomplished by pretreating cultures for 2 h with 45–90 μg of rabbit antitransferrin receptor IgG in PBS at 4°C. All experiments were accompanied by appropriate controls in which cultures were incubated with 45 μg of FITC-Tf + 500 μg of unconjugated Tf to assess nonspecific binding. Cultures were mounted live in 10 mM para-phenylenediamine in 90% glycerol at pH 8.6 (24). The cultures were examined under a Zeiss fluorescence microscope (Oberkochen, Federal Republic of Germany); photographs were taken by using a ×40 objective at an exposure setting of 22 s with Ilford HP-5 film (Ciba-Geigy, Basildon, Essex, England).

RESULTS

The morphological development of the dissociated chick spinal cord cultures was similar to that described previously (25). After 7 d in vitro, numerous well-differentiated neurons lying atop a non-neuronal cell monolayer could be observed using a Zeiss Invertoscope under phase-contrast optics. Despite the presence of these non-neuronal cells, neuronal perikarya and their processes were clearly apparent.

Synthesis and Turnover of Protein Precipitable by Antitransferrin Antibodies

Control experiments were carried out to optimize the concentrations of goat heteroclonal, rabbit heteroclonal, or mouse monoclonal anti-chicken transferrin antibodies for immunoprecipitation of transferrin. Precipitation was quantitative at antisera dilutions of 1:4, 1:4, and 1:10,000, respectively. Inasmuch as these experiments showed that the specific activity of ^{3}H-labeled neuronal proteins increased linearly for at least 120 min (Fig. 2), the absence of L-leucine from the labeling medium for relatively short periods did not adversely affect either the viability of the cells or their capacity to synthesize proteins de novo.

To detect transferrin synthesized de novo, cultures were labeled for varying time periods in the presence of L-^{3}H-amino acids, and the protein was precipitated from the *post*-1,000 *g* supernatant with either rabbit heteroclonal, goat heteroclonal, or mouse monoclonal anti-chicken transferrin antibodies. As the experiment in Fig. 3 indicates, incorporation of label into immunoreactive protein was linear for at least 30 min (k_s = 0.015 min^{-1}; r = 1.0) and was diminished 95% by preincubating cultures for 4 h with puromycin (20 μg/ml; Fig. 3) or by 94% by preincubating for 1 h with cycloheximide (10^{-5} M; not shown). Other experiments showed that ~7% of the counts incorporated into protein appeared in radiolabeled immunoprecipitates. A linear incorporation of labeled amino acids into immunoprecipitable protein was observed regardless of whether rabbit heteroclonal, goat heteroclonal, or mouse monoclonal anti-chicken
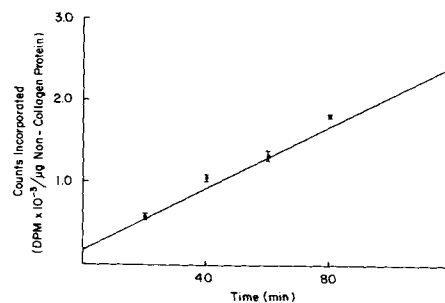

FIGURE 2   Total protein synthesis in cultures of embryonic chicken spinal neurons. Cultures of dissociated spinal neurons (11-d-old) were washed thrice with L-leucine-free DME and 1.0 ml of this medium was added to each culture dish. 10 μCi L-^{3}H-amino acids was then added to each culture. The labeling reaction was stopped at timed intervals by aspirating the medium, washing the cultures thrice with APB (see Materials and Methods) and precipitating cellular proteins with 1.0 ml of ice-cold 10% TCA. The dishes were then reextracted with 10% TCA, washed three times with ethanol-ether (3:1) and air-dried. 1 ml of 0.1 N NaOH was then added to each dish and noncollagen protein was solubilized overnight (26). Counts incorporated into noncollagen protein were determined in duplicate by counting portions of the soluble alkaline extract in 10 ml of Aquasol using a Searle Mark III liquid scintillation counter (Des Plaines, IL) at 50% efficiency. Noncollagen protein was determined by the method of Lowry et al. (27). Each point represents the mean ± SE of four determinations. Least squares analysis showed that the data were fitted to a straight line (r = 0.98).
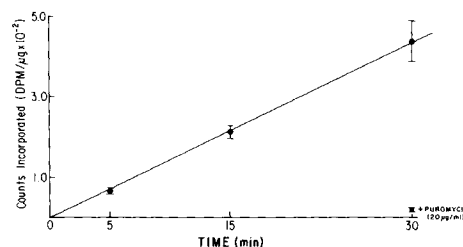

