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Identification of a truncated form of the nerve growth factor receptor

(Schwann cell/axotomy/development/urine composition/plasma composition)

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ABSTRACT **Schwann cells express growth factor (NGF) receptors on their cell surface in response to axotomy, a phenomenon that can be demonstrated both *in vivo* and *in vitro*. The predominant form of the NGF receptor on Schwann cells exists as an ≈80-kDa band, as determined by NaDodSO$_4$/PAGE. We demonstrate that cultured Schwann cells shed a truncated (50-kDa) form of the NGF receptor (NGF-Rt) into their medium. Other cell types that shed the NGF-Rt into medium include a rat schwannoma and, to a lesser extent, PC12 cells and superior cervical ganglion neurons. NGF-Rt was not found in media conditioned by mixed neuron/glia cultures from various brain regions, or anterior pituitary cells derived from rat. *In vivo*, NGF-Rt was present in neonatal rat urine, and its presence was developmentally regulated: levels were high in postnatal day-1 rat urine and declined to low, but detectable, levels by weeks 4 and 8. NGF-Rt was also found in amniotic fluid and in the stomach contents of fetal rats. Maternal urine (pre- and postnatal) had slightly elevated NGF-Rt levels over normal adult urine. NGF-Rt was detected in rat plasma and showed developmental regulation similar to that found for urine. In addition, a 77-kDa receptor species was detected in plasma during early development. Finally, NGF-Rt was significantly elevated in the urine of adult rats with bilateral sciatic nerve lesions. These findings suggest that the developmentally regulated release of NGF-Rt, present in plasma and other body fluids, plays a regulatory role in nervous system development.**

Nerve growth factor (NGF) exerts its effects on NGF-dependent neurons by interacting with specific, high-affinity receptors located on the neuronal cell surface (1–5). NGF receptors have been extensively characterized on peripheral sympathetic neurons (6, 7), neural crest-derived sensory neurons (8), as well as a variety of tumor cell lines (9–11). NGF receptors have also been localized to neurons, predominantly cholinergic, of the central nervous system (12–15). In addition to their neuronal localization, NGF receptors have recently been identified on cultured ganglionic Schwann cells (16–18) and on rat peripheral nerve Schwann cells both *in vivo* and *in vitro* (19, 20). Schwann cells in the distal portion of lesioned adult sciatic nerve increase their NGF receptor content with time after injury (19). Likewise, Schwann cells isolated from neonatal rat sciatic nerve increase their NGF receptor number with time in culture (20). These experiments indicate that Schwann cells express NGF receptors on their cell surface in response to loss of axonal contact. We have proposed that expression of NGF-binding sites on the Schwann cell surface sequesters NGF (which is probably secreted by the Schwann cell) and thus guides NGF-dependent axons during regeneration and possibly development (19, 20).

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In these studies we have discovered that Schwann cells and, to a lesser extent, PC12 cells and superior cervical ganglion (SCG) neurons, shed a truncated form of the NGF receptor (NGF-Rt) into their medium when maintained in culture. In addition, NGF-Rt appeared in amniotic fluid, neonatal urine, and neonatal plasma samples in surprisingly high amounts; NGF-Rt levels declined in body fluids as development proceeded. Finally, lesion of adult peripheral nerve, which induces Schwann cell NGF receptor levels (19), resulted in rapid increases in urinary NGF-Rt levels that persisted for at least 2 weeks. NGF-Rt *in vitro* and *in vivo* is characterized here, and its potential biological significance is discussed.

MATERIALS AND METHODS

Cell Culture. Schwann cells from postnatal day 1 (P1) rats were cultured by the method of Brockes *et al.* (21) modified as described (20). SCG neurons and PC12 cells were cultured as described (22). The JS-1 cell is a rat schwannoma and was the gift of D. Schubert and J. LeBeau (Salk Institute, La Jolla, CA). JS-1 cells were grown in 10% fetal calf serum (Hazelton, Denver, PA)/1% glutamine/89% Dulbecco's modified Eagle's medium (GIBCO) containing penicillin (100 international units/ml) and streptomycin (100 μg/ml). Mixed cultures of neurons and glial cells from several brain regions (septum, cortex, hippocampus, and cerebellum) were cultured essentially as described (23).

