Identification, Purification, and Characterization of Truncated Forms of the Human Nerve Growth Factor Receptor*

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We report the presence of truncated forms of the nerve growth factor receptor (NGFR) in the conditioned medium of the human melanoma cell line A875 and in human urine and amniotic fluid. Radioiodinated nerve growth factor (125I-NGF) specifically bound to NGFR, was chemically cross-linked. After immunoprecipitation, labeled receptor species were visualized by autoradiography following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. NGFRs were purified from human adult male urine or a mixture of human amniotic fluid and infant urine by using a combination of either ion exchange chromatography (adult) or ammonium sulfate precipitation (infant) and immunoaffinity chromatography. Typical yields were about 1 μg/liter of adult urine and 75 μg/liter of amniotic fluid/infant urine. The purified proteins, with molecular masses of 45, 40, and 35 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%), were confirmed to be NGFRs by amino-terminal sequencing and were designated NGFRt-1, NGFRt-2, and NGFRt-3, respectively. The isoelectric points of these three species ranged from 3.3 to 3.95 and displayed intraspecies heterogeneity; subsequently, amino acid residues covalently modified with sialic acid-containing carbohydrates were documented. The binding affinities of these species for nerve growth factor were comparable to that of the low affinity cell surface receptor. The potential to isolate milligram quantities of human NGFRs allows for model studies of the physicochemical structure of the intact receptor and the generation of polyclonal antibodies to study the biological functions of the NGF receptor.

Nerve growth factor (NGF) is a neurotrophic factor that promotes the survival and function of sympathetic and neural crest-derived sensory neurons subsequent to specific interaction with high affinity receptors present on the plasma membrane (1, 2). In addition, specific nerve growth factor receptors (NGFR) are demonstrable in brain, particularly on cholinergic neurons (3–5), as well as on nonneuronal derivatives of the neural crest including pheochromocytoma cells (6), melanoma cells (7), Schwann cells (8), and neurofibroma cells (9). During development, the receptor is also observed on many tissues not previously considered NGF targets (10–12).

The human NGFR cDNA has been cloned and sequenced (13). The NGFR is a member of the family of membrane glycoproteins containing multiple cysteine-rich domains (e.g. low density lipoprotein receptor, epidermal growth factor receptor, and insulin receptor (14–16)). The structural and biological significance of this architectural motif is unknown. A prerequisite to addressing these issues is the isolation of sufficient quantities of native receptor. Although NGFR has recently been purified from human melanoma cells in culture (17), yields were not sufficient to permit extensive study. Our laboratory has shown by using the monoclonal antibody (mAb) 192-IgG, specifically directed against rat NGFR, that Schwann cells, the JS-1 Schwannoma cell line, PC12 cells, and superior cervical ganglion neurons all elaborate a truncated form of the nerve growth factor receptor (NGFRt) into the extracellular medium when these cells are maintained in culture (18). Rat NGFR, was also demonstrated in amniotic fluid, urine, and plasma with levels elevated in younger animals and significantly diminished with increased age. This time course parallels the developmental decrease in tissue expression of NGFR (12). Although the biological function of NGFR, is unknown, its presence in physiological fluids of experimental animals suggests the possibility that such fluids might serve as preparatively feasible sources of NGFR-like species.

In this report, we have used the mAb ME20.4-IgG (9), specifically directed against the human NGFR (based on its capacity to inhibit binding of 125I-NGF to intact receptor-positive cells and to immunoprecipitate specifically the 125I-NGF affinity-labeled receptor) to demonstrate NGFR, in the conditioned medium of cultured A875 human melanoma cells as well as in human amniotic fluid, infant urine, and adult urine. We utilized adult urine or a combination of amniotic fluid and infant urine as a preparative source of human NGFR. We describe the purification of human NGFRs in hundred-microgram quantities by immunoaffinity chromatography, and we verify the purification by electrophoresis, iodination, and amino-terminal sequencing. The determination of approximate isoelectric points and an analysis of the glycosylated nature of NGFR, are also presented. Analysis of the specificity and affinity that these truncated species have for ligand reveals similarities to the intact low affinity NGFR and strengthens the potential for their use in studying the cell-associated receptor (19).