FIGURE 3   Synthesis of protein precipitable by rabbit antitransferrin antibodies in cultures of dissociated spinal neurons. In this experiment, 7-d-old neuronal cultures were incubated in the presence of ^{3}H-amino acids as described in Materials and Methods and the legend to Fig. 2. Some cultures had been preincubated in the presence of either puromycin (20 μg/ml) for 4 h or cycloheximide (10^{-5} M) for 1 h. At timed intervals the cells were washed thrice with APB, harvested, and homogenized in 1.0 ml of APB. A portion of each homogenate was used for determining noncollagen protein, and the supernatant that was obtained by centrifuging the remainder of the homogenate at 1,000 *g* for 20 min was incubated overnight at 4°C with an equal volume of rabbit antitransferrin antibodies in PBS (final titer, 1:4). The immunoprecipitates formed were collected by centrifugation and processed for liquid scintillation counting as described in Materials and Methods. Values represent the mean ± SE of triplicate determinations. The data were fitted to a straight line by least squares analysis (r = 1.0). Puromycin inhibited incorporation into immunoreactive protein by 95% whereas cycloheximide inhibited incorporation by 94% (not shown).

transferrin antibodies were used for immunoprecipitation.

To estimate the turnover of immunoreactive protein, we pulse-labeled neuronal cultures for 30 min with 10 μCi L-^{3}H-amino acids, the cultures were washed several times with 85% DME/15% horse serum, and antitransferrin-precipitable protein was determined in cells and in the culture medium at timed intervals. As shown in the experiment in Fig. 4, the

turnover of immunoreactive protein was linear with a half-life ($t_{1/2}$) of ~8–10 h. About 10–12% of this precipitable protein was released into the culture medium in a linear fashion and this release occurred during only the initial 90 min after pulse-labeling (not shown). Thereafter, the rate of release decreased to zero. During this latter stage, intracellular degradation appeared to account for the continued linear turnover of immunoreactive protein.

The Immunoprecipitated Protein Synthesized by Neuronal Cultures Is Not Transferrin

To test the specificity of the anti-chicken transferrin antibodies, we separated some radioactive immunoprecipitates obtained after labeling neuronal cultures for 30 min with [^{14}C]-leucine on 12.6% SDS gels, stained the gels with Coomassie Blue, and fluorographed the dehydrated electropherograms. As shown in Fig. 5 A, Coomassie Blue staining revealed a prominent protein band, which co-migrated with a transferrin standard (lane S; $M_r = 84,000$). However, fluorographs of such gels (Fig. 5 B) showed no radioactivity in the transferrin region of the gel, but did reveal a single, prominent band with a M_r of 56,000. To determine whether this 56,000-M_r protein represented a degradative fragment of transferrin, we added ^{14}C-labeled transferrin to unlabeled neuronal homogenates and precipitated the protein by the standard immunoprecipitation technique. As shown in Fig. 5 B, the labeled immunoprecipitated protein migrated at an M_r of 84,000 with no apparent degradation into a 56,000-M_r fragment. To determine which cells were immunoreactive to antitransferrin antibodies, we fixed and stained cultures by the PAP technique. As shown in Fig. 5 C, only neurons and their processes reacted appreciably with the antitransferrin antibodies.

Radiolabeled Transferrin Receptors Co-precipitate with Unlabeled Transferrin

The transferrin obtained by immunoprecipitating the radiolabeled proteins synthesized by neuronal cultures was not
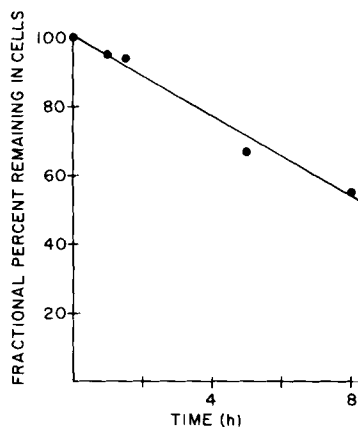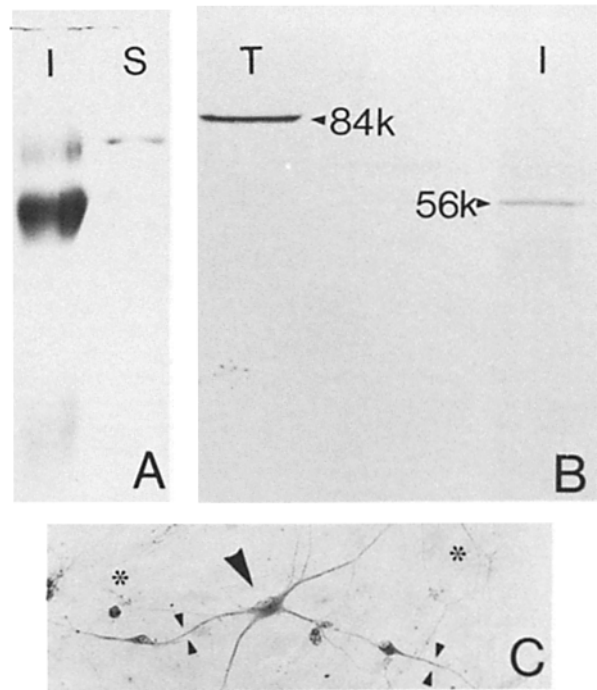