Animal Treatments. Timed-pregnant rats and various aged rats (all Sprague–Dawley) were obtained from Sasco (Madison, WI). Urine from adult rats was obtained over 3- to 24-hr periods by using metabolic cages. Urine from younger rats (P1–P14) was obtained from bladder puncture with a 25-gauge needle. Amniotic fluid was also extracted from embryos with a 25-gauge needle. All blood samples were collected from the trunk of decapitated rats and treated with heparin. Plasma was separated from blood cells by centrifugation at 10,000 × *g* for 2 min. Urine and plasma samples were stored at −20°C until assayed for NGF receptor species. NGF-Rt was stable for at least 3 mo when so stored. Bilateral sciatic nerve lesions were done in 6-week-old rats. Rats were anesthetized with pentobarbital (50 mg/kg), and sciatic nerves were exposed. Nerves were cut at the level of the tendon of the obturator internus muscle, and wounds were closed with 3-0 silk suture. Three-hour urine samples were collected from rats at 1, 4, 7, and 14 days postlesion and stored at −20°C until assayed for NGF-Rt.

Abbreviations: E, embryonic day; P, postnatal day; NGF, nerve growth factor; NGF-Rt, truncated form of nerve growth factor receptor; SCG, superior cervical ganglion; ^{125}I-NGF, ^{125}I-labeled NGF.

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Assay of NGF Receptor Species. NGF (2.5S) was purified from male mouse submaxillary glands (24). NGF was iodinated (25) to obtain specific activities ranging from 1500–3000 cpm/fmol. NGF receptor was assayed and quantitated on Schwann cells, PC12 cells, JS-1 cells, and SCG neurons by using a described two-site immunoprecipitation assay (19, 20, 26). Soluble NGF receptor species were assayed in conditioned media, urine, amniotic fluid, stomach contents, and plasma samples by a modification of this method. Briefly, fluids were centrifuged twice at 10,000 × *g* for 3 min, or filtered through a 0.2-μm filter before assay. Various amounts (5–100 μl) of media, urine, or plasma were added to radioiodinated NGF (^{125}I-NGF) (2 nM, final concentration) to a final volume of 200 μl. Samples were buffered with 20 mM phosphate-buffered saline containing 0.1% bovine serum albumin. Nonspecific binding was determined by incubating the identical sample with a 300-fold excess of unlabeled NGF. ^{125}I-NGF was allowed to bind for 1 hr at room temperature or on ice; temperature of the binding reaction had no apparent effect on binding signal or on amount of nonspecific binding. ^{125}I-NGF was crosslinked to receptor species with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) followed by immunoprecipitation with the anti-rat NGF receptor monoclonal antibody, 192-IgG (27) as described (19, 20). Receptor species were resolved on NaDodSO$_4$/polyacrylamide gels and visualized autoradiographically by using Kodak X-OMAT AR-5 film (Eastman Kodak). In some experiments data were analyzed quantitatively by excising portions of the gel that corresponded to autoradiogram bands and determining their radioactivity in a Beckman gamma counter (model 5500). Data were expressed as a fraction, relative to highest level counted (in each fluid). NGF-Rt was reliably detectable in as little as 5 μl of P1 urine and 40 μl of P1 plasma.

Materials. Na^{125}I (100 mCi/ml, 1 Ci = 37 GBq) was obtained from Amersham. Mouse myeloma protein, MOPC 21, was obtained from Organon Teknika (Malvern, PA). MOPC 21 is an intact IgG1 immunoglobulin and was used as a nonspecific control antibody to compare with 192-IgG, which is also an IgG1 immunoglobulin. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from Pierce. All other reagents were obtained from Sigma.

