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The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor

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Abstract

Nerve growth factor (NGF) promotes neuronal survival and differentiation and stimulates neurite outgrowth. NGF is synthesized as a precursor, proNGF, which undergoes post-translational processing to generate mature β-NGF. It has been assumed that, *in vivo,* NGF is largely processed into the mature form and that mature NGF accounts for the biological activity. However, we recently showed that proNGF is abundant in CNS tissues whereas mature NGF is undetectable, suggesting that proNGF has biological functions beyond its role as a precursor. To determine whether proNGF exhibits biological activity, we mutagenized the precursor-processing site and expressed unprocessed, cleavage-resistant proNGF protein in insect cells. Survival and neurite outgrowth assays on murine superior cervical ganglion neurons and PC12 cells indicated that proNGF exhibits neurotrophic activity similar to mature 2.5S NGF, but is approximately fivefold less active. ProNGF binds to the high-affinity receptor, TrkA, as determined by cross-linking to PC12 cells, and is also slightly less active than mature NGF in promoting phosphorylation of TrkA and its downstream signaling effectors, Erk1/2, in PC12 and NIH3T3-TrkA cells. These data, coupled with our previous report that proNGF is the major form of NGF in the CNS, suggest that proNGF could be responsible for much of the biological activity normally attributed to mature NGF *in vivo.*

Keywords: apoptosis, nerve growth factor, neurite outgrowth, neurotrophin, p75, TrkA.

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Nerve growth factor (NGF), a neuronal growth and survival-promoting protein, was originally purified from the mouse submandibular gland and has been extensively characterized from this tissue (Fahnestock 1991). Two promoters and alternative splicing of murine transcribed NGF produces four different mRNA transcripts, two major and two minor, with the mature NGF moiety located at the 3′ terminus (Selby *et al*. 1987; Racke *et al*. 1996). Subsequent translation and signal peptide cleavage produces 32–34 and 25 kDa proNGF species (Darling *et al*. 1983). Mouse NGF is highly homologous to human NGF (Ullrich *et al*. 1983). In the mouse, rat, and human central nervous system, the 32–34 kDa proNGF molecule predominates, with little or no mature NGF present (Fahnestock *et al*. 2001).

ProNGF is post-translationally processed in the *trans*-Golgi network by proteases that recognize the dibasic and tetrabasic cleavage sites flanking the sequence for mature NGF (Greene *et al*. 1968; Edwards *et al*. 1988b; Seidah *et al*. 1996). Additional processing and glycosylation sites in the proNGF protein produce various high-molecular-weight forms, peptides, and intermediates whose biological properties and roles are still unclear (Seidah *et al*. 1996; Darling *et al*. 1983; Dicou *et al*. 1997; Reinshagen *et al*. 2000; Lee *et al*. 2001). Mature NGF, also known as β-NGF or 2.5S NGF depending on the isolation procedure (Mobley *et al*. 1976), is a dimer of a 13.2 kDa molecule and was previously thought to be the only biologically active form of NGF.

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Abbreviations used: BS^3, bis(sulfosuccinidyl)-suberate; DRG, dorsal root ganglion; FCS, fetal calf serum; MAP, mitogen-activated protease; NGF, nerve growth factor; SCG, superior cervical ganglion; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Neuronal uptake and retrograde transport of secreted NGF is initiated by the binding of NGF to one or both of its receptors. The single membrane-spanning receptor tyrosine kinase, TrkA, is the high affinity receptor for NGF, signaling neuronal survival, differentiation and growth. The transmembrane, common neurotrophin receptor, $p75^{NTR}$, is involved in apoptosis and in modulation of TrkA signaling (Friedman and Greene 1999). Both the N-terminus of mature NGF and the beta-hairpin loops at the top of the NGF dimer are important for high-affinity binding to TrkA (Kahle *et al*. 1992; Woo *et al*. 1995; Kullander *et al*. 1997). Because of the importance of the N-terminus, it might be expected that proNGF, with an N-terminal extension compared to mature NGF, would be sterically hindered from binding to TrkA.

The main epitope responsible for NGF binding to $p75^{NTR}$ includes the positively charged residues Lys-32, Lys-34 and Lys-95 within the beta-hairpin loops. Mutagenesis of these residues abolishes $p75^{NTR}$ binding, however, the mutated NGF molecules retain TrkA binding ability and bioactivity (Ibanez *et al*. 1992). Another determinant involved in NGF binding to $p75^{NTR}$ includes Asp-72 to Asn-77, a region of highly conserved residues that form a hydrophilic loop (Ryden *et al*. 1997). C-Terminal residues of NGF also have some effect on NGF bioactivity, perhaps mostly via an effect on structural stability (Kruttgen *et al*. 1997).

NGF mRNA is synthesized in many regions of the brain, most highly within the hippocampus and cortex of both human and rat brain (Korsching *et al*. 1985; Phillips *et al*. 1990). Previous studies using specific two-site enzyme-linked immunosorbent assays (ELISAs) have mapped NGF protein in relatively high levels to the hippocampus, cortex, and septum of rat and human brain (Scott and Crutcher 1994). Western blot analysis, however, shows that the NGF-like immunoreactivity is likely due to proNGF and not mature NGF (Fahnestock *et al*. 2001). This suggests that proNGF may have a biological function distinct from its role as a precursor molecule.

Numerous studies have been conducted to identify the role of proNGF in relation to mature NGF. That the NGF precursor has little or no biological activity was widely assumed on the basis of experiments by Edwards *et al*. (1988a) demonstrating that the neurite outgrowth activity of proNGF on chick dorsal root ganglion (DRG) neurons increased from 10- to 20-fold after processing to mature NGF with trypsin. The pro segment was thought only to aid in folding of mature NGF (Suter *et al*. 1991; Rattenholl *et al*. 2001a,b). In contrast, a number of other investigators over the years have demonstrated that either the full-length proNGF or intermediate forms exhibit neurite outgrowth and survival activity (Ibanez *et al*. 1992; Saboori and Young 1986; Lakshmanan *et al*. 1989; Suter *et al*. 1991; Chen *et al*. 1997; Rattenholl *et al*. 2001a). Surprisingly, a mutated, cleavage-resistant form of proNGF was shown to promote apoptosis in primary SCG neurons and smooth muscle cells (Lee *et al*. 2001). This proNGF binds $p75^{NTR}$ with high affinity but has negligible ability to bind TrkA. Our laboratory has also constructed a cleavage-resistant mouse NGF cDNA. However, the construct is different from that of Lee *et al*. (2001). In this report, we tested our proNGF for biological activity in neurite outgrowth and survival assays on murine superior cervical ganglion neurons, for neurite outgrowth on PC12 cells, and for its ability to bind to and activate TrkA receptors and the downstream mitogen-activated-protein (MAP) kinase signal transduction pathway in PC12 cells and NIH 3T3-TrkA cells.