FIGURE 5 Synthesis of a protein of 56,000 mol wt by neuronal cultures that co-precipitates with transferrin and the immunocytochemical localization of transferrin in cultured neurons. (A) Immunoprecipitates from neuronal cultures labeled in the presence of L-[^{14}C]leucine for 30 min were separated by SDS gel electrophoresis and the proteins were visualized with Coomassie Blue. The immunoprecipitate in lane I shows three major bands that correspond to: top—transferrin, middle–heavy chains of IgG, and bottom—light chains of IgG. Notice that transferrin in lane I co-migrates with a transferrin standard in lane S. (B) An electrophoretic gel obtained as in A above was dehydrated and fluorographed for 14 d as described in Materials and Methods. The fluorograph of the immunoprecipitate (lane I) had no radioactive band corresponding to transferrin ($M_r = 84,000$). However, a prominent band was visualized at an $M_r = 56,000$. Lane T shows an immunoprecipitate obtained by mixing an unlabeled neuronal homogenate containing [^{14}C]transferrin (28,000 dpm) with goat antitransferrin IgG. Notice that the ^{14}C-labeled transferrin was precipitated by antibody and that no faster-migrating, degradative fragments were generated during the immunoprecipitation procedure. (C) A spinal cord culture was fixed and stained by the peroxidase–antiperoxidase technique using rabbit antitransferrin serum as described in Materials and Methods. The culture was photographed using bright-field optics at a magnification of 100. Notice the localization of reaction product in a spinal neuronal cell body (large arrowhead) and its processes (small arrowheads). Contaminating non-neuronal cells (*) are not stained by the antiserum. Control cultures incubated with preimmune serum showed no reaction product in neuronal cells (not shown). k, $\times 10^{-3}$ (molecular weights).

FIGURE 4 Turnover of protein precipitable by goat antitransferrin antibodies in neuronal cultures. 7-d-old neuronal cultures were labeled with ^{3}H-amino acids for 30 min as described in Materials and Methods. Cultures were then washed thrice with 85% DME–15% horse serum, and ^{3}H-protein precipitable by goat antitransferrin antibodies was determined in cells and in the incubation medium at timed intervals. The fractional percentage of counts within cells as shown here decayed in a linear fashion with an estimated turnover time ($t_{1/2}$) of 8.6 h. Each value represents the mean of duplicate determinations.

itself radioactively labeled (Fig. 5 B), even though transferrin was present in abundance within cultured neurons as evidenced by the peroxidase–antiperoxidase technique (Fig. 5 C). Also, the antiprotease mixture (APB) used to homogenize cultures appeared to be of a suitable composition to prevent the degradation of ^{14}C-labeled transferrin into either a 56,000-M_r fragment or other still smaller fragments (Fig. 5 B). Additional control experiments showed that these observations were unchanged by using citrate buffer at pH 4.2 to homogenize the cultures in that citrate has been observed to have a stabilizing effect on transferrin (28). Also, the results were not affected by increasing the concentration of unlabeled L-leu-

cine in the labeling medium, by adding other protease inhibitors such as phenylmethylsulfonyl fluoride to the homogenizing mixture or by using various other lots of anti-chicken transferrin antibodies. In view of these negative findings, we wondered whether the 56,000-M_r protein represented a receptor for transferrin (14) which coprecipitated with transferrin in the presence of antitransferrin antibodies (29). Therefore, we attempted to isolate the putative neuronal transferrin–56,000-M_r protein (receptor) complex on immobilized monoclonal antitransferrin IgG and to purify the receptor protein by differential desorption at alkaline pH in the presence of 0.5% Triton X-100 (protocol 1, Fig. 1; see reference 20).

Purification of the Neuronal Transferrin Receptor by Selective Desorption from Antitransferrin IgG

Fig. 6 shows a step-elution profile of neuronal proteins desorbed from mouse monoclonal anti-chicken transferrin IgG (2E12/47) conjugated to Sepharose 4B (protocol 1, Fig. 1). The pooled, stippled fraction eluted by 20 mM borate/NaOH, pH 10.0 containing 0.5 M NaCl and 0.5% Triton X-100 (Fig. 6, peak A) was found to contain a highly purified protein of M_r 56,000 as judged by SDS gel electrophoresis (Fig. 6, inset). After the appearance of peak A, proteins remaining absorbed to the column shown were stripped from the IgG using 50 mM potassium phosphate/phosphoric acid, pH 2.0, containing 150 mM NaCl (see reference 20). The pooled, stippled fraction eluted by this buffer (Fig. 6, peak B) was found to contain several proteins on SDS gel electrophoresis (Fig. 6, inset); one of these proteins co-migrated with a transferrin standard (not shown), while another appeared to
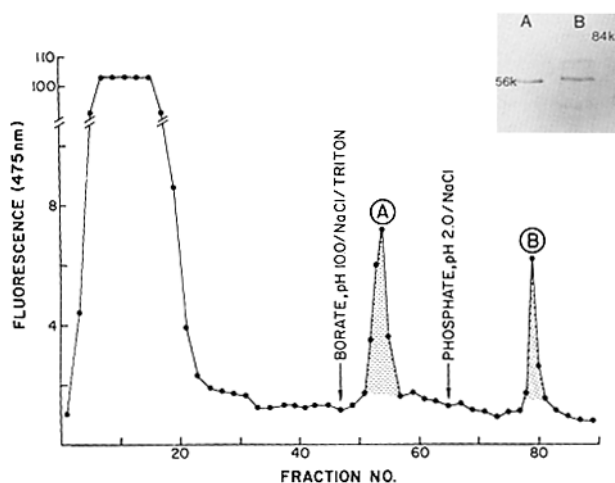