RESULTS

During the experiments characterizing NGF receptors on Schwann cells (20) we discovered that conditioned medium from primary Schwann cell cultures assayed positively for a NGF receptor-like species. The cellular NGF receptor species found on SCG neurons (Fig. 1*A*, lane 1) and Schwann cells (Fig. 1*A*, lane 3) are shown. Note that, as described (20), the ^{125}I-NGF crosslinked receptor species on Schwann cells migrated slightly more slowly than did the neuronal receptor, although both had apparent molecular masses of ≈90 kDa. The predominant crosslinked binding site in the medium represented a smaller, or truncated, form with a mean molecular mass of 63 kDa (Fig. 1*A*, lane 5). Subtracting a monomer of NGF (13 kDa) from cellular and truncated forms of the receptor yielded apparent molecular masses of ≈77 kDa and 50 kDa, respectively. In some experiments Schwann cell-conditioned medium contained both the 50- and 77-kDa NGF receptor species but, invariably, the predominant form was the 50-kDa band; in many cases only the 50-kDa band was seen. Conditioned media from other cell types was also assayed for NGF-Rt. The JS-1 cell line, a rat schwannoma, has NGF receptors on its cell surface (Fig. 1*B*, lane 9). JS-1 cells, like their normal Schwann cell counterparts, shed NGF-Rt into their medium (Fig. 1*A*, lane 7). Conditioned media from PC12 cells and SCG neurons also contained NGF-Rt (Fig. 1*A*, lanes 9 and 11, respectively); however, SCG neurons consistently largely shed the 77-kDa species

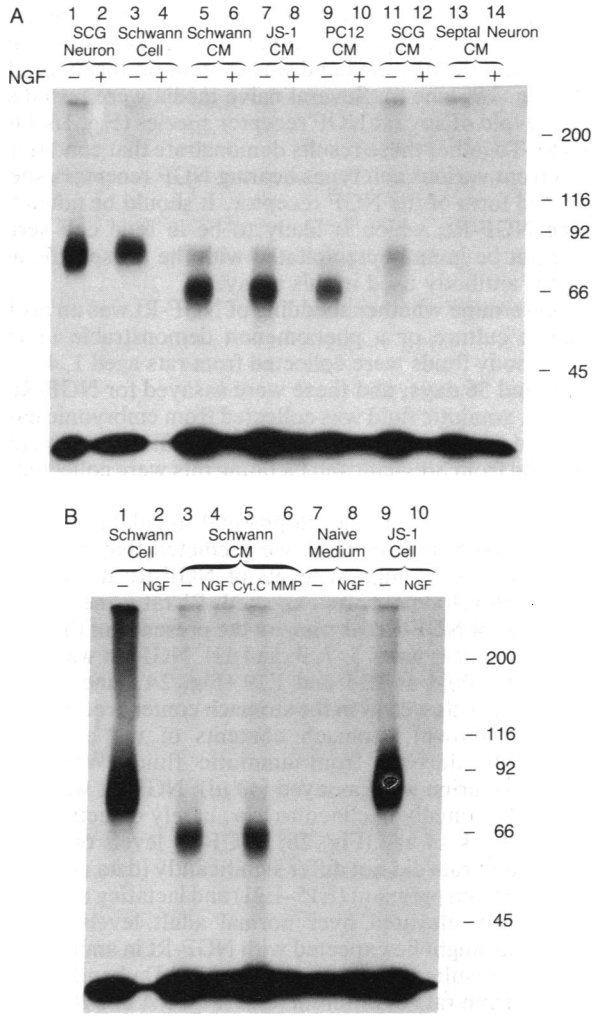

FIG. 1. (*A*) Identification of NGF-receptor species in various cultured cells and cell-conditioned media (CM). Samples were prepared as described and separated on NaDodSO$_4$/7% polyacrylamide gels; subsequent autoradiograms were generated as described. Even-numbered lanes correspond to nonspecific binding of material in the lanes at left. For cells (lanes 1–4), bands correspond to a mean molecular mass of 77 kDa. The predominant species in media migrates at 50 kDa. The ^{125}I-NGF crosslinked to NGF receptor species is dimeric; upon denaturing gel electrophoresis, a monomer of ^{125}I-NGF dissociates from the crosslinked complex, resulting in the radioactivity seen at the leading edge of the gel (bottom). Gel autoradiograms are representative of four independent experiments. Molecular mass markers are at right. (*B*) Characterization of ^{125}I-NGF crosslinked to NGF-Rt from Schwann cell-conditioned medium. Binding was blocked by an excess of NGF but not by cytochrome *c* (Cyt.C). Immunoprecipitation with the mouse myeloma protein (MMP) yielded no receptor species on the autoradiogram. Results are compared with Schwann cell NGF receptor (lanes 1 and 2) and JS-1 cell receptor (lanes 9 and 10).

and shed only small amounts of the 50-kDa (truncated) form. NGF-Rt was not detectable in conditioned media from mixed neuron/glial cultures from rat cerebral cortex, hippocampus, septum, or cerebellum as cultured in these experiments (see Fig. 1*A*, lane 13). Possibly NGF-Rt is shed by central neurons or glia, but levels are significantly lower than peripheral NGF receptor-bearing cells. Also, conditioned medium from rat anterior pituitary cells (NGF-receptor negative) grown in primary culture was devoid of NGF-Rt (data not shown).