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† The abbreviations used are: NGF, 2.5 S nerve growth factor; NGFR, nerve growth factor receptor; mAb, monoclonal antibody; NGFRt, truncated nerve growth factor receptor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Cell Culture and Antibodies—The human melanoma cell line A875 was a gift from Dr. George Todaro (Oncogen) and was grown in McCoy's 5A medium (Mediatech) supplemented with 10% fetal calf serum (JR Scientific). The pheochromocytoma cell line, PC12, was maintained as described (20). Hybridoma cell lines 192 (21) and ME20.4 (9) were obtained from Charles Chandler (Pfizer) and the American Type Culture Collection (HB 5873), respectively, and maintained in Dulbecco's modified Eagle's medium (Mediatech) with 20% fetal calf serum. These cells were used for induction of ascitic tumors in mice as described (21). The associated monoclonal antibodies were purified from ascites by using a Bio-Rad monoclonal antibody purification system.

Specimen Collection—Adult urine was obtained from normal male volunteers. Infant urine was supplied by local pediatric intensive care units (Children's Hospital and St. John's Mercy Medical Center, St. Louis, MO). Amino acid fluid was provided primarily from The Genetics Institute (Pasadena, CA), Jewish Hospital amniocentesis center (St. Louis, MO), and St. John’s Mercy Medical Center cytogenetics center (St. Louis, MO). Additional amino acid fluid was occasionally provided by other cytogenetics centers.

Immunoprecipitation and Lectin Precipitation—NGF-binding proteins were affinity labeled by using modifications of a described protocol (22). Briefly, NGFR on cell surfaces was assayed by incubating prewashed cells with 2 nm 125I-NGF in 20 mM phosphate-buffered saline (PBS) for 1 h at 4°C. Nonbinding was determined by subtracting the presence of a 200-400-fold excess of unlabeled NGF. NGF was chemically cross-linked to receptor species by incubating 20 min with 20 mM 1-ethyl-3-(3-dimethylaminopropyl)carboimidate (Pierce Chemical Co.), and unreacted sites were quenched with addition of Triton/HC1 buffer, pH 7.4, at a final concentration of 50 mM. After washing, the cells were solubilized in the presence of 1.3% Mase (m/v) 1-0-n-octyl β-D-glucopyranoside (n-octyl glucoside, Boehringer Mannheim) for 1 h. Subsequently, samples were centrifuged in a Beckman Microfuge 12 at 12,000 X g for 5 min; 5 μg of mAb ME20.4-IgG was added to the supernatant. After 1 h, 20 μl of a 10% (m/v) suspension of formalin-fixed Staphylococcus aureus cells (Pansorbin, Calbiochem), previously saturated with anti-mouse IgG antibody, was added. After a 1-h incubation, the mixture was centrifuged and the pellet washed three times with PBS, 0.1% (m/v) bovine serum albumin, 0.1% (v/v) Triton X-100, 500 mM sucrose. A final wash in PBS, 0.1% (v/v) Triton X-100 followed. Soluble NGFRs in conditioned media, urine, and amino acid fluid were assayed similarly except the detergent solubilization with subsequent centrifugation was omitted. Prior to assay, these samples were clarified by centrifugation at 12,000 X g.

Lectin precipitation followed essentially the same protocol except that per gent agglutinin immunomobilized on Sepharose 6MB (Sigma) replaced the anti-NGFR and Pansorbin incubations. After purification of NGFRs, forms, the relative binding affinities of these species were assayed by immunoprecipitation in the presence of 1 mNGF and varying amounts of unlabeled NGF. Washed precipitates were suspended in 0.5% (m/v) Triton/HC1 buffer, pH 7.4, containing 0.2% (m/v) sodium deoxydosed (SAS) and 2% (v/v) β-mercaptoethanol, boiled 5 min, and clarified. The supernatant was used for SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