Experimental procedures

Construction of pVL-NGF (R-1G)

A full-length murine NGF cDNA was inserted into pVL1392 at the Pst I site. Site-directed mutagenesis of the NGF cDNA was completed using the QuikChange™ kit (Stratagene, La Jolla, CA, USA), with primers encoding a cytosine-to-guanine point mutation at position 633 in the murine NGF mRNA sequence (GenBank accession number K01759). This resulted in an arginine-to-glycine substitution at the −1 position in the proNGF polypeptide, which mutated the cleavage site used for processing the 34-kDa proNGF molecule to the 13.2-kDa mature form. The sequence of the mutated pVL-NGF (R-1G) was verified by restriction enzyme digestion (the C-to-G mutation in the NGF gene results in the loss of a *Hae II* restriction enzyme cleavage site), and by sequencing both strands.

Recombinant *Baculovirus* and NGF protein production

Calcium-phosphate cotransfection of AcRP23 lacZ linearized baculovirus DNA (BD Pharmingen, Franklin Lakes, NJ) with pVL-NGF (wild-type, cleavable proNGF) or pVL-NGF (R-1G) DNA into Sf9 insect cells was performed according to the manufacturers' protocol, except that Grace's supplemented media (Invitrogen Life Technologies, Burlington, ON, Canada) was used during cultivation of the recombinant viruses, instead of TMN-FH.

Recombinant wild-type NGF and proNGF (R-1G) baculoviruses, as well as wild-type (empty) baculovirus as a negative control, were plaque-purified and amplified in Sf9 cells to produce high titer viral stocks. Cells infected with viral stocks were incubated at 27°C in Sf-900 II serum-free medium (Invitrogen LifeTechnologies), the medium was changed after 24 h, and the conditioned medium containing secreted protein was harvested by centrifugation on day three. All assays with the exception of one SCG neurite outgrowth assay (described below) used conditioned medium that was concentrated, dialyzed, and quantified by ELISA and western blotting (see below).

Purified murine 2.5S NGF was prepared from male mouse salivary glands as previously described (Mobley *et al*. 1976; Petrides and Shooter 1986; Fahnestock *et al*. 2001). Affinity purified rabbit polyclonal antibodies raised against 2.5S NGF were prepared as described previously (Van der Zee *et al*. 1995).

Western blotting for NGF and ProNGF

Western blotting was as previously described (Fahnestock *et al*. 2001). The primary antibody was affinity-purified rabbit anti-NGF IgG, at a dilution of 1 : 1000. The secondary antibody was an

HRP-conjugated donkey anti-rabbit IgG (Amersham Biosciences, Baie d'Urfé, QC, Canada) at a dilution of 1 : 5000. For blocking of NGF immunoreactivity to determine non-specific binding by the antibodies, the primary antibody was preincubated with fivefold (by weight) excess 2.5S NGF.

NGF ELISA

NGF-immunoreactive protein levels were measured by two-site, sandwich ELISA using our affinity-purified rabbit polyclonal antibody, following protocols from Boehringer Mannheim, with the fluorescent substrate 4-methylumbelliferyl β-D-galactoside. The polyclonal antibody recognized proNGF (R-1G) and NGF with roughly equal affinity as estimated by western blotting. Purified 2.5S NGF of known concentration (determined by spectrophotometry) was treated in parallel with proNGF (R-1G), and served as an internal standard. Samples were measured at three different dilutions, each in triplicate.

Size exclusion chromatography

Insect cell conditioned medium was concentrated 40-fold by Centriprep® 10 (Millipore Corp., Mississauga, Ontario, Canada). NGF levels were verified by ELISA, and molecular mass was checked by western blotting. Samples were loaded on a Sephadex G-75 column (1 × 45 cm, Amersham Biosciences) in 25 mM sodium phosphate pH 5.6, 0.1 M NaCl, 4 M urea. The column was eluted with the same buffer, and 1-mL fractions were collected and assayed by western blotting. Fractions containing proNGF (R-1G) were dialyzed against RPMI 1640 medium to remove urea and analyzed by ELISA prior to use in survival and neurite outgrowth assays. Wild-type baculovirus-infected insect cell conditioned medium was treated similarly, and the same fractions were pooled for use as a negative control. Assays of total protein (Bio-Rad Laboratories, Hercules, CA, USA) and NGF-immunoreactive protein (ELISA) demonstrated that size exclusion chromatography resulted in an eightfold purification of proNGF (R-1G). ProNGF (R-1G) represented 11% of the total protein in these samples.

Insect cell conditioned medium containing proNGF (R-1G) was adjusted to 0.5 M NaCl pH 7.4 and was passed through a 1 × 4 cm anti-NGF affinity chromatography column. The column was washed with 0.5 M NaCl in 0.02 M phosphate buffer, pH 7.4, and eluted with 0.5 M acetic acid, pH 2.5. Immunoreactive fractions were quantified by ELISA, pooled, and stored for 4 months in acidic solution before dialysis against RPMI 1640 for use in the neurite outgrowth bioassay. Pooled material was checked by western blotting, and approximately half of this material was found to be cleaved to the size of 2.5S NGF. This material was used for only one neurite outgrowth assay shown in Fig. 3(b).

Bioassay of neuronal survival with neurite outgrowth

Neurotrophic activity was assayed by determining survival of neurite-bearing cells in cultures of dissociated superior cervical ganglion (SCG) neurons from one-day old mouse pups, using a modification of previously described methods (Coughlin *et al*. 1981; Coughlin and Collins 1985). For bioassays of neurite outgrowth and survival, infection supernatants were made 2% in fetal calf serum (FCS) (Invitrogen Life Technologies) and dialyzed (cut-off of 3500 Da) at 4°C overnight in three changes of 20 volumes of RPMI 1640 containing 5% FCS. Dialyzed material was concentrated twofold by Centriprep® 10, and the concentrated samples that contained some precipitate were then clarified and sterilized by filtration. Final NGF concentrations were verified by ELISA, and molecular mass was checked by western blotting. Both conditioned medium and Sephadex-G-75-purified material (see above) were used for these assays.

Dissected ganglia were rinsed in calcium- and magnesium-free Puck's Saline G, incubated for 30 min in 0.25% trypsin (Difco Laboratories, Detroit, MI, USA), and then incubated for 5–10 min in 2 mg/mL of collagenase (Sigma, St. Louis, MO, USA) in Puck's Saline G. Ganglia were then rinsed once with 10% FCS, and cells were dissociated by repetitive pipetting in 10% FCS. Approximately 30 000 cells (neuronal + non-neuronal) were obtained from each ganglion, similar to that previously reported (Coughlin and Collins 1985). For all experiments, approximately 20 000 cells were plated onto each 35 mm collagen- and lysine-coated dish. Maximal survival of neurite-bearing cells after 2 days in culture reached nearly 27%, with approximately half the cells after 1–2 days in culture being non-neuronal cells as previously reported (Coughlin and Collins 1985). Cells were treated with proNGF (R-1G), wild-type NGF, or with conditioned medium from insect cells infected with wild-type (empty) baculovirus, similarly concentrated, dialyzed and filtered as described above. Round, intact, phase-bright cells exhibiting neurite outgrowth were counted after 2 days. Each sample was assayed in triplicate over a concentration curve and compared to a standard curve derived from a known concentration of 2.5S NGF that was similarly dialyzed, concentrated and filtered. The average number of cells bearing sprouted neurites was counted over 2% of the dish surface. In this bioassay, a surviving neuron is defined as a phase bright cell bearing a neurite that is longer than twice the diameter of the cell body.

