The Unprocessed C-terminal Dipeptide of Recombinant β-Nerve Growth Factor Determines Three Stable Forms with Distinct Biological Activities*

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The processing of polypeptide neurotrophins in the nervous system is poorly understood. In this paper, we provide information on the effects of C-terminal processing of nerve growth factor. Three forms of recombinant mouse β-nerve growth factor (rNGF) were produced and isolated from insect cells infected with a recombinant baculovirus. The three purified forms of rNGF exhibited distinct biological activities and differed in their abilities to compete with high affinity binding of native β-nerve growth factor (mNGF). However, they were chemically and structurally indistinguishable from each other. All three forms of rNGF differed from mature mNGF from mouse submaxillary gland in that the C-terminal Arg-Gly dipeptide had not been proteolytically removed. Removal of the C-terminal dipeptide by γ-NGF peptidase treatment converted the three forms into a single form identical with mature mNGF. The above results demonstrate that a single polypeptide of rNGF, due to the presence of a C-terminal dipeptide, exhibits three stable dimeric protein conformations with distinct biological activities. The apparent lack of γ-NGF peptidase in the nervous system raises the possibility that the biologically significant form of NGF may differ from mature mNGF; such a difference may be of physiological relevance.

Nerve growth factor (NGF), a target-derived neurotrophic factor, supports the survival and differentiation of sympathetic neurons, neural crest-derived sensory neurons, and central cholinergic neurons of basal forebrain (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Thoenen et al., 1987). Although NGF released from target tissue has exceedingly low abundance and can only be reliably detected by highly sensitive assays (Barde, 1989), the discovery of a rich source of NGF from male mouse submaxillary gland has greatly aided the understanding of its biological role and its mechanism of action in the past 35 years (Cohen, 1960; Levi-Montalcini, 1987).

NGF exists in male mouse submaxillary gland as a β-NGF subunit of the 7 S NGF complex, which also includes an α-NGF subunit and a γ-NGF subunit with a stoichiometry of αβγγ (Varon et al., 1967a, 1967b, 1968; Smith et al., 1969; Bradshaw, 1978; Palmer and Neet, 1980). Although α-NGF has no clearly defined function so far (Isackson et al., 1984, 1987), γ-NGF is a potent arginine-esteropeptidase that has been implicated in the processing of NGF precursors (Thomas et al., 1981; Berger and Shooter, 1977; Edwards et al., 1988; Jongstra-Bilen et al., 1989). β-NGF itself is a 26.5-kilodalton dimer protein linked by noncovalent forces and contains all the biological activities ascribed to NGF (Bothwell and Shooter, 1977; Angeletti and Bradshaw, 1971; Greene et al., 1971). The amino acid sequence of β-NGF has been determined (Angeletti and Bradshaw, 1971; Angeletti et al., 1973). Cloning of β-NGF based on its amino acid sequence revealed that mature β-NGF is a proteolytic product of precursor NGFs cleaved at both N and C termini (Scott et al., 1983; Ullrich et al., 1983). Since the predicted sequences show only 2 additional amino acids past the C terminus of the mature β-NGF, the physiological significance of such proteolytic cleavage is unclear.

Besides binding to the α- and γ-NGF subunits in the 7 S NGF complex of mouse submaxillary gland, NGF exhibits a complex pattern of interactions with specific NGF receptors on cell surfaces (Sutter et al., 1979; Landreth and Shooter, 1980; Schechter and Bothwell, 1981; Woodruff and Neet, 1986). Such interactions have resulted in two classes of binding where the high affinity binding is required for the initiation of biological responses (Zimmermann et al., 1978). Two distinct NGF receptors, the low affinity NGF receptor and the trk proto-oncogene that encodes a tyrosine kinase receptor, have been identified (Johnson et al., 1986; Radeke et al., 1987; Large et al., 1989; Kaplan et al., 1991a, 1991b; Klein et al., 1991). How the class of high affinity binding is generated remains to be clarified (Klein et al., 1991; Hempstead et al., 1991). Besides the complexity it displayed in NGF receptor binding, NGF also possesses remarkable neuronal specificities. NGF belongs to a family of structurally related neurotrophins that include brain-derived neurotrophic factor and the recently identified neurotrophin-3 (Barde et al., 1982; Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990; Ernfors et al., 1990; Rosenthal et al., 1990; Jones and Reichardt, 1990). Neurotrophins share over 50% of amino acid identity and bind to low affinity receptor (Rodriguez-Tebar et al., 1990; Squinto et al., 1991), yet they act on overlapping, but nonetheless distinct neuronal populations (Snyder, 1991).

Little information is available on the structural requirements of NGF for its complex binding interactions, its neuronal specificities, and its biological functions. Such infor-
mation is essential for the understanding of the underlying molecular mechanism of the NGF neurotrophic effect. Structure-function relationships also are valuable to the practical application of NGF as a potential therapeutic drug in Alzheimer's disease treatment (Pheips et al., 1989). Mutational analysis has proven to be a powerful way of studying protein structure/function relationships. Toward this goal, we have expressed rNGF in insect cells. In this study, we have purified and characterized the rNGF produced in insect cells and compared it with mature mNGF. Three forms have been found with differing biological activities that depend on a C-terminal dipeptide extension. Such results have significance for understanding mechanistic and regulatory processes of NGF action.