FIGURE 6   Step-elution profile of neuronal proteins from mouse monoclonal antitransferrin IgG conjugated to Sepharose 4B. Brain and spinal cord tissues (25 g) were harvested from 13-15-d-old chicken embryos and homogenized in 10 mM potassium phosphate, pH 7.5 containing 150 mM NaCl and 0.1% Triton X-100. The "floating pellet" was obtained as described in Fig. 1, and was applied to a column of monoclonal antitransferrin IgG conjugated to Sepharose 4B. The step elution was developed by using the buffers described in Fig. 1, protocol 1 at a flow rate of 20 ml/h. The column fractions were screened for protein by using the fluorescamine assay. The pooled, stippled peak A was found to contain a highly purified protein that migrated at an M, of 56,000 on SDS gel electrophoresis (inset lane A). Peak B contained several proteins including one that co-migrated with a transferrin standard and another that migrated slightly more slowly than the 56,000-mol-wt protein (inset lane B).

FIGURE 7   Double-immunodiffusion precipitin reaction of goat antitransferrin serum with the transferrin receptor and with transferrin. Center well, goat antitransferrin serum diluted 1:3 with PBS; lanes 1, 3, and 5, transferrin receptor (peak A of Fig. 5; 100 µg/ml); wells 2, 4, and 6, transferrin (peak B of Fig. 5; 100 µg/ml).
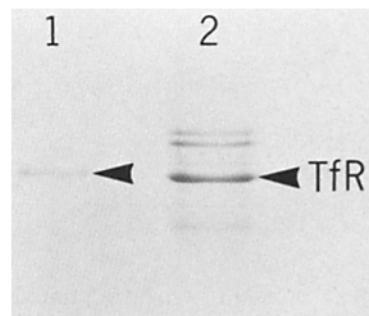

FIGURE 8   SDS gel electrophoresis of purified transferrin receptors. Transferrin receptors were purified from embryonic chicken neural tissue by the two-column procedure summarized in Fig. 1, protocol 2. (Lane 1) Wash 4, column 2 (chicken transferrin affinity column), ~1 µg of protein. (Lane 2) Wash 4, column 1 (mouse monoclonal anti-chicken transferrin IgG column), ~10 µg of protein. (Arrowheads) Transferrin receptors (M_r 56,000).

co-migrate with the 56,000-M_r protein in peak A.

The respective proteins in peaks A and B were tested for cross-reactivity against antitransferrin IgG by double immunodiffusion in agar (Fig. 7). The 56,000-M_r protein in peak A (receptor) failed to cross-react with mouse monoclonal anti-chicken transferrin IgG (not shown), or goat heteroclonal anti-chicken transferrin IgG (Fig. 7, wells 1, 3, and 5). However, as expected, the transferrin in peak B formed a line of precipitation with either antibody (e.g., Fig. 7, wells 2, 4, and 6).

Binding of the 56,000-M_r Protein Receptor to ^{125}I-Transferrin and Its Distribution on Neurons

Although the 56,000-M_r protein could be readily purified using the elution scheme shown in protocol 1, Fig. 1, the resulting protein was devoid of biological activity as evidenced by its failure to bind to immobilized transferrin or to ^{125}I-transferrin. This was probably a result of denaturation due to the high pH (10.0) required to dissociate the 56,000-M_r protein from transferrin bound to very high affinity monoclonal IgG. As a result, the active protein was purified from brain homogenates using protocol 2, Fig. 1. As this procedure used urea as a dissociating agent, the 56,000-M_r protein recovered from the first affinity column (mouse antitransferrin IgG) could be further purified by affinity chromatography on immobilized transferrin (Fig. 8). The affinity-purified 56,000-M_r protein bound radioiodinated transferrin as demonstrated by the gel filtration experiment shown in Fig. 9. Here, the receptor-^{125}I-transferrin complex was eluted from the column at a molecular weight estimated to be 130,000 (see reference 14).