Specificity of the neurite outgrowth activity was determined by addition to the cultures of 100–200 ng/mL of affinity-purified rabbit antibodies raised against murine 2.5S NGF. Supernatants from the neurite outgrowth assay were collected after 2 days and assayed using western blotting, as described above, to determine whether any cleavage of proNGF (R-1G) occurred during the assay.

PC12 cells were obtained from the ATCC (Washington DC) and were grown as monolayer cultures in RPMI 1640 supplemented with 10% horse serum and 5% calf serum (Invitrogen Life Technologies) in a humidified atmosphere of 5% CO_2 at 37°C. For use in bioassays, PC12 cells were primed (Greene and Tischler 1976; Seeley *et al*. 1983): medium was changed to supplemented RPMI 1640 with 50 ng/mL 2.5S NGF, and the cells were grown for 10–14 days before harvesting for use in the neurite outgrowth assay. Washed cells were plated onto 35 mm collagen-lysine-coated tissue culture dishes at 15 000 cells/dish, samples were added, and neurite-bearing cells were counted after 2 days as described above.

Dose–response curves and statistical analyses were generated using the Prism 3 program (GraphPad Software, Inc, San Diego, CA, USA).

Neuronal survival

Murine SCG cells were prepared as described above, except cells were preplated for 2.5–4.5 h to remove non-neuronal cells prior to plating on collagen-polyornithine-coated 12 mm glass cover slips. Cells were treated for 24 h with 3 ng/mL 2.5S NGF, wild-type NGF,

or proNGF (R-1G), a concentration producing maximal neurite outgrowth in the bioassay described above, or with an equivalent volume of baculovirus-infected insect cell conditioned medium, similarly concentrated and dialyzed. Cells were fixed in 1% paraformaldehyde overnight at 4°C, and endogenous peroxidase was inhibited with 3% H_2O_2. Cells were stained with an ApopTag® Plus Peroxidase *in situ* Apoptosis Detection kit (Chemicon Serologicals Corp., Norcross, GA, USA) for apoptotic cells or with an anti-TrkA antibody (Chemicon International, Temecula, CA, USA) for determination of the percentage of neurons in each sample. Incubations with anti-TrkA antibody were performed overnight at room temperature in TBS-Tx (10 mM Tris pH 7.5, 100 mM NaCl, 0.4% Triton X-100 + 3% goat serum) at a dilution of 1 : 300, followed by incubation for 1 h at room temperature in goat anti-rabbit EnVision +™ (Dako) at a dilution of 1 : 50 in TBS-Tx. Diaminobenzidine substrate was used for detection.

All cover slips were counterstained with methyl green, mounted in Permount, and 4% of the cover slip total area was counted under bright field illumination. Differences between groups were determined by one-way ANOVA followed by *post hoc* Tukey tests.

TrkA cross-linking

Iodination of 2.5S NGF and proNGF (R-1G) was performed using the lactoperoxidase method (Ross *et al*. 1998). The ^{125}I-NGF and ^{125}I-proNGF (R-1G) obtained (typically 80–120 cpm/pg total protein) was separated from free iodine by size exclusion chromatography on a PD-10 column (Amersham Biosciences). For chemical cross-linking, 10^6 PC12 cells were suspended in 1 mL of HKR buffer (10 mM HEPES, pH 7.35 containing 125 mM NaCl, 4.8 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 g/L glucose and 1 g/L bovine serum albumin) and 10^7 cpm of iodinated protein and incubated for 2 h at 4°C. Cells were cross-linked using 0.4 mM bis(sulfosuccinidyl)-suberate (BS^3) for 30 min at 25°C, washed two times with Tris-buffered saline (10 mM Tris−0.9% NaCl pH 7.35), and the resulting pellet was suspended in 1 mL lysis buffer for TrkA immunoprecipitation as previously described (Ross *et al*. 1998). The radiolabeled TrkA-immunoreactive proteins were solubilized in reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, normalized for lane load (based on cpm), separated on a 7.5% SDS–PAGE gel, and visualized by autoradiography.

TrkA-MAP kinase activation

For TrkA and MAP kinase phosphorylation assays, insect cell conditioned medium was concentrated 40-fold by Centriprep® 10, NGF levels were verified by ELISA, and molecular mass was checked by western blotting before use. PC12 cells (10^6 cells per well) were grown for 24 h in RPMI + 10% horse serum, 5% fetal bovine serum, switched to serum-free medium for 24 h, and serum-free medium was replaced 2 h prior to stimulation. NIH3T3-TrkA cells (8×10^5) were grown for 24 h in Dulbecco's modified Eagle's medium + 10% fetal calf serum, switched to 2% fetal calf serum for 24 h, and the medium replaced 2 h prior to stimulation. Cells were stimulated for 5 min according to the company's instructions (Cell Signalling Technology, Inc.) with various concentrations of proNGF(R-1G), 2.5S NGF, or control (empty) baculovirus-infected insect cell supernatant that had been similarly concentrated, dialyzed and serially diluted. Cells were lysed in 150 μL/well cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylene glycol-bis(β-amino-ethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL each aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF), chilled on ice for 15 min, and centrifuged for 10 min at 4°C, 16 000 *g* in an Eppendorf microcentrifuge. Supernatants were assayed for protein using the DC protein assay (Bio-Rad Laboratories). Thirty micrograms of protein from each supernatant was loaded onto 10% SDS–PAGE gels. Western blotting was carried out as described (Fahnestock *et al*. 2001; Xu *et al*. 2002). The blots were probed with either antibody against phosphorylated $p44/42^{MAPK}$ (anti-ERK1/2, Thr202/Tyr204; Cell Signalling Technology, Inc., Mississauga, Ontario, Canada), against phosphorylated $p140^{TrkA}$ (Cell Signalling Technology), against total $p44/42^{MAPK}$ (Cell Signalling Technology), or against total TrkA (Chemicon). Cell Signalling Technology antibodies were used at a dilution of 1 : 1000, according to the manufacturer's instructions, while the total TrkA antibody was used at a dilution of 1: 2000.

Signal intensity was determined by densitometry using a ScanJet 3970 (Hewlett-Packard, Palo Alto, CA, USA) and Scion Image software (Scion Corp., Frederick, MD, USA). Dose–response curves and statistical analyses were generated using the Prism 3 program (GraphPad Software, Inc.).

Results

Construction and expression of cleavage-resistant proNGF [proNGF (R-1G)]

A cytosine-to-guanine mutation in the murine NGF gene was incorporated by site-directed mutagenesis of pVL-NGF. The mutation was verified by the loss of a *Hae II* restriction enzyme cleavage site and by sequencing of both strands. Recombinant wild-type NGF baculovirus and proNGF (R-1G) baculovirus were produced by cotransfection with linearized baculovirus DNA, plaque-purified, and amplified in Sf9 insect cells. Infection of Sf9 cells with wild-type NGF or proNGF (R-1G) viruses resulted in the production and secretion of NGF or proNGF (R-1G) protein into serum-free medium.