Further characterization of NGF-Rt in Schwann cell-conditioned media showed that addition of excess unlabeled NGF in the ^{125}I-NGF binding step completely blocked binding (Fig. 1*B*, lane 4), whereas 1 μM cytochrome *c* did not

block binding (Fig. 1*B*, lane 5). When NGF–NGF receptor complexes were immunoprecipitated with the mouse myeloma protein (MMP), no band was evident on the autoradiogram (Fig. 1*B*, lane 6). Several naive media were tested and found devoid of any rat NGF receptor species (Fig. 1*B*, lanes 7 and 8). Together these results demonstrate that conditioned media from various cell types bearing NGF receptors shed a truncated form of the NGF receptor. It should be noted that bovine NGF-Rt, which is likely to be in fetal calf serum, would not be immunoprecipitated with the rat-specific anti-receptor antibody used in this assay.

To determine whether shedding of NGF-Rt was an artifact of tissue culture or a phenomenon demonstrable *in vivo*, various body fluids were collected from rats aged 1, 4, 7, 10, 14, 28, and 56 days, and these were assayed for NGF-Rt. In addition, amniotic fluid was collected from embryonic day 15 (E15) and E19; blood was also collected at E19. Finally, urine and blood from pregnant and lactating rats were collected and assayed for NGF-Rt. From previous studies in this laboratory (20, 26) investigating developmental regulation of NGF receptors on Schwann cells, we predicted that body fluids would contain the highest levels of NGF-Rt at the earliest stages of development. As expected, P1 rat urine contained high levels of NGF-Rt, as seen by the presence of the 50-kDa species (Fig. 2*A*, lanes 5, 7, 9, and 11). NGF-Rt was present in amniotic fluid at E15 and E19 (Fig. 2*A*, lanes 1 and 3, respectively), as well as in the stomach contents of rats of E19 (data not shown); stomach contents of the embryo are presumably derived from amniotic fluid. When equal amounts of urine were assayed (40 μl), NGF-Rt was highest at P1 and gradually declined to low, barely detectable levels at 4 and 8 wk of age (Fig. 2*B*). NGF-Rt levels of male and female adult rats did not differ significantly (data not shown). The urine from pregnant (E15–E21) and lactating rats (P1–P4) was slightly elevated over normal adult levels (data not shown), as might be expected with NGF-Rt in amniotic fluid.

Similar results were seen with the 50-kDa band in plasma samples from rats of different ages (Fig. 3 *A* and *B*). NGF-Rt levels were highest in fetal plasma at E19 with diminishing levels at P1 through adulthood. As with urine, maternal plasma samples were slightly higher in NGF-Rt content than normal adult plasma samples (see Fig. 3 *A* and *B*). Interestingly, the binding of ^{125}I-NGF to the putative 50-kDa protein in adult plasma samples (4 and 8 wk old) was not blocked by excess unlabeled NGF, indicating possible developmental changes in this protein by adulthood or, alternatively, that the 50-kDa protein is not an NGF receptor species and that NGF binds nonspecifically to a 50-kDa protein found in adult, but not neonatal rat blood. Also present in the plasma chromatogram was a 77-kDa protein species, detectable at P1, peaking at P7, and subsiding to nondetectable levels by adulthood (Fig. 3 *A* and *B*). This band is reminiscent of the *intact* receptor (cell surface receptor, 77 kDa). Exactly what cell type is responsible for the appearance of this protein in plasma is uncertain. The 77-kDa species is presumably NGF receptor because it specifically binds NGF (Fig. 3*A*, lanes 3–10) and is recognized by 192-IgG. This higher-M_r species was not seen in urine, presumably because the glomerulus would not filter it.