SDS gel electrophoresis demonstrated that the 56,000-M_r transferrin receptor present in DRG cultures could be precip-
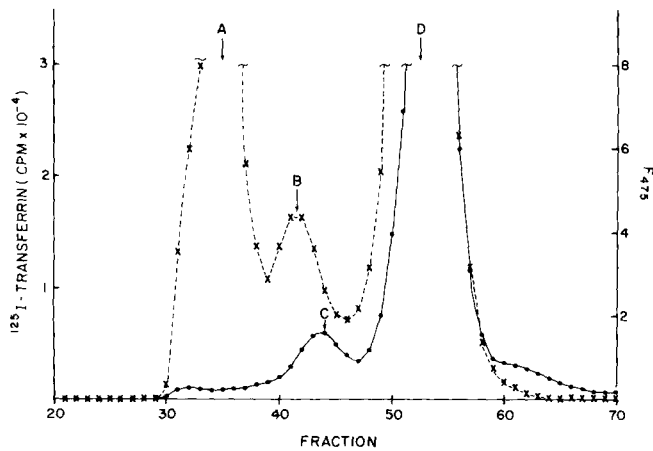
FIGURE 9 Elution profile of ^{125}I-transferrin/transferrin-receptor complexes from superfine Sephadex G-200. Wash 4 of column 2, containing ~50 ng of transferrin-receptor protein (see Fig. 1, protocol 2), was added to 81 ng of ^{125}I-Tf (4.6 μCi/μg) and stored overnight at 4°C. The protein mixture was then separated by gel filtration on a calibrated, 1 × 50-cm column of superfine Sephadex G-200 at a flow rate of 2.0 ml/h that used equilibration buffer (Fig. 1) as the eluent. Fractions of 0.35 ml were collected and counted in a γ counter (●———●). The unlabeled proteins used to calibrate the column were measured by the fluorescamine assay as described in Materials and Methods (x———x). Peak C, which represents the ^{125}I-Tf/TfR complex, was eluted from the column at a K_{av} of 0.125 (130,000 mol wt). Approximately 8 ng of ^{125}I-Tf was bound to the receptor in peak C. A-blue dextran (2,000,000 mol wt); B-goat IgG (150,000 mol wt); C-Tf/TfR complex (130,000 mol wt); D-Tf (80,000 mol wt).
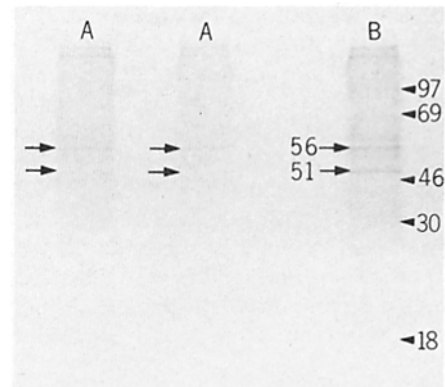

FIGURE 10 Co-migration of labeled transferrin receptors precipitated by mouse monoclonal anti-chicken transferrin IgG or by rabbit anti-chicken transferrin receptor IgG. DRG neurons were cultured as described previously (6) and labeled with [^{14}C]leucine for 1 h as described in Materials and Methods. The labeled neurons were washed and homogenized in APB buffer, and the supernatant was collected after centrifugation at 1,000 g for 20 min. Aliquots of supernatant were mixed with either mouse monoclonal (2E 12/47) anti-chicken transferrin IgG (final concentration, 1:20,000) or rabbit anti-chicken erythrocyte transferrin receptor IgG (23; final concentration, 100 μg). After incubation overnight at 4°C, the precipitates were collected by centrifugation. The pellets were denatured in a lysis buffer and electrophoresed as described in Materials and Methods. The dried gel was fluorographed using Kodak X-Omatic film for 30 d at −70°C. Notice the presence of the receptor band that migrates at 56,000 mol wt in both immunoprecipitates (lanes A and B, top arrow). The faster-migrating band at 51,000 mol wt (lower arrow) was unidentified, but occasionally appeared in other immunoprecipitates. Lane A, precipitate obtained with mouse antitransferrin IgG; Lane B, precipitate obtained with rabbit antireceptor IgG. The migration of known standards is indicated in relation to lane B. $M_r \times 10^{-3}$.

itated from culture homogenates using rabbit anti-chicken erythrocyte transferrin receptor IgG (Fig. 10). As a result of the apparent homology between the neuronal and erythrocytic forms of the transferrin receptor in the chicken, rabbit anti-chicken erythrocyte transferrin receptor IgG (14) was used to localize the transferrin receptors on chicken neurons in culture. This antibody had been previously shown to react with the receptor protein present on the surface of reticulocytes since it agglutinated embryonic chicken red blood cells, a process which could be inhibited by preincubation with the $F_{ab'}$ fragments of this IgG, to cause complement-mediated hemolysis and to inhibit uptake of ^{55}Fe-Tf by these cells. As shown in Fig. 11, PAP immunocytochemical localization of transferrin receptors revealed a diffuse reaction product on the plasma membranes of cultured neurons. Control cultures incubated with preimmune rabbit IgG showed no reaction and are therefore not shown. Contaminating, non-neuronal cells were also stained by rabbit antireceptor IgG, but the intensity of this staining was much less than that of neurons.