SDS–PAGE gels of the conditioned medium demonstrated a band of increased staining intensity at the expected molecular weight of proNGF (R-1G) compared to conditioned medium from wild-type baculovirus-infected insect cells (Fig. 1a). In wild-type (cleavable) NGF-infected insect cell conditioned medium, a band corresponding to the size of mature NGF and co-migrating with mouse submandibular gland 2.5S NGF was also detected (Fig. 1a). Western blotting with an anti-NGF antibody (Fig. 1b) showed that Sf9 cells expressed and secreted NGF proteins. Cells infected with wild-type (cleavable) NGF virus secreted a proNGF polypeptide of 34 kDa as well as the mature β-NGF of 13 kDa (lane 1) which co-migrated with purified mouse submandibular gland 2.5S NGF (lane 3). The infection

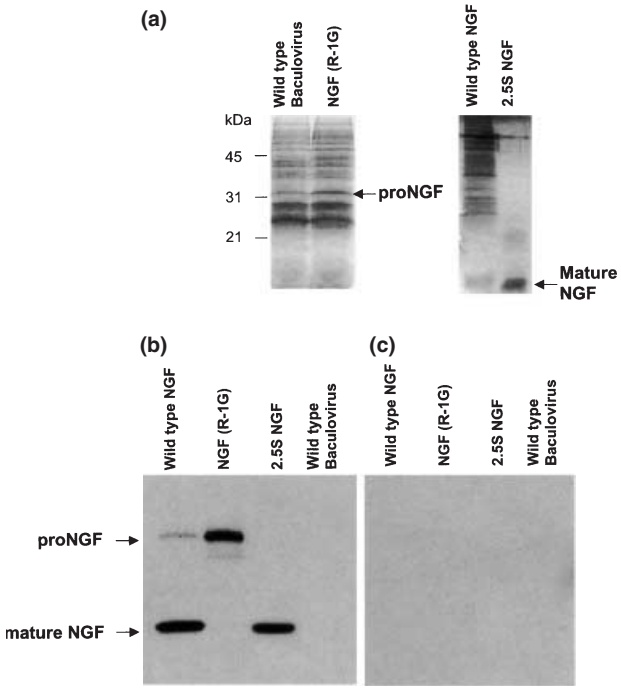

Fig. 1 SDS–PAGE and western blotting of proNGF (R-1G) and wild-type NGF expressed in a baculovirus/insect cell system. Samples were prepared as described in Experimental procedures. (a) Silver-stained SDS–PAGE gel showing conditioned medium from control cells infected with wild-type (empty) baculovirus, conditioned medium from cells expressing proNGF (R-1G), conditioned medium from cells expressing wild-type (cleavable) NGF and mouse submandibular gland 2.5S NGF. (b) Western blots of samples as described in (a). The primary antibody was affinity-purified rabbit anti-NGF IgG, at a dilution of 1 : 1000. The secondary antibody was an HRP-conjugated donkey anti-rabbit IgG at a dilution of 1 : 5000. (c) To control for non-specific binding, the primary antibody was preincubated with fivefold excess 2.5S NGF.

supernatant from the proNGF (R-1G) virus (lane 2) also exhibited a very strong proNGF band, a very faint band at 27 kDa probably representing translation of the short transcript (Selby *et al*. 1987; Edwards *et al*. 1988a), but lacked any bands of lower molecular weight, indicating that cleavage of the mutant precursor did not occur, and that β-NGF was not secreted by these cells. Cells infected with wild-type baculovirus (lane 4) produced no NGF-immunoreactive material. Blocking of NGF immunoreactivity with excess 2.5S NGF resulted in the disappearance of all the bands, verifying their specificity (Fig. 1c).

Survival-promoting activity of proNGF (R-1G)

To determine the effect of proNGF (R-1G) on survival of dissociated murine superior cervical ganglion (SCG) neurons, apoptosis was assayed following 24 h in culture with 3 ng/mL mouse 2.5S NGF, cleavable recombinant NGF (mostly mature NGF, see Fig. 1), and cleavage-resistant recombinant proNGF (R-1G). All three samples supported the survival of SCG neurons, with no significant differences between the three groups in either total cell number or percent apoptotic cells (one-way ANOVA and *post hoc* Tukey test, $p > 0.05$) (Table 1). TrkA staining verified that 93% of the cells were neurons. Conditioned medium from insect cells infected with wild-type baculovirus, similarly concentrated, dialyzed, and serially diluted, did not support cell survival to the same extent (Table 1). The number of surviving cells in this condition was roughly half the number surviving in the presence of proNGF (R-1G) or wild-type NGF [$p < 0.003$ for proNGF (R-1G) versus baculovirus and $p < 0.001$ for wild-type NGF versus baculovirus], whereas the percent apoptotic cells in baculovirus-infected insect cell conditioned medium was roughly between three and five times greater than in proNGF (R-1G)- or wild-type NGF-treated cells [$p < 0.05$ for proNGF (R-1G) versus baculovirus and $p < 0.01$ for wild-type NGF versus baculovirus].

Neurite outgrowth activity of proNGF (R-1G)

The neurite outgrowth bioassay was carried out on both SCG neurons and PC12 cells to determine the relative level of activity of proNGF (R-1G) compared to mature NGF. Wild-type NGF and proNGF (R-1G) promoted the robust growth of neurites from SCG and PC12 cells, as did 2.5S NGF (Fig. 2). Baculovirus-infected insect cell conditioned medium did not support any neurite outgrowth, demonstrating that the neurite outgrowth activity in the wild-type and proNGF (R-1G) samples is a property of the recombinant expressed protein rather than the conditioned medium (Fig. 2). The neurite outgrowth activity of wild-type NGF and proNGF (R-1G) were completely blocked by the addition of 100–200 ng/mL of affinity-purified rabbit antibodies raised against murine 2.5S NGF (data not shown).

As shown in Fig. 3(a), proNGF (R-1G) is approximately one-fifth as active as mature NGF in eliciting sprouting from SCG neurons, but the cells are equally capable of reaching maximal survival and sprouting with the proNGF (R-1G) protein as with the wild-type (cleavable) NGF expressed in insect cells. The EC_{50} for neurite outgrowth activity of 2.5S

Table 1 Survival of murine superior cervical ganglion neurons

Sample[a] (3 ng/mL)	Alive cells[b] (number in 4% of cover slip total area) (±SEM)	Percentage apoptosis (±SEM)
2.5S NGF	175.25 (7.3)	11.22 (2.7)
proNGF (R-1G)	191.5 (8.3)	9.76 (2.6)
Wild-type NGF	220.25 (8.25)	6.17 (2.0)
Baculovirus CM[c]	91.75 (8.5)	30.97 (5.0)

[a]$n = 4$ in each group. [b]Ninety-three percent of cells were TrkA$^+$ by immunostaining. [c]Conditioned medium from wild-type baculovirus-infected insect cells was concentrated, dialyzed and diluted for the assay in parallel with the other samples. ELISAs and western blotting verified this material contained no NGF.