From *in vitro* and *in vivo* studies with NGF-Rt we presently believe that Schwann cells are the major cell type secreting or shedding NGF-Rt. To further investigate Schwann cell involvement in elaborating NGF-Rt, sciatic nerves of adult rats were sectioned, thereby inducing Schwann cells in the nerve distal to the section to express high levels of NGF binding sites on their cell surface shortly after lesion. Three-hour urine samples were collected from adult (6 wk) sciatic nerve-lesioned rats at 1, 4, 7, and 14 days postlesion. Urine samples were assayed for NGF-Rt along with prelesioned urine samples from the same rats. Fig. 4 shows that a significant increase in NGF-Rt was seen as early as 1 day postlesion and persisted for 2 wk after injury.

DISCUSSION

Results of this study show that Schwann cells, the JS-1 schwannoma cell line, and, to a lesser extent, SCG neurons and PC12 cells shed a truncated form of NGF receptor into their tissue culture media. We suggest that the receptor originates from the cells, as several tested naive media were negative for rat NGF-Rt. Further, the media from rat cells not bearing NGF receptors were devoid of NGF-Rt. The 50-kDa band is NGF receptor-like because the binding of NGF is specific (displaced by an excess of unlabeled NGF but not cytochrome *c*), and the band is recognized by the NGF receptor-specific monoclonal antibody 192-IgG but not by a nonspecific IgG1 antibody. The low-affinity NGF receptor, the only species present on rat Schwann cells, is known to be trypsin sensitive. Potential trypsin cleavage sites are near the base of the extracellular domain of the NGF receptor (28, 29).

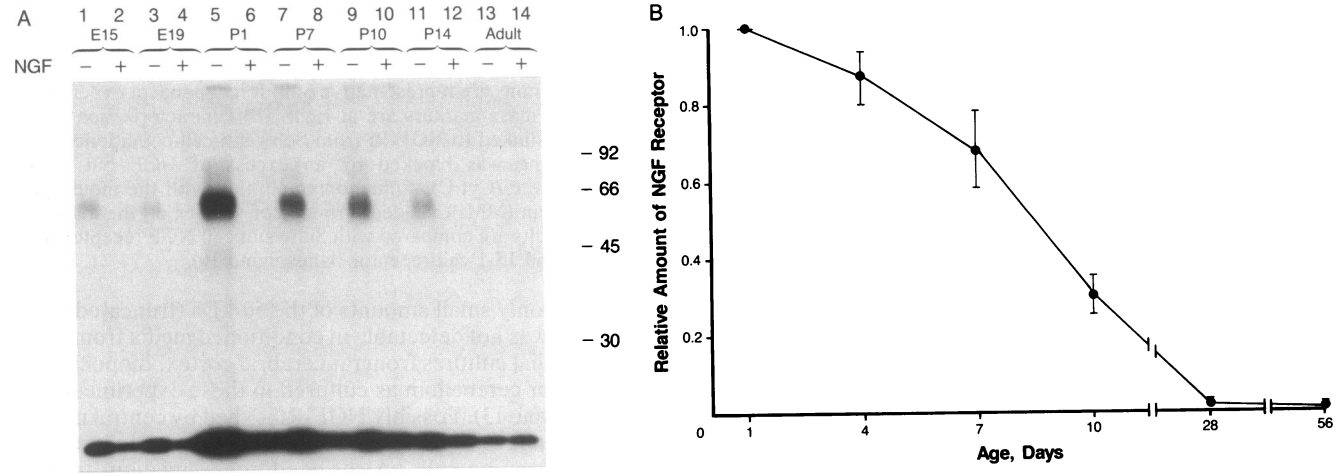

FIG. 2. (*A*) Demonstration of ^{125}I-NGF-labeled NGF-Rt in amniotic fluid and in urine of rats of different ages. Samples were collected by using a 25-gauge needle, centrifuged 10,000 × *g*, and affinity labeled with 2 nM (final) ^{125}I-NGF. Each lane was developed with 40 μl of amniotic fluid or urine. NGF-Rt was determined by separation on $NaDodSO_4$/10% polyacrylamide gels. Molecular mass standards are indicated at right. (*B*) Quantitative analysis of urine NGF-Rt levels from Fig. 2*A*. Gels were excised under the areas corresponding to bands on the autoradiogram and radioactivity was estimated in a gamma counter. Values are the mean ± SEM of three to seven independent determinations and are expressed relative to P1 urine.