Purification of NGFR—Purification from adult urine was performed by a combination of ion exchange and immunofluorescence chromatographies. Eighty to 100 ml of urine was collected in 4-liter aliquots. To each aliquot approximately 15 ml of 5 N NaOH was added to alkalinize the sample to pH 8.0. These aliquots were maintained at 4°C for at least 10 h but not more than 5 days to precipitate the proteins. The addition of 10% (m/v) sodium deoxydosed (SAS) and 2% (v/v) β-mercaptoethanol was included. The supernatant was used for SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

Electrophoresis—Receptor species in isolation or cross-linked to 125I-NGF were resolved by SDS-PAGE (28) by using Bio-Rad and Pharmacia reagents. Purified NGFRs were silver stained with GELOCODE (Pierce Chemical Co.). Cross-linked species were visualized autoradiographically by using Kodak X-Omat AR-5 film (Eastman Kodak). For quantitative determination of the displacement of 1 mNGF by unlabeled NGF, the ports of gel corresponding to autoradiographic bands were excised, and the amount of radioactive activity was determined in a Beckman γ-counter (model 5500). Two-dimensional PAGE was performed by using isoelectric focusing of immunopurified NGFR species for the first dimension and SDS-PAGE for the second dimension. Immediately after sampling, focused, sample lanes were excised with the backing plate attached, and gel strips were incubated in 62.5 mM Tris/HCl, pH 6.8, containing 2.3% (m/v) SDS and 2% (v/v) β-mercaptoethanol, at room temperature for 5 min with constant agitation. The strips were then immersed in 1% agarose containing the same buffer atop the SDS-PAGE stacking gel.

Electroelution—Immunopurified NGFR species were separated from one another by SDS-PAGE. 125I-NGFR, in an adjacent lane served as a tracer. Gel areas containing sample to be amino-terminally sequenced were excised by using a model Ektachem-E (Bio-Rad). Electroelution-purity grade SDS (Bio-Rad) was recrystallized twice prior to use, as recommended (25).

Treatment of 125I-NGF, with Human Serum or Glycosidases—For analysis of the effects of human serum on the electrophoretic mobility of 125I-NGF, dialyzed for 24 h against PBS containing 0.04% (m/v) sodium azide before storage. Purification from a mixture of amino acid fluid and infant urine typically involved a starting volume of 2-4 liters. Ammonium sulfate (390 g/liter of starting material) was slowly added to the chilled sample (4°C) with constant stirring. The sample was maintained at 4°C overnight with continued stirring and was centrifuged at 18,000 X g in a Sorvall GSA rotor. The pellet was resuspended in 200-400 ml of 20 mM ammonium acetate, pH 7.8, and this was dialyzed against 4 liters of the same buffer with three buffer changes. The dialysate was then lyophilized and reconstituted in 25-50 ml of 20 mM HEPS, pH 7.4, NGFR, was purified using a model ME20.4-Sepharose noaffinity column as described previously. The immunoaffinity column was prepared by using CNBr-activated Sepharose 4B (Sigma) derivatized with ME20.4-IgG. Purification efficiency was determined by using trace amounts of 125I-NGFR. Protein determinations were performed by the Folin-Ciocalteau (Lowry) method (23) for concentrated protein samples (urine, amino acid fluid) and by a 215 nm/225 nm spectrophotometric method (24) for purified NGFR samples.

Amino-terminal Sequencing and Compositional Analysis—Automated Edman degradation chemistry was used to determine the amino-terminal protein sequence. An Applied Biosystems, Inc., model 477A gas phase sequencer was employed. The Oase sequencing procedure was performed. The respective phenylthiohydantoin derivatives were identified by reverse phase high performance liquid chromatography analysis in an on-line fashion employing an Applied Biosystems, Inc., model 120A PTH Analyzer fitted with a Brownlee PTH C18 column (inner diameter, 2.1 mm). Compositional analysis was performed on a Beckman 6300 high performance analyzer.