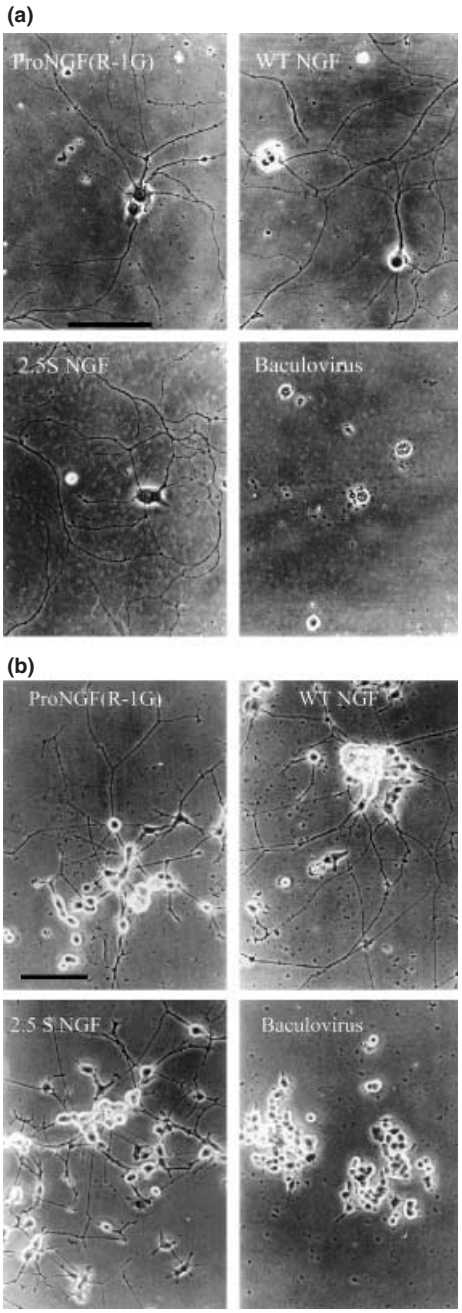

Fig. 2 Neurite outgrowth assays. Dissociated murine SCG neurons (a) or PC12 cells (b) were used to assay neurite outgrowth as described in Experimental procedures. Representative fields from cells treated with 3 ng/mL proNGF (R-1G), wild-type (cleavable) NGF, 2.5S NGF, or baculovirus-infected insect cell medium. Scale bar = 100 μm.

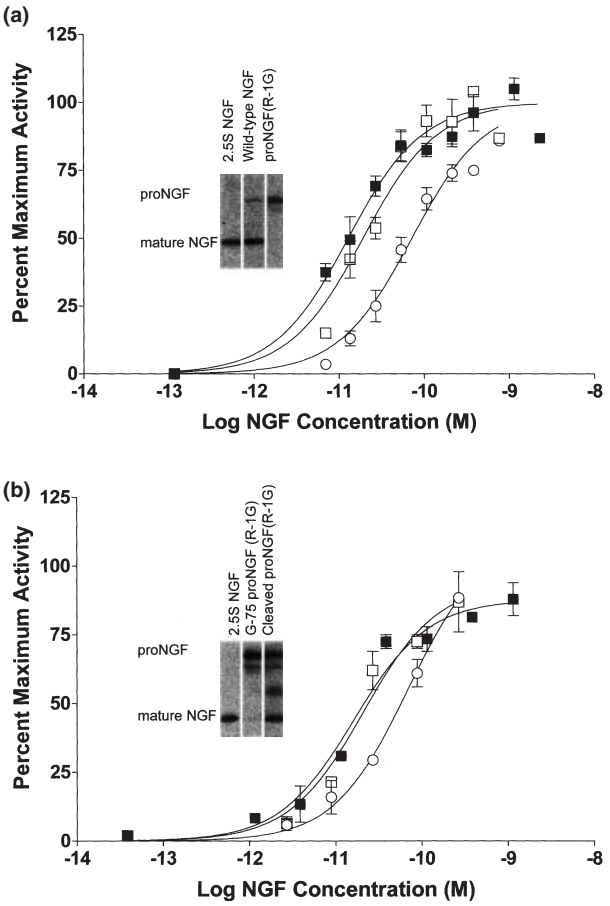

Fig. 3 Neurite outgrowth activity on SCG neurons. Dissociated SCG neurons from one-day old mouse pups were used to assay neurite outgrowth activity of baculovirus-infected insect cell supernatants as described in Experimental procedures. (a) Each sample was assayed in triplicate over a concentration curve and compared to a standard curve derived from a known concentration of purified mouse 2.5S NGF that was similarly dialyzed, concentrated and filtered. Data is aggregated from two separate assays. In each experiment, maximum (100%) activity was taken from the 2.5S NGF curve. A few of the high-concentration points without error bars are single values only. Error bars represent standard error of the mean (SEM). ■ = 2.5S NGF; □ = wild-type NGF expressed in insect cells; ○ = proNGF (R-1G). Inset shows western blot of 2.5S NGF (lane 1), wild-type (cleavable) NGF (lane 2) and proNGF (R-1G) (lane 3). (b) As in (a), but using proNGF (R-1G) subjected to purification or cleavage. All points determined in duplicate in a single experiment. ■ = 2.5S NGF; ○ = proNGF (R-1G) purified by Sephadex G-75 chromatography; □ = purified proNGF (R-1G) partially processed to the mature form. Inset shows western blot of 2.5S NGF (lane 1), proNGF (R-1G) purified by Sephadex G-75 chromatography (lane 2), and proNGF (R-1G) partially processed to the mature form (lane 3).

NGF was 1.2×10^{-11} M. The concentration curve for dialyzed 2.5S NGF was the same as the control, undialyzed 2.5S NGF (data not shown), ensuring that no NGF was lost during dialysis and filtration of the samples. The EC_{50} for neurite outgrowth activity of wild-type (cleavable) proNGF expressed in insect cells, which contained a mixture of native proNGF and mature NGF (see inset), was only slightly less active than 2.5S NGF, with an EC50 of 2.0×10^{-11} M. The EC_{50} for proNGF (R-1G), however, was 6.5×10^{-11} M, 5.4 times lower than for mature NGF ($p < 0.05$). Wild-type

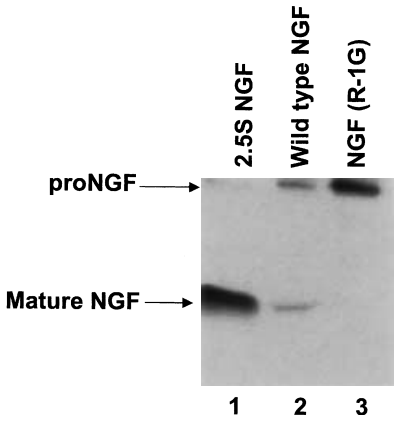

Fig. 4 Lack of processing of proNGF (R-1G) during survival and neurite outgrowth assays. Following a 2-day incubation for survival and neurite outgrowth bioassays, SCG neurons supplied with murine 2.5S NGF still had an excess of NGF in the culture medium (Lane 1). Cells supplied with wild-type NGF (Lane 2) or with proNGF (R-1G) (Lane 3) did not further process either proNGF or mature NGF.