To demonstrate further the surface localization of the 56,000-M_r transferrin receptor, we carried out binding and internalization studies on living DRG neurons in culture using Tf conjugated to FITC. After preincubation with 1% preimmune serum, FITC-Tf was bound to the surface membranes of DRG neurons at 4°C to yield a fine granular rim of surface fluorescence (23; Fig. 12A). When these cultures were tranferred to a 37°C incubator, the fluoresceinated Tf was internalized and appeared as distinct intracellular granules in a vesicular pattern (23; Fig. 12B). By contrast, if cultures were preincubated with antitransferrin receptor IgG for 2 h at 4°C,
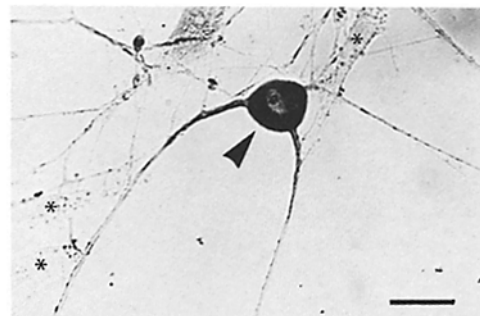

FIGURE 11 Immunocytochemical localization of transferrin receptors on a chick embryonic DRG neuron. DRG neurons were cultured as described previously (6). Transferrin receptors were localized on aldehyde-fixed cultures by the PAP technique as described in Materials and Methods. The primary antibody was rabbit anti-chicken erythrocyte transferrin receptor IgG at a concentration of 100 μg/ml PBS (23). Notice the intense staining on the cell body (arrowhead) and processes of a DRG neuron. The non-neuronal cells present (*) reacted very weakly as compared to the staining observed on DRG or spinal cord neurons (not shown). Cultures incubated with preimmune IgG showed no reaction product and are not shown here as a result. Bar, 20 μm; magnification, 400.

surface Tf receptors were blocked as no binding or internalization of FITC-Tf was evident (Fig. 12C). These results appeared to confirm the surface localization of the 56,000-M_r transferrin receptor illustrated in Fig. 11.
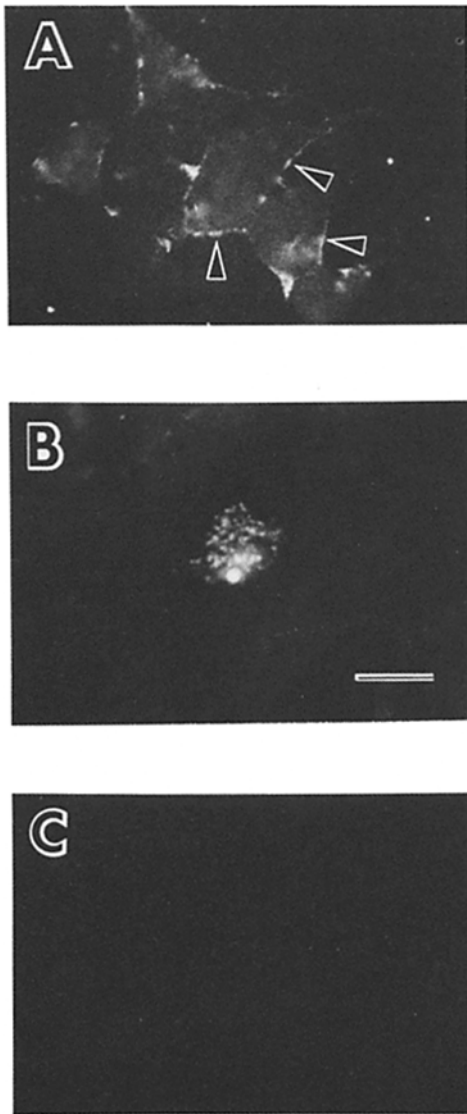
FIGURE 12 Antitransferrin receptor IgG blocks surface binding of FITC-Tf to embryonic DRG neurons. DRG neurons were cultured on collagen-coated glass coverslips as described in Material and Methods. The cultures were preincubated in either 1% preimmune serum or in 45–90 μg of rabbit antitransferrin receptor IgG in PBS for 2 h at 4°C. For binding studies, DRG cultures were incubated in the presence of 29–86 μg of FITC-Tf at 4°C for 2 h. For internalization studies, receptors were saturated with FITC-Tf for 30 min at 4°C and then transferred to a 37°C incubator for 90 min. After binding or internalization studies, cultures were washed with PBS/1% BSA and mounted in 10 mM para-phenylenediamine in 90% glycerol, pH 8.6. (A) Fluorescence photomicrograph of FITC-Tf bound to a cluster of DRG neurons at 4°C after preincubation in preimmune serum. Note the granular rim of surface fluorescence indicated by the arrowheads. (B) FITC-Tf internalized by a DRG neuron at 37°C after preincubation in preimmune serum. Note the vesicular pattern of internalized FITC-Tf. (C) Absence of FITC-Tf binding to a DRG neuron at 4°C after preincubation in 45 μg of rabbit antitransferrin receptor IgG. Control experiments were carried out in the presence of 500 μg of unconjugated Tf in order to assess the nonspecific binding of FITC-Tf. Bar, 20 μm; all micrographs shown were taken at the same magnification (400).