Purification of NGFR—Purification from adult urine was performed by a combination of ion exchange and immunofluorescence chromatographies. Eighty to 100 ml of urine was collected in 4-liter aliquots. To each aliquot approximately 15 ml of 5 N NaOH was added to alkalinize the sample to pH 8.0. These aliquots were maintained at 4°C for at least 10 h but not more than 5 days to precipitate the proteins. The aliquots were then filtered over gauze followed by a Whatman No. 4 filter before loading onto 500 ml of DEAE-Sepharose Fast Flow (Pharmacia Biotech Inc.). After the column was prepared by using CNBr-activated Sepharose 4B (Sigma) derivatized with ME20.4-IgG. Purification efficiency was determined by using trace amounts of 125I-NGFR. Protein determinations were performed by the Folin-Ciocalteau (Lowry) method (23) for concentrated protein samples (urine, amino acid fluid) and by a 215 nm/225 nm spectrophotometric method (24) for purified NGFR samples.
Identification of NGF-binding Species in the Conditioned Medium of A875-cultured Human Melanoma Cells—Incubation of 2 nM $^{125}$I-NGF with the conditioned medium of A875 cells followed by cross-linking with 1-ethyl-3-(3-diethylammoniopropyl)carbodiimide and immunoprecipitation with the anti-human NGFR mAb ME20.4 resulted principally in the labeling of a 66-kDa species (Fig. 1, lane 3). Minor $^{125}$I-NGF-labeled molecular complexes were also visualized at 50 and 85 kDa. The labeling was specific since it was eliminated by the addition of excess nonradioactive NGF (Fig. 1, lane 4). We presumed this 66-kDa molecule to be analogous to the truncated receptor, and so named it NGFR.  The entire membrane-associated receptor was identified in ME20.4-IgG immunoprecipitates of solubilized A875 cells affinity labeled with $^{125}$I-NGF (Fig. 1, lanes 1 and 2). As shown by others (9), the principal species specifically labeled in this case has a mass of $\approx85$ kDa. Minor species were also detected at approximately 190, 110, 66, and 55 kDa, all of which were specific for NGF binding. When the molecular mass of monomeric NGF (13 kDa) is taken into account for the most intensely labeled NGFR species, an apparent molecular mass of $\approx83$ kDa for the truncated receptor and $\approx72$ kDa for the intact receptor is obtained. Further controls demonstrated that (a) a molar excess of unlabeled cytochrome c (a molecule similar in size and isoelectric point to NGF) did not attenuate the labeled receptor signal; (b) the labeled species could not be immunoprecipitated by the mAb 192-IgG specific for rat NGFR; (c) no labeled species could be immunoprecipitated from nonconditioned medium for A875 cells; and (d) ME20.4-IgG failed to immunoprecipitate rat NGFR on PC12 cells or NGFR, from PC12 conditioned medium (data not shown).

In addition, the $^{125}$I-NGF-labeled NGFR, present in the conditioned medium of A875 cells was specifically precipitable by wheat germ agglutinin (Fig. 1, lanes 5 and 6), a lectin recognizing N-acetyl-β-D-glucosamine- or N-acetylneuraminic acid-containing oligosaccharides. The apparent molecular mass of this lectin-precipitated species was equivalent to that associated with the immunoprecipitated species. Since wheat germ agglutinin can bind to the cell surface-associated NGFR (22), it appears that the 66-kDa-labeled complex is a truncated form of the NGFR which maintains at least one lectin binding site.

Identification of NGFR, in Human Amniotic Fluid and Urine—The existence of a human NGFR, was demonstrated not to be strictly an in vitro phenomenon. Examination of human amniotic fluid and urine by immunoprecipitation after $^{125}$I-NGF affinity labeling revealed the presence of specific NGF-binding molecules (Fig. 2). However, unlike the tissue culture condition, three distinct NGFR species were detected in amniotic fluid (panel A) and infant urine (panel B) with apparent molecular masses of 66, 60, and 50 kDa (includes mass of monomeric NGF) when analyzed on a 7% polyacrylamide gel. Because the apparent molecular masses varied according to the gel's acrylamide content, a phenomenon typically seen with glycoproteins (29), we labeled these specific NGF-binding proteins as NGFR-1, NGFR-2, and NGFR-3, respectively. All amniotic fluids tested (obtained...
from pregnancies ranging from 14 to 35 weeks) and urines from infants less than 2 years old were shown to contain this triad of NGF-binding molecules. The relative autoradiographic intensity of labeled molecules within this triad was not consistent from sample to sample, although typically the NGF-NFR,-1 complex was present in the greatest amount (data not shown). Furthermore, there was no discernible temporal relation of labeling intensity of NGF-binding molecules in amniotic fluids throughout gestation (data not shown). However, with increasing postnatal age, the autoradiographic intensity of immunoprecipitable NGF-binding species in urine diminished. By adulthood, only the NFR,-1 species was faintly detectable above background (Fig. 2C).