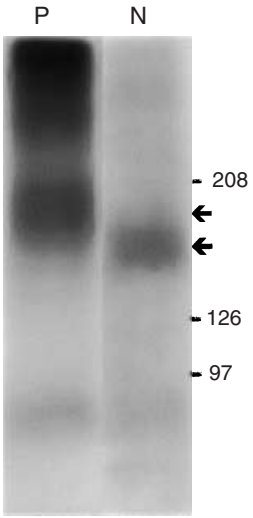

Fig. 5 Cross-linking of ^{125}I-NGF and ^{125}I-proNGF (R-1G) to TrkA. Chemical cross-linking of ^{125}I- proNGF (R-1G) to PC12 cells and subsequent immunoprecipitation with anti-TrkA revealed specific labeling of a protein with a molecular weight consistent with a proNGF-TrkA complex (lane P), which ran with a higher molecular weight than a control 2.5S NGF-TrkA complex (lane N). Molecular weight markers in kDa are indicated on the right. Arrows point to cross-linked complexes. Cross-linking of ^{125}I-baculovirus conditioned medium gave no signal (30-day exposure, data not shown). Lane P exposed for 30 days, lane N exposed for 7 days.

baculovirus-infected insect cell conditioned medium exhibited no neurite-outgrowth-promoting activity (Fig. 2a,b).

Molar concentrations for survival and neurite outgrowth assays were determined assuming dimers for all forms of NGF, including proNGF (R-1G) as has been previously shown (Rattenholl *et al*. 2001a; Fahnestock *et al*. 2003). Differences between curves are based on quantification of proNGF (R-1G) by ELISA and western blotting using pure 2.5S NGF as a standard, and are therefore not exact.

When further purified by Sephadex G-75 chromatography, proNGF (R-1G), like the raw supernatants, exhibited roughly one-fifth of the activity of 2.5S NGF (Fig. 3b), arguing that its activity is not due to a contaminating protein in the insect cell conditioned medium. The EC_{50} for proNGF (R-1G) purified by Sephadex G-75 chromatography was 7.7×10^{-11} M compared to 1.7×10^{-11} M for 2.5S NGF, a 4.5-fold difference. When proNGF (R-1G) was partially processed to the size of mature NGF, its activity was increased (EC_{50} of 2.2×10^{-11} M), demonstrating that processing of proNGF (R-1G) leads to mature NGF exhibiting the same biological activity as 2.5S NGF (Fig. 3b).

ProNGF (R-1G) elicits neurite outgrowth from PC12 cells with similar activity (Fig. 2b and data not shown).

The supernatants from the SCG neurite outgrowth bioassay were collected following a 2-day incubation in cell culture and assayed by western blotting (Fig. 4). Neurons supplied with murine 2.5S NGF (lane 1) still had an excess of NGF in the culture medium after two days. Cells supplied with baculovirus-produced wild-type NGF (lane 2) or with proNGF (R-1G) (lane 3) did not further process either the precursor or the mature NGF, suggesting that it is indeed the proNGF protein that has biological activity in the NGF (R-1G) sample. Longer exposures of the western blots showed that the amount of mature NGF in both starting material and medium harvested after 2 days was less than 2.5% of the total, an amount insufficient to account for neurite outgrowth at all concentrations.

ProNGF (R-1G) binding to TrkA

Cross-linking of ^{125}I-2.5S NGF to PC12 cells followed by immunoprecipitation with anti-TrkA antibody produces a product of approximately 150 kDa, representing a complex of 2.5S NGF bound to $p140^{TrkA}$ (Fig. 5, lane N). ^{125}I-proNGF (R-1G) also binds to PC12 cells, producing a product of approximately 170 kDa (Fig. 5, lane P). The slightly increased molecular mass of the complex is consistent with cross-linking of the 34-kDa proNGF (R-1G) to $p140^{TrkA}$. A higher molecular weight complex (>250 kDa) is also produced by cross-linking of proNGF (R-1G) to PC12 cells, however, this complex has not yet been characterized. ^{125}I-labeled control vector baculovirus-infected insect cell conditioned medium in concentrations up to 100 μg/mL protein did not interfere with ^{125}I-NGF cross-linking to $p140^{TrkA}$ (data not shown).

ProNGF (R-1G) activation of TrkA and the Ras-MAP kinase signal transduction pathway

Like 2.5S NGF, proNGF (R-1G) is active in promoting phosphorylation of TrkA and p44/42 MAP kinase

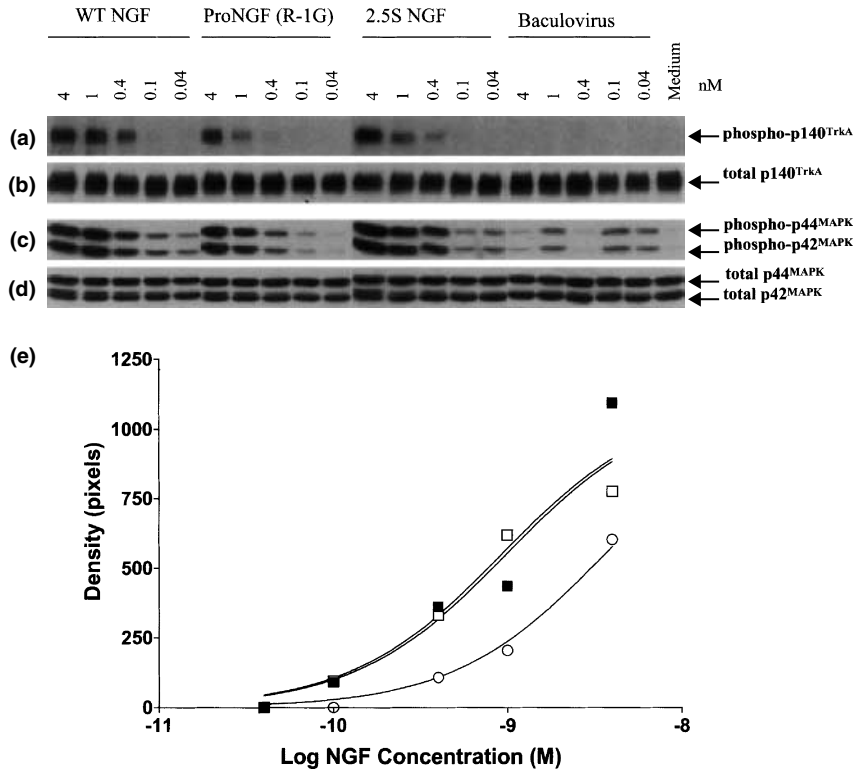

Fig. 6 ProNGF (R-1G) activates TrkA and the MAP kinase signal transduction pathway in PC12 cells. Following a 5-min stimulation with medium, baculovirus negative control (supernatant from wild-type baculovirus-infected insect cells not carrying the NGF gene), WT NGF, proNGF (R-1G), or 2.5S NGF, PC12 cells were lysed and analyzed by western blotting using antibodies against phosphorylated TrkA, total TrkA, phosphorylated Erks (MAP kinase), and total Erks as described in Experimental procedures. (a) $p140^{TrkA}$ is phosphorylated in cells activated by WT NGF, proNGF(R-1G) and 2.5S NGF in a dose-dependent manner, but not in control cells. (b) Total TrkA protein is relatively unchanged by any treatment. (c) $p42/44^{MAPK.}$ (Erk1/2) phosphorylation is increased in a dose-dependent manner in cells exposed to WT NGF, proNGF (R-1G) and 2.5S NGF compared to control cells. (d) Total $p42/44^{MAPK.}$ protein is unchanged by any treatment. (e) Quantification of TrkA activation for proNGF (R-1G) compared to wild-type NGF and 2.5S NGF. ■ = 2.5S NGF; □ = wild-type NGF; ○ = proNGF (R-1G).