DISCUSSION

Because of the numerous trophic effects of transferrin upon skeletal muscle and the selective localization of this protein

in neuronal cell bodies and processes, we had anticipated that this protein might represent a myotrophic agent synthesized and secreted by neurons that directly regulated the metabolic processes of skeletal muscle (e.g., references 2 and 4). In fact, Stamatos et al. (9) recently reported the synthesis of transferrin by embryonic chicken spinal cord neurons in culture (see also reference 10). However, our present study has shown that transferrin is not synthesized by cultures of spinal cord neurons (Fig. 5 B). Instead, these cultures synthesize a protein of M_r 56,000, which co-precipitates with transferrin in the presence of specific antitransferrin antibodies and which appears to represent the neuronal receptor for transferrin. Evidence supporting the contention that this 56,000-M_r protein represents the transferrin receptor includes the facts that: (a) this 56,000-M_r protein was purified from neuronal tissue by differential desorption from either immobilized antitransferrin IgG (Fig. 6) or immobilized transferrin (Fig. 8); (b) this protein did not represent a degradation product of transferrin (Fig. 5 B); (c) this 56,000-M_r protein itself did not react with mouse monoclonal or goat heteroclonal IgG (Fig. 7), but did react with rabbit anti-chicken transferrin receptor IgG (Fig. 10; see reference 14); (d) this protein bound ^{125}I-transferrin (Fig. 9); and (e) this protein could be localized on the surface of cultured neurons by immunocytochemistry using rabbit anti-chicken erythrocyte transferrin-receptor IgG (Fig. 11; see reference 14), or by studies of the IgG blocking of surface receptors (Fig. 12). In light of this evidence, it seems reasonable to assume that this 56,000-M_r protein does indeed represent a neuronal receptor or binding protein for transferrin.

The transferrin receptor from human HeLa cells has a turnover time of ~10–12 h (30). This value is in close agreement with the turnover time (8–10 h) that we observed in the chicken by using immunoprecipitation (Fig. 4). However, this $t_{1/2}$ is probably only a close estimate of the actual turnover time because the antibodies used in immunoprecipitations were directed towards transferrin and not the receptor per se. Also, we were initially unable to observe the receptor protein in immunoprecipitates separated by SDS gel electrophoresis and stained with Coomassie Blue (Fig. 5 A), although the radioactive protein was clearly demonstrated by fluorography (Fig. 5 B). It seems likely that the 56,000-M_r protein could not be resolved on Coomassie Blue-stained gels because the heavy chain of IgG migrated as a large smear of protein with an M_r of ~55,000. Thus, the receptor protein was apparently obscured by the extensive heavy chain band of IgG. However, the receptor protein can be readily visualized by Coomassie Blue in the absence of IgG and migrates at an M_r of 56,000 (e.g., inset, Fig. 6).

Several plasma proteins including transferrin have been localized within neuronal tissues (31–33). Schachter and Toran-Allerand (34) have shown that these intraneuronal proteins are not synthesized by neuronal tissue (however, see reference 10). Instead, these proteins appear to reach their target neurons by uptake followed by retrograde transport (see also references 31 and 33). Transferrin is known to be transported into cells by binding to high-affinity receptors localized on the cell surface (35–39) followed by receptor-mediated endocytosis (e.g., reference 35). Therefore, our observations that the transferrin receptor is synthesized both rapidly and in abundance (7% of incorporated label) by cultured neurons and co-precipitates with antitransferrin antibodies (implying that this receptor is present as a receptor-ligand complex) may help to explain the intraneuronal local-

ization of transferrin that we have observed (6) and the apparent abundance of transferrin in peripheral nervous tissue (40).

A surprising aspect of this study concerns the quantitative difference in the cytological levels of immunoreactive transferrin (Fig. 5C) or transferrin receptors (Fig. 11) in neurons as contrasted to the relatively low levels observed in contaminating non-neuronal cells. The immunocytochemical visualization of transferrin was performed on intact cells fixed in absolute methanol using high-affinity rabbit anti-chicken transferrin antibodies (Fig. 5C). Because of the de-lipidating properties of methanol, the immunocytochemical pattern observed probably reflects any transferrin that had been internalized by these cells as well as transferrin that had been associated with the plasma membrane. The results shown in Fig. 5 agree with our previous study on the immunocytochemical localization of transferrin in adult or embryonic chicken neurons, which also demonstrated increased immunoreactivity within sectioned or intact neuronal tissues as compared with low levels of reactivity in muscle, liver, or, stromal elements (6). These results also appear to reflect our observation that transferrin comprises ~2.6% of soluble axonal protein (40). By contrast, the immunocytochemical visualization of transferrin receptors was performed on intact cells fixed in cold 4% paraformaldehyde-0.1% glutaraldehyde in 100 mM sodium cacodylate, pH 7.2, using rabbit anti-chicken erythrocyte transferrin receptor IgG (Fig. 11; reference 14). These antibodies were shown to agglutinate embryonic chicken erythrocytes, a process that could be inhibited by preincubation with the $F_{ab'}$ fragments of this IgG, to cause complement-mediated red cell lysis and to inhibit the uptake of ^{55}Fe-Tf by these cells (14). Because the neuron shown in Fig. 11 was an intact cell fixed with cold aldehyde fixative, and as no attempt was made to solubilize the cell membrane during the PAP procedure, the immunocytochemical pattern observed probably reflects surface receptors. This is more convincingly demonstrated by the inhibition of surface fluorescence shown on the neuron in Fig. 12C which was pretreated with antitransferrin receptor IgG and then incubated in the presence of FITC-Tf at 4°C. Therefore, the difference in the staining intensity visible in Fig. 11 may reflect a quantitative difference in the number of surface transferrin receptors on neurons although this supposition will have to be investigated further at either the electron microscopical level or by isotopic binding experiments.