**Purification of Human NFR,-1**—Initial purification efforts used adult urine as starting material since this was readily available in large volumes. Purification, employing a combination of ion exchange chromatography and immunoaffinity chromatography, was performed as described under “Experimental Procedures.” Seven separate preparations resulted in yields of ≈1 μg/liter of urine. A silver-stained 10–15% gradient polyacrylamide gel of the immunoaffinity column eluate is shown (Fig. 3A) revealing two distinct bands at ≈45 and 35 kDa. This eluate was subjected to amino-terminal sequence analysis and gave the following sequence: Lys-Glu-Ala-X-Pro-Thr-Gly-Leu-Tyr-X-(Gly)-(Ser,Asp)-(Gly)-(Glu). Here, X represents undefined residues (cysteines cannot be assigned without prior alkylation, and modified amino acids may not be identified), and residues in parentheses signify tentative assignments with recoveries too low for a definitive designation. The sequence was compared with the human NFR,-1 cDNA nucleotide-derived sequence (13) of Lys-Glu-Ala-Cys-Pro-Thr-Gly-Leu-Tyr-Thr-His-Ser-Gly-Glu, which demonstrated that the isolated protein is a truncated portion of the NFR containing the amino terminus. No interfering signals were detected during each cycle, suggesting that either (a) both molecular species shared the same amino-terminal sequence; (b) one species was chemically blocked at the amino terminus; or (c) one species gave residue signals too low to detect. A further indication of purity was provided by iodination of 20 μg of this sample followed by SDS-PAGE and autoradiography. Only two bands, corresponding in molecular mass to the two silver-stained proteins, were visible (data not shown).

Because infant urine and amniotic fluid contain significantly more NGFR,-1 than adult urine (see Fig. 2) and because previous work (18) indicated that rat urine on postnatal day 1 is a 50–100-fold more concentrated source of NFR,-1 than on postnatal day 28, we purified human NGFR,-1 from a mixture of infant urine (both male and female in origin) and amniotic fluid by using a combination of ammonium sulfate precipitation and immunoaffinity chromatography. From eight successive purifications, we obtained an average yield of ≈75 μg of NFR,-1/liter of starting material. Protein determinations of NFR,-1 were based on the spectrophotometric method of Waddell (24), the accuracy of which was verified by Lowry assay (23) and amino acid compositional analysis. NFR,-1 yields represent 0.001–0.002% of total adult urinary protein or total amniotic fluid protein. Estimations of the efficiency of purification were made by using 125I-NFR,-1 tracer. In two consecutive preparations, 51.3 and 33.3% of incorporated label added to the starting solution were recovered. Assuming a 40% recovery, we estimate that NFR,-1 in pooled samples of amniotic fluid is at a concentration of ≈190 ng/ml. A silver-stained 12% polyacrylamide gel of the immunoaffinity column eluate from one preparation is shown (Fig. 3B). Here, three species are evident with molecular masses of 45, 40, and 35 kDa. Iodination, SDS-PAGE, and subsequent autoradiography revealed the sample to be virtually free from contaminating proteins (Fig. 3C). These three species were successfully electroeluted separately after SDS-PAGE (12%) and displayed amino-terminal sequence identities to the amino-terminal cDNA-derived sequence (see above).