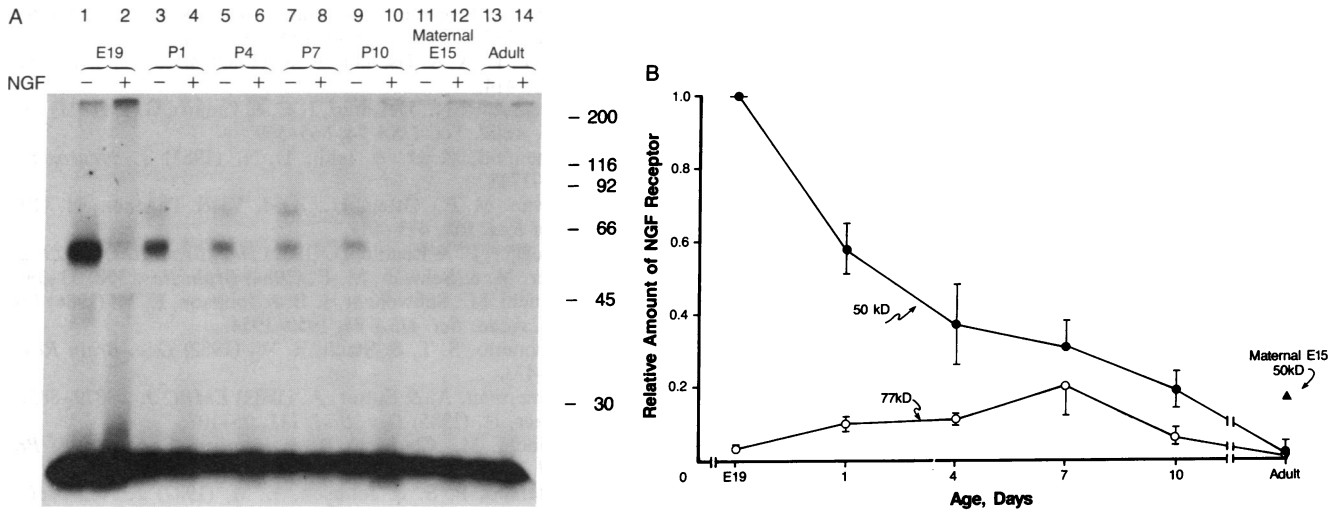

FIG. 3. (*A*) Analysis of NGF receptor species in the plasma of embryonic, neonatal, and adult rats. Binding, crosslinking, and immunoprecipitation assays were done on samples pooled from three rats at each age (except adult, $n = 1$). Each lane was developed with 100 μl of pooled plasma samples. Samples were separated on 9% acrylamide gels under reducing conditions. Notice that, in addition to the 50-kDa band, a 77-kDa band appears at ≈P1–P10 and subsides by adulthood. (*B*) Quantitative analysis of the 50-kDa (●) and 77-kDa (○) NGF-binding species in plasma samples described in *A*. Data were quantitated as in *Materials and Methods*. Values represent the mean ± SE of three to four independent determinations. Data for the 50-kDa and 77-kDa receptor species are expressed relative to the 50-kDa species of E19 plasma (=1.0). kD, kDa.

We suggest that NGF-Rt is most likely derived from proteolytic cleavage. Considering the ratio of the purported extracellular to transmembrane/cytoplasmic portions of whole NGF receptor, cleavage at this site would produce a soluble fragment (NGF-Rt) consistent with observed molecular mass.

The appearance of NGF-Rt in body fluids (urine and plasma) confirms that NGF-Rt appearance is not an artifact of tissue culture. But what cell source(s) contributes NGF-Rt to these fluids? Previous results from this laboratory suggest that Schwann cells are the major contributing cell. In studies to characterize developmental regulation of NGF receptors, peripheral nerve was found to contain high levels of NGF receptor early in development (E20–P10), gradually declining to low, barely detectable levels by adulthood (26). It was suggested that most receptors in nerve are probably located on Schwann cells (19, 20) and that NGF receptor expression on Schwann cells is suppressed as Schwann cell–axon interactions mature. In related studies Schwann cells were isolated from the sciatic nerve from E19 to P3 and assayed for NGF receptor levels (20). Significantly fewer binding sites were found on Schwann cells isolated from older animals, again indicating a developmental regulation of NGF receptor levels on Schwann cells. Interestingly, the time course of Schwann cell NGF receptor loss determined in the above studies closely parallels the decreased urine and plasma NGF-Rt levels seen during development in this study (see Figs. 2*B* and 3*B*).