The transferrin receptor from human tissues or human cell lines is a glycoprotein that migrates at an M_r of 90,000–100,000 on SDS gels (20, 37, 41, 42). No ready explanation can be given to account for the smaller size (M_r 56,000) of the chicken neuronal transferrin receptor especially in that this protein was purified by a procedure similar to that used to isolate this receptor from human placenta (20). On certain occasions, immunoprecipitates separated by gel electrophoresis revealed a second labeled protein band which migrated more quickly than the 56,000-M_r protein (M_r 51,000; Fig. 10). Although compelling evidence suggests that the 56,000-M_r protein contains the binding moiety for transferrin (Figs. 5, 6, 8, 9; see also reference 14), it may be that this smaller polypeptide represents a degradation product of the 56,000-M_r protein because no serine or sulphydryl protease inhibitors were present in the tissue homogenization buffer (14), or this 51,000-M_r protein might be a structural filamentous protein involved in membrane anchorage of the receptor. Clearly, the

identity of this 51,000-M_r band must await further investigation. In any case, the fact that the transferrin-binding moiety of the transferrin receptor in neurons (56,000 mol wt) is of an almost identical molecular weight to that in chicken reticulocytes (59,000 mol wt, reference 14), and the fact that antibodies against this erythrocytic receptor cross-react with the 56,000 mol wt protein on the surface membrane of neurons suggest that the transferrin receptor in chicken tissues may have a uniquely different composition from that of the human and other mammals. It seems clear that the physicochemical parameters of this chicken receptor protein, including a clarification of the identity of the 51,000 mol wt band, will have to be studied in more detail before any further comparisons can be made to the mammalian receptor.

The protocol for purifying transferrin receptors described by Seligman et al. (20) depends upon two major features: (a) the use of detergent in the extraction buffer in order to solubilize the membrane-bound receptor; and (b) maintenance of a tight binding interaction between transferrin and its receptor on the immunoabsorption column until the specific alkaline desorption step. These authors used Triton X-100 to solubilize the ligand–receptor complex from placental tissue although recent data suggest that 0.4% SDS is far superior for this purpose (29). We too found that complete extraction of the 56,000 mol wt protein from neuronal tissues required the presence of detergent because most detergent-free buffers were almost totally ineffective for this purpose. However, we have obtained preliminary data that demonstrates that 10 mM citrate buffer, pH 4.2, extracts a significant proportion of the receptor protein from neuronal tissue (for rationale, see reference 28). If the resulting membrane pellet is reextracted with equilibration buffer, a further pool of receptors is obtained. These data appear to support the view that neuronal transferrin receptors may exist in two separate pools: an intracellular pool extractable by citrate, and a membrane-bound pool extractable only with detergent. For desorbing transferrin receptors from the immunoabsorption column (Fig. 1, protocol 1), we chose borate buffer at pH 10.0 over glycine/NaOH because glycine interfered with the fluorescent assay used in constructing elution profiles. However, as either borate or glycine/NaOH appeared to denature the receptor, we ultimately used a urea/Triton buffer for desorptions (Fig. 1, protocol 2). We are now experimenting with a variety of other desorbents in the hope of obtaining active receptors in higher yields.

Transferrin appears to have very pronounced growth-regulating properties because blocking the transferrin receptor of human tumor cell lines with antireceptor monoclonal antibodies dramatically reduces cell growth (43). In fact, the presence of the transferrin receptor on the surface of normal or neoplastic cells undergoing division appears to correlate with cellular proliferation (39). In light of these findings, it remains to be seen what role transferrin plays in neuronal differentiation and metabolism (32) and why this protein is required in such abundance by neuronal tissues (6, 40). This appears to be a highly relevant question since it was recently reported that transferrin is an essential component for the differentiation and maintenance of sensory neurons in culture (44). In this regard, unpublished studies in our laboratory have demonstrated that cultured chicken DRG neurons internalize ^{125}I-transferrin by a specific, receptor-mediated endocytotic mechanism (Markelonis, G. J., T. H. Oh, L. P. Park, P. Azari, and S. R. Max, unpublished). In view of the

fact that transferrin selectively appears in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis (45), a degenerative disease of motor neurons, it may be that this protein plays some important yet unrecognized role in neuronal survival.

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