An iodinated sample of these proteins immunopurified from amniotic fluid and infant urine was subsequently analyzed by two-dimensional electrophoresis under reducing or nonreducing conditions (Fig. 4). In either experiment, NFR,-1, -2, and -3 displayed acidic isoelectric points with ≈5–10 isoforms apparent within each species. NFR,-3 migrated in two major

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**Fig. 3. Identification of purity of NFR,-1 by affinity chromatography.** Panel **A** demonstrates silver staining of two NFR,-1 species (NFR,-1 and -2) isolated from adult human urine. Load was 15 ng, and separation was 10–15% gradient SDS-PAGE. Panel **B** is a silver-stained 12% polyacrylamide denaturing gel of NFR,-1 species (NFR,-1, -2, and -3) isolated from human amniotic fluid/infant urine. Panel **C** demonstrates an autoradiogram of 12% polyacrylamide denaturing gel used to separate 125I-labeled NFR,-1 isolated from human amniotic fluid. Molecular mass markers (in kDa) are as indicated.

**Fig. 4. Two-dimensional gel analysis of 125I-NFR,-1 purified from amniotic fluid/infant urine.** Nonreduced sample (106 cpm) was isoelectrically focused on a horizontal slab gel as described under "Experimental Procedures." A lane was removed and annealed to the top of an SDS-containing molecular sieving gel for size separation. An autoradiogram of the dried gel is depicted with positions of NFR,-1 labeled. Mean pI values are 3.3 for NFR,-1, 3.6 for NFR,-2, 3.8 for NFR,-3a, and 3.95 for NFR,-3b. Species tentatively assigned NFR,-4 and -5 are indicated. The more acidic end of the gel is on the left, and heavier molecular mass species are at the top. NFR,-1 isoforms are labeled, and the inset demonstrates a miniaturized isoelectric focusing gel used for assigning pI values.
isoform groupings. Identical results were obtained for NGFR, focused under nonreduced nondenaturing conditions, indicating absence of a carbamylation artifact. Mean pI values for each species were 3.3 for NGFR-1, 3.6 for NGFR-2, and 3.8 and 3.95 for NGFR-3. We have designated the more acidic grouping for NGFR-3, NGFR-3a and the more basic grouping NGFR-3b. Upon prolonged exposure, two other acidic isoform groupings were noted having apparent molecular masses of 30 and 20 kDa. We have tentatively named these NGFR-4 and -5, respectively, but offer them no further consideration at this time.

Further evidence that all three major species are indeed NGFR, was based on 125I-NGF affinity-labeled immunoprecipitation studies. When these were performed with the amniotic fluid/infant urine-purified proteins, all three protein species were specifically labeled and had apparent molecular masses of 60, 50, and 45 kDa (mass of monomeric NGF included), as determined by SDS-PAGE (12%). These species were presumed to be NGFR-1, -2, and -3 specifically immunoprecipitated from amniotic fluid.

To estimate the apparent binding affinities of the three species for NGF, we performed an experiment in which 100 ng of the NGFR, preparation was bound to 1 nM 125I-NGF in the presence of varying amounts of unlabeled NGF (ranging from 0 to 128 nM). The areas of the gel corresponding to autoradiographically labeled bands were isolated, their radioactivity determined, and results plotted (Fig. 5). The unla-beled NGF displaced 125I-NGF with an IC50 of approximately 15 nM in each of the NGFR, species. The data indicate that the binding affinities of the NGFR, species approximate that previously ascribed to the cell-associated low affinity receptor (30).

Relationships of NGFR-1, -2, and -3—A purified sample of NGFR, (8.6 μg), isolated from amniotic fluid and infant urine, was radioiodinated to a specific activity of 1955 cpmlfmol and resulted in the labeling of all three bands present on silver-stained gels. Incubations of this sample at 35 °C with 1% neuraminidase treatment. An aliquot of the two samples above was loaded on human NGFR, species.

A variant sample of NGFR, (3.3 μg), isolated from amniotic fluid and infant urine, was radioiodinated to a specific activity of 1955 cpmlfmol and resulted in the labeling of all three bands present on silver-stained gels. Incubations of this sample at 35 °C with 1% neuraminidase treatment. An aliquot of the two samples above was loaded on human NGFR, species.