(ERK1/2) in PC12 cells in culture (Fig. 6). ProNGF (R-1G) promoted rapid phosphorylation of TrkA and p44/42 MAP kinase without changing the total amounts of these proteins (Fig. 6a–d). When measured by densitometry, the EC_{50} for 2.5S NGF activation of TrkA was 0.93, for wild-type NGF 0.99, and for proNGF (R-1G), 3.63. ProNGF (R-1G) is therefore approximately fourfold less active than wild-type or 2.5S NGF in phosphorylating TrkA in PC12 cells (Fig. 6e), consistent with its activity in the neurite outgrowth assay on PC12 and SCG neurons. ProNGF (R-1G) also promoted phosphorylation of TrkA in NIH3T3-TrkA cells, which do not express $p75^{NTR}$ (Fig. 7). ProNGF (R-1G) was less active than mature NGF in these assays, consistent with its effects on neurite outgrowth (Fig. 7c). The EC_{50}s calculated from the titration of TrkA phosphorylation were 0.32 nM for wild-type NGF and 4.0 nM for proNGF (R-1G), an eightfold difference.

Discussion

A cytosine-to-guanine mutation in the murine NGF cDNA was designed to convert the arginine residue at the −1 position in the proNGF polypeptide to a glycine residue, thereby destroying the basic cleavage site required to process proNGF to mature NGF. Recombinant wild-type (cleavable) NGF or cleavage-resistant proNGF [proNGF (R-1G)] baculoviruses were used to infect Sf9 insect cells, which secreted the appropriate NGF protein. Baculovirus/insect cell recombinant protein expression systems have repeatedly been shown to express high levels of correctly processed recombinant NGF, yielding fully active protein (Barnett *et al.* 1990; Buxser *et al.* 1991). Also, Sf9 cells make no endogenous neurotrophins and were cultured in serum-free media during infections, allowing for easy isolation of secreted recombinant NGF protein. The mutation ensured that there was no mature NGF in the proNGF (R-1G) sample,

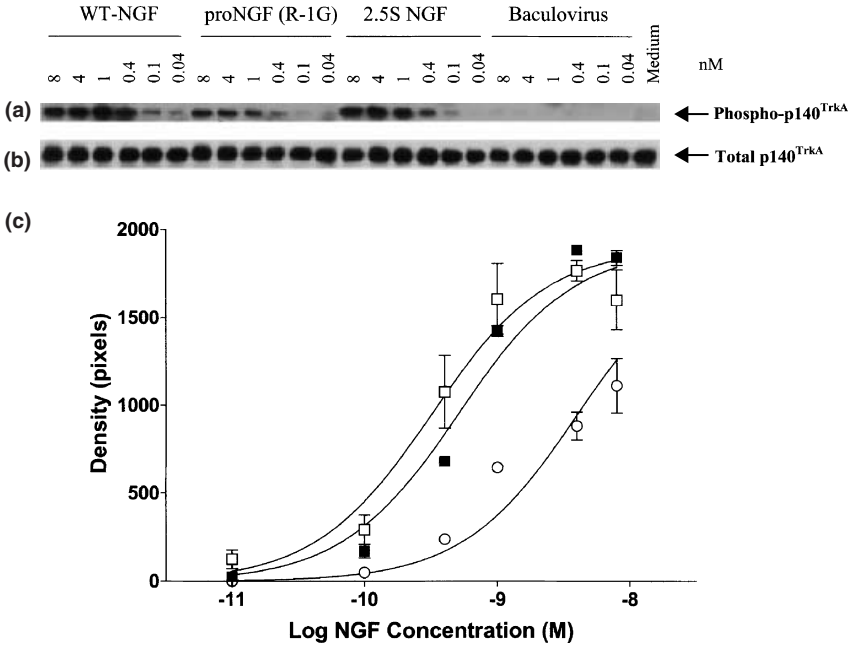

Fig. 7 ProNGF (R-1G) activates TrkA in NIH3T3-TrkA cells. Legend as in Fig. 6. (a) $p140^{TrkA}$ is phosphorylated in cells activated by WT NGF, proNGF(R-1G) and 2.5S NGF in a dose-dependent manner, but not in control cells. (b) Total TrkA protein is relatively unchanged by any treatment. (c) Comparison of the relative TrkA-activating activity of proNGF (R-1G) versus wild-type NGF and 2.5S NGF as determined by densitometry of western blots. ■ = 2.5S NGF; □ = wild-type (cleavable) proNGF expressed in insect cells; ○ = proNGF (R-1G). Error bars represent standard error of the mean (SEM).

allowing us to ascertain whether proNGF has biological activity without the confounding influence of contaminating mature NGF.

Murine postnatal day 1 SCG neurons require added NGF for survival in culture (Coughlin and Collins 1985). We used this assay to demonstrate that proNGF (R-1G) exhibits cell survival activity similar to that of mature NGF, and rescues these neurons from apoptosis at roughly the same concentration as mature NGF. Furthermore, concentration curves using murine submandibular gland 2.5S NGF, wild-type NGF from baculovirus-infected insect cell supernatant and proNGF (R-1G) viral infection supernatant demonstrated that proNGF (R-1G) exhibits approximately 20% of the neurite outgrowth activity of 2.5S NGF on both SCG neurons and PC12 cells. ProNGF (R-1G) can attain maximum cell survival at higher concentrations. The neurite outgrowth activity was specific, as determined by blocking all the proNGF (R-1G) activity with an antibody to 2.5S NGF.

We measured the concentrations of proNGF (R-1G) using an ELISA based on a standard curve of 2.5S NGF. The antibody used in the ELISA recognizes both proNGF and mature NGF with roughly similar affinities, based on western blots. In addition, we used size exclusion chromatography over a calibrated Sephadex G-75 column in the presence of 4 M urea to determine that proNGF (R-1G) exists as a ~64 kDa dimer in solution (Fahnestock *et al*. 2003), in agreement with the data of Rattenholl *et al*. (2001a). Therefore, we can be confident that our calculations represent a fair estimate of proNGF (R-1G) biological activity.

Previous studies have suffered from the possibility that wild-type proNGF added to mammalian cell cultures may be processed by the cells to form mature NGF, resulting in the biological activity seen in some assays (Saboori and Young 1986). Our mutant proNGF [proNGF (R-1G)] removes this argument, as the precursor is not readily cleaved to form β-NGF. Mature NGF routinely represented less than 2.5% of the NGF-immunoreactive material in our samples, an amount incapable of producing the biological activities measured over the range of concentrations used in our assays. Neuronal cells might use alternate cleavage sites present in the proNGF molecule to produce biologically active intermediates or other peptides (Darling *et al*. 1983; Dicou *et al*. 1997; Lee *et al*. 2001). Western blotting of the conditioned media from neurite outgrowth assays demonstrated that the cells did not process the added recombinant proNGF (R-1G) in the conditioned medium. Our cross-linking data, showing a 170 kDa complex, further argues against proteolytic cleavage of proNGF on the surface of cells giving rise to NGF that would subsequently bind to TrkA. These data taken together substantiate the finding that the activity seen in the proNGF (R-1G) cultures is due to unprocessed proNGF.