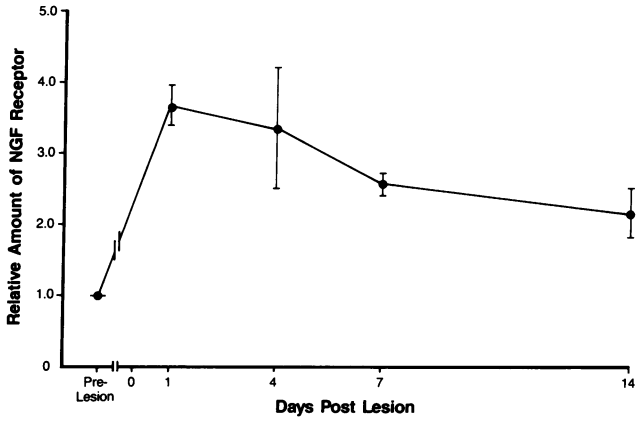

FIG. 4. Effect of bilateral sciatic nerve lesion on urinary NGF-Rt in adult rats. Six-week-old rats were used in all experiments. Surgery was done as described in the text. Urine was collected on the designated days for 3 hr. NGF-Rt was assayed in 300 μl of adult urine; data are expressed relative to prelesioned controls (=1.0) and are the mean ± SE of four rats at each time point.

Further evidence that Schwann cells contribute to urine and plasma NGF-Rt levels came from lesion studies. Lesion of adult peripheral nerve results in dramatic increases in Schwann cell NGF receptor levels distal to the lesion site (19). Urinary NGF-Rt levels were significantly elevated after sciatic nerve section with a time course similar to, but somewhat faster, than that of Schwann cell NGF receptor induction after injury (19). The more rapid rise in urinary NGF-Rt may be receptor generated by degenerating sympathetic and sensory axons within the lesioned nerve. Why the 77-kDa (intact) receptor appears in plasma from days P1 through P10 is yet unanswered. Possibly the 77-kDa NGF receptor species is derived from neuronal sources. Aside from normal cell death, axons of the sciatic nerve (30) and sympathetic trunk (31) undergo spontaneous degeneration in the postnatal rat. This process commences near birth, and axon numbers stabilize by P6 (31). This time course agrees well with the developmental regulation of the plasma 77-kDa NGF receptor species seen in our studies. Alternatively, the 77-kDa species in blood may reflect neuronal or glial membrane turnover that pertains to neonatal myelination (32).

What is the biological significance of NGF-Rt shed by Schwann cells and other NGF receptor-bearing cells? (*i*) Possibly the shedding of NGF-Rt is a mechanism by which the cell turns over this particular protein. Although the cell would appear to be wasting valuable protein by shedding NGF-Rt, this event is not without precedent. For example, sympathetic neurons release norepinephrine from storage vesicles and, in doing so, they also release vital proteins such as dopamine-β-hydroxylase and chromogranins (for review, see ref. 33) that apparently are not recovered by the cell. (*ii*)

A second alternative is that NGF-Rt may serve as a "sink" for NGF protein elaborated by Schwann cells or targets of NGF-dependent neurons, thus providing a mechanism by which NGF can be regulated. (*iii*) Conversely, NGF-Rt could serve as a carrier protein, carrying NGF to other targets. Similar roles are also suggested for the truncated forms of insulin-like growth factor (34) and epidermal growth factor (35) receptors. (*iv*) Finally, NGF-Rt could serve as a substratum molecule analogous to the extracellular matrix molecules laminin and fibronectin (for review, see ref. 36). The extracellular domain of the NGF receptor is very negatively charged (28, 29) making it a possible attractant to positively charged molecules involved in cell adhesion or growth cone migration.

In addition to these possible physiological functions for NGF-Rt, two practical implications deserve comment. First, attempts to measure or establish the presence of NGF in blood have been complicated by the fact that NGF-binding proteins may exist in blood. NGF-Rt could be such a binding protein, particularly in samples from developing animals and in tissue culture sera. Secondly, efforts to produce monoclonal antibodies to the NGF receptor have yielded few positive results, and those antibodies have proven species specific. One explanation for this difficulty in raising antibodies to the NGF receptor is that high NGF-Rt levels during development may make animals immunologically tolerant to the protein.

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