FIG. 5. Determination of relative apparent binding affinities of NGFR-1, -2, and -3 isolated from amniotic fluid and infant urine. 100 ng of NGFR, was assayed in each tube in the presence of 1 nM 125I-NGF and various amounts of unlabeled NGF. After cross-linking and immunoprecipitation, samples were resolved by SDS-PAGE (12%), and areas of the gel corresponding to autoradiographically labeled bands were isolated and their radioactivity measured. Ordinate values represent counts per minute (cpm) of a gel area corresponding to the specific gel zone in the lane containing no unlabeled NGF. The experiment was repeated a second time with essentially identical results. IC50 values for NGFR-1, -2, and -3 occur at 16, 15, and 21 nM unlabeled NGF, respectively.

FIG. 7. Demonstration of the presence of sialic acid residues on human NGFR, species. Panel A displays the increase in apparent molecular mass after neuraminidase treatment. 125I-NGFR, was incubated for 12 h at 35 °C in buffer containing (+) or not containing (−) neuraminidase. Samples were boiled in denaturing electrophoresis buffer, which lacked reductant, prior to separation on SDS-PAGE (12%). Molecular mass markers (in kDa) are indicated on the left. Panel B displays the cathodic shift of NGFR, isoforms after neuraminidase treatment. An aliquot of the two samples above was loaded at the cathodic end (bottom) of a 5% polyacrylamide isoelectric horizontal slab gel and focused for 3 h. Values for pH are marked at intervals on the right.
Truncated NGF Receptor Purification and Characterization

Truncated NGFR, with neuraminidase resulted in an unexpected increase in apparent molecular mass (Fig. 6). This result was verified and more clearly demonstrated on SDS-PAGE when the sample was boiled but not reduced (Fig. 7A). Presumably, a low level of binding of SDS to the heavily glycosylated NGFR, coupled with the loss of negative charges carried by sialic acid residues resulted in this increase in apparent molecular mass. Fig. 7B demonstrates support for this hypothesis. Isoelectric focusing of 125I-NGFR reveals a dramatic cathodic shift when sample was pretreated with neuraminidase, as above, indicating the removal of many negatively charged sugars.

**Discussion**

The phenomenon of release of surface proteins from intact cells incubated in a buffered serum-free medium has been described (31). Both the insulin receptor (32) and the growth hormone receptor (33) have been detected in cultured human lymphocyte-conditioned medium, being spontaneously solubilized without the use of detergents. Although these soluble proteins exist as intact receptors closely resembling their membrane-bound counterparts, there is also precedent for the existence of truncated forms of membrane-associated proteins detectable in the extracellular milieu. Fragmented portions of the epidermal growth factor receptor (34), neural cell adhesion molecule (35), and the major histocompatibility Qa-2 antigen (36) are thought to be generated by alternative splicing of a single transcribed gene product, whereas those of the interleukin 2 receptor (37) and the complement receptor type 2 protein (38) are believed to be products of cell-derived proteases. The significance of NGFR, elaboration is unclear. Possible reasons for its appearance in extracellular fluid include: (a) a normal pathway for general protein turnover; (b) a mechanism by which NGF levels can be regulated (NGFR serving as a sink and/or carrier protein); or (c) a separate biological role(s).

Results of this study indicate that there exists a human correlate of NGFR, elaboration studied previously in rat (18). Our study was enabled by the availability of the human-specific anti-NGFR mAb ME20.4-IgG, which can precipitate the NGFR,125I-NGF complex. When this immunoprecipitation was performed on a sample of conditioned medium from the human melanoma cell line A875 and the precipitate was analyzed by autoradiography subsequent to SDS-PAGE (7%), a 86-kDa species (NGFR,125I-NGF complex) was specifically labeled. Specificity is delineated by displacement with excess unlabeled NGF, lack of displacement by excess unlabeled cytochrome c, and an inability to visualize such a species upon testing of nonconditioned medium or on immunoprecipitating conditioned medium with the rat-specific antireceptor mAb 192-IgG. (Presumably, nonconditioned medium, supplemented with fetal calf serum, contains bovine NGFR; however, this is not recognized by ME20.4.)