It has been suggested that because proNGF (R-1G) is expressed in insect cell conditioned medium, there may be contaminating materials present that are responsible for its activity. Any contaminating material in our baculovirus preparations is insufficient to produce neurite outgrowth (Fig. 2) or to activate TrkA (Fig. 6a and 7a). Baculovirus conditioned medium alone is not responsible for the high molecular weight material (>250 kDa) in cross-linking studies, as cross-linking of baculovirus conditioned medium to PC12 cells gave no signal. Furthermore, proNGF (R-1G) purified by size exclusion chromatography in the presence of urea (Fig. 3b) results in material with the same neurite outgrowth activity as the unpurified material (Fig. 3a).

Processing of this material to mature NGF restores the level of neurite outgrowth activity to that of 2.5S NGF (Fig. 3b). These data, taken together, show convincingly that the neurotrophic activity seen in our assays is not due to a contaminating substance in the baculovirus medium.

These results are in striking contrast to those of Lee *et al*. (2001), who demonstrated apoptosis in primary SCG neurons and in smooth muscle cells upon exposure to cleavage-resistant proNGF. Lee *et al*. reported that their proNGF bound with high affinity to $p75^{NTR}$ but exhibited negligible binding to TrkA. Therefore, we tested whether our proNGF (R-1G) could bind to the high-affinity NGF receptor TrkA and activate TrkA signaling. We demonstrate here that cross-linking of PC12 cells in the presence of proNGF (R-1G) produces a TrkA-immunoprecipitable product of roughly 170 kDa, consistent with a complex of $p140^{TrkA}$ and 34-kDa proNGF (R-1G). ProNGF (R-1G) is cross-linked to PC12 cells at roughly the same specific activity as mature NGF. Furthermore, proNGF (R-1G) promotes the phosphorylation of TrkA and of p44/42 MAP kinase (ERK1/2), a key signal transduction step downstream of TrkA, in PC12 cells. The amount of 2.5S NGF or proNGF (R-1G) required to induce TrkA phosphorylation in our study is consistent with the amount used in other studies of PC12 cells (D'Ambrosi *et al*. 2000). The activity of proNGF (R-1G) in these assays is fourfold less than that of mature NGF, consistent with the results of our neurite outgrowth assays.

We also demonstrate activation of TrkA by proNGF (R-1G) in NIH3T3-TrkA cells, which do not express $p75^{NTR}$ These data, together with the TrkA cross-linking experiments, support the conclusion that proNGF (R-1G) elicits neurotrophic activity by binding to and activating TrkA. It further rules out the possibility that the biological effects of proNGF (R-1G) are mediated solely by binding to $p75^{NTR}$. The phosphorylation of TrkA by proNGF (R-1G) in NIH3T3-TrkA cells is approximately eightfold less efficient than TrkA phosphorylation mediated by wild-type NGF. The reduction in activity when compared to TrkA activation in PC12 cells could be due to the lack of $p75^{NTR}$ in NIH3T3-TrkA cells; $p75^{NTR}$ is known to increase the affinity of NGF for TrkA (Davies *et al*. 1993; Mahadeo *et al*. 1994).

Our cleavage-resistant proNGF differs from that of Lee *et al*. (2001) in several key structural features and in the expression and purification systems used. First, our proNGF contains a single amino acid substitution, an R-to-G substitution, at the −1 position, and no C-terminal alterations. Our single substitution was designed to perturb the molecule as little as possible by substituting a small amino acid, glycine, for an arginine residue that forms part of the tetrabasic cleavage site. ProNGF from Lee *et al*. (2001) contains four separate amino acid substitutions and a polyhistidine tag. Polyhistidine tags have been shown to interfere with protein refolding, stabilization and biological activity (Ledent *et al*. 1997; Lawrence *et al*. 2001). This may be particularly true if the N- and C-terminal ends interact in any way. The pro segment of NGF is important for proper folding of the molecule (Suter *et al*. 1991; Rattenholl *et al*. 2001a,b), whereas the C-terminus is important for stability and biological activity (Drinkwater *et al*. 1993; Kruttgen *et al*. 1997). Furthermore, the C-terminus of NT-3 is a key domain for interactions of NT-3 with $p75^{NTR}$ (Urfer *et al*. 1994), suggesting this might also be true for NGF. It is therefore possible that a C-terminal polyhistidine tag might change both inter- and intramolecular interactions of proNGF.

Lastly, the nickel columns commonly used to purify histidine-tagged proteins can cause oxidation and promote proteolysis by contaminating metalloproteases (Ramage *et al*. 2002). ProNGF from Lee *et al*. (2001) is susceptible to cleavage by a variety of proteases including plasmin and matrix metalloprotease-7, whereas native proNGF is stable in tissue, as shown by its detection in a variety of sources including postmortem human brain (Fahnestock *et al*. 2001). Studies are underway to compare the Lee proNGF and ours.

ProNGF is the dominant form of NGF in the central nervous system and is the only detectable form in many normal tissues (Fahnestock *et al*. 2001). This suggests, in accordance with this report and with several others (Saboori and Young 1986; Lakshmanan *et al*. 1989; Chen *et al*. 1997), that the biological role of proNGF *in vivo* is neurotrophic, not apoptotic. It has been suggested that the accumulation of proNGF in Alzheimer's disease (Fahnestock *et al*. 2001) may be responsible for increased neuronal death in this illness (Chao and Bothwell 2002). However, proNGF accumulation and neuronal death are equally consistent with atrophy of basal forebrain cholinergic neurons from other causes, their loss of TrkA (Salehi *et al*. 1996), and their subsequent inability to take up and transport neurotrophic (pro)NGF.

Given the structural data implicating the N-terminus of mature NGF in TrkA binding (Kahle *et al*. 1992; Woo *et al*. 1995), further studies on the binding of proNGF to TrkA will be required to clarify the mechanism of proNGF binding. However, our data offers clear evidence that at least one form of proNGF activates TrkA and acts as a neurotrophic molecule. Then, what is the role of proNGF? We propose that native proNGF is neurotrophic *in vivo*. Although mature NGF appears not to be present in most neural tissues (Fahnestock *et al*. 2001; Fahnestock *et al*. 2003), this does not exclude the possibility that a rapid amplification of proNGF's neurotrophic effects could be achieved by processing of proNGF to mature NGF. This may serve as a mechanism to increase neurotrophic activity quickly and where needed following injury. In support of this hypothesis, proNGF processing enzymes are up-regulated in response to sciatic nerve injury (Marcinkiewicz *et al*. 1999).

In summary, we have constructed and expressed a cleavage-resistant proNGF molecule. The recombinant

proNGF protein is not processed during biological assays and is capable of supporting cell survival, promoting neurite outgrowth, and binding to and activating the TrkA receptor and its major signal transduction pathway. ProNGF is the major form of NGF in the CNS, and therefore our demonstration that it possesses all the biological activities normally attributed to mature NGF (albeit up to fivefold less active) calls into question our assumptions about the biologically relevant NGF molecule. It is very possible that proNGF is responsible for much of the biological activity normally attributed to mature NGF *in vivo*.

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