As is the case in the rat, human NGFR, can also be immunoprecipitated from extracellular fluids, namely urine and amniotic fluid, with amounts of immunoprecipitable NGFR-NGF complex being significantly higher in samples from developmentally young humans and declining with increasing age. Because NGFR, is presumably heavily glycosylated (>10% by mass), a value for its apparent molecular mass, as determined by SDS-PAGE, will be critically dependent on acrylamide concentration (29). As this concentration increases, the electrophoretic mobility of a glycoprotein increases with reference to nonglycosylated standards, and a true molecular mass is asymptotically approached. Because of this, we have used the designations NGFR,1, NGFR,2, and NGFR,3 for the 45-, 40-, and 35-kDa species identifiable on SDS-PAGE (12%). In amniotic fluid and in the urine of infants, all three NGFR, species were immunoprecipitated, with NGFR,1 typically being most prevalent. This finding differs from the in vivo rat model in which only one species can be visualized. We suspect this disparity reflects a difference in the antigenic binding site on the receptor molecule between ME20.4-IgG and 192-IgG. The former antibody may recognize a site on the receptor molecule very near the NGF-binding site (as its presence precludes NGF binding), whereas the latter antibody may bind at some distance from the ligand-binding pocket. We cannot, however, exclude the possibility that this difference is in part, or totally, species related. In urine from human adults, only NGFR,1 remains detectable using the affinity-labeled immunoprecipitation assay. However, purification from adult urine produces two species of truncated receptor: NGFR,1 and NGFR,2, with the former being consistently more prevalent. Such a purification yields ~1 μg/liter of starting material.

A more abundant source of NGFR, has been amniotic fluid and infant urine from which we have purified ~75 μg/liter of starting material. This process resulted in the isolation of three protein species that we assume are the same three visualized autoradiographically after immunoprecipitation of 125I-NGF affinity-labeled specimens (i.e. NGFR,1,2, and -3). Verification that the protein species isolated were true NGFR, molecules is based upon amino-terminal sequence data of purified preparations which match cDNA-derived sequence data for the amino-terminal portion of the human NGFR.

The determination of protein content was based on the spectrophotometric method of Waddell (24). This method has proven to be the most advantageous for its simplicity, sensitivity, and lack of protein destruction. Its accuracy was verified both by Lowry analysis (23) and amino acid compositional analysis. The Bradford spectrophotometric assay (39) was not useful since NGFRs appear to be poor Coomassie Blue binders (as evidenced by their resistance to this stain on electrophoretic gels). This quality is common to acidic glycoproteins, a class of molecules to which the NGFR belongs. Two-dimensional gel electrophoretic data indicated that the isoelectric points of the purified proteins ranged from 3.3 to 3.95. Although these values are more acidic than the value of 5 first determined for the membrane-associated receptor (40), 86% of the net negative charge is distributed over the extracellular domain (13). This also ignores the presence of negatively charged carbohydrate residues. Further evidence that these isolated species are heavily glycosylated proteins includes their shifts in electrophoretic mobility after treatment with endoglycosidases, their appearance on polycrylamide gels as ill-defined bands, their characteristic reddish color upon silver staining, and their increase in apparent electrophoretic mobility with increasing acrylamide concentrations (29). The presence of abundant sialic acid residues is also demonstrated by the cathodic shift of NGFR, when isoelectrically focused after treatment with neuraminidase. This confirms the determi-
nation of sialic acid residues on intact NGFR from PC12 cells (41).

At this point, we cannot surmise why a difference exists between the number of immunoprecipitable species in vivo as compared with in vitro. In agreement with molecular studies indicating the existence of only one NGFR message (42), we suggest that NGFR-1 is most probably generated from proteolytic cleavage of the intact cell surface receptor. Potential serine protease cleavage sites are present near the juncture of the receptor’s extracellular and transmembrane domains. NGFR-1 species did not generate smaller species in culture medium or upon incubation with human serum or amniotic fluid. Differences among NGFR species, however, appear not to be strictly carbohydrate related. Because all three species share the same amino terminus, we propose that proteolytic processing at the carboxyl terminus constitutes the major difference in their apparent molecular masses. Furthermore, results from glycosidase treatment experiments suggest residue Asn-32, the only asparagine identifiable by cDNA se-

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