**EXPERIMENTAL PROCEDURES**

NGF—7 S NGF complex was prepared from the mouse submaxillary gland of adult male Swiss Webster mice according to Stach et al. (1977). The mouse β-NGF and γ-NGF subunits were purified as previously described (Woodruff and Neet, 1986). The mouse β-NGF used in this study was further purified by Mono S FPLC (see below). Because of their notorious stickiness and great loss upon long-term storage at low concentration, mNGF and rNGF were quantitated (mNGF as standards) by the micro-BCA method (Pierce Chemical Co.) immediately before each bioassay.

**Production of Recombinant Baculovirus Containing the Full-length NGF Gene**—The 964-base pair Smal-Fstl fragment of a plasmid (kindly provided by Scott et al. (1983)) that contains the full-length prepro-NGF gene was inserted into the transfer vector pVL1393 (Invitrogen). Co-transfection of the resultant plasmid with wild type Autographa californica nuclear polyhedra virus viral DNA into the Spodoptera frugiperda 21 insect cells (kindly provided by Cephalon Inc.) and subsequent identification of the recombinant baculovirus were carried out according to Summers and Smith (1987). Two recombinant baculoviruses were isolated by plaque purification. One of them, RVNGF5, was used for expression of rNGF.

**Expression and Purification of rNGF**—200 ml of SF21 cells were grown in a spinner flask (50 rpm/min) at 28 °C until the cell density reached 2 × 10^6 cells/ml. Cells collected were infected with recombinant baculovirus, RVNGF5, at a multiplicity of infection over 1 plaque-forming unit/cell for 1 h and grown for an additional 72 h in 200 ml of Excell-400 media (J. R. Scientific, Inc.). Postinfection media collected after centrifugation at 5000 × g was run through an immunoaffinity column made of 10 mg of a monoclonal antibody. Purified protein showed a single band around 13 kilodaltons with purity of about 95% as judged by SDS-PAGE under the reducing condition (Fig. 1A). The initial yield of the amino acid sequence was 101 pmol with an average repetitive yield of 92.8 pmol.

**Mono S FPLC—rNGF and mNGF were further purified through Mono S FPLC (5 cm, Pharmacia LKB Biotechology Inc.) with buffer A of 10 mM NaH2PO4, pH 7.0, and buffer B of 10 mM NaH2PO4, pH 7.0, 1 M NaCl. A flow rate of 0.5 ml/min and an elution profile of a buffer of 20 mM Tris, pH 8.0, and then a buffer of 50 mM Tris, pH 9.0, 0.4 M NaCl and then 10 mM Tris, pH 8.0. rNGF was eluted with 0.1 M glycine, pH 2.5, and fractions were adjusted to pH 7.0 by 2 M Tris immediately.

**N-terminal Amino Acid Sequence**—The 13-kilodalton rNGF protein separated in SDS-PAGE was blotted on a polyvinylidene difluoride membrane and cut out for amino acid sequencing. The initial yield of the amino acid sequence was 101 pmol with an average repetitive yield of 92.8 pmol.

**RESULTS**

**Expression and Characterization of rNGF**—The expression of rNGF in the recombinant baculovirus infected insect cells was monitored by defined medium PC12 bioassay. The bioactivity in the culture media appeared 2 days postinfection and reached the maximal level of 1 mg eq of rNGF per liter of culture 3 days postinfection. This activity is inhibitable by rabbit anti-NGF antiserum. No biological activity was found from culture media harvested from wild type baculovirus-infected insect cells. The rNGF was enriched by a CM52 cation exchange column and was further purified through an immunoaffinity column coupled with an anti-NGF monoclonal antibody. Purified protein showed a single band around 13 kilodaltons with purity of about 95% as judged by SDS-PAGE under the reducing condition (Fig. 1A). This band was also detectable by Western blot with rabbit anti-NGF anti-
serum. N-terminal amino acid sequence analysis of the 13-kilodalton protein eluted from the SDS-PAGE produced a single amino acid sequence that matches the N-terminal amino acid sequence of mNGF (Ser-Ser-Thr-His-Pro-Val-Trp-Val-Phe-His-Met-Gly). Thus, rNGF produced in insect cells is biologically and immunologically active, has a size similar to that of mNGF, and is properly processed to the mature mNGF at its N terminus.

rNGF Exhibits Three Different Forms by Mono S FPLC—Unlike behavior of other proteins in affinity chromatography, rNGF was eluted from the immunoaffinity column in a very broad peak (8-ml fractions collected from the 2-ml immunoaffinity column). When a buffer (50 mM sodium acetate, pH 4.0, 0.15 M NaCl) of relatively higher pH was used, a significant portion of rNGF, as high as 50%, was eluted from the immunoaffinity column. In defined medium PC12 bioassay, the immunopurified rNGF had a specific biological activity 2-fold lower than that of mNGF and differed from mNGF in the shape of their dose response curves as well. These results prompted us to further characterize the immunopurified rNGF by additional chromatographic methods. When the immunopurified rNGF was applied to Mono S FPLC and eluted with a 0–1 M NaCl salt gradient, three distinct peaks at 0.30, 0.36, and 0.62 M of NaCl gradient, respectively, were observed (Fig. 1B). To ascertain that the peaks observed in Mono S FPLC were really different and stable, fractions containing each individual peak were collected and run over the Mono S FPLC again. Although peak I and III were eluted at the same salt gradient as that in the original run, peak II appeared to be sharper and was eluted at a higher salt gradient around 0.7 M. The peak I and peak III were stable to rechromatography on Mono S, whereas the stability of peak II is less clear. Each form of rNGFs was named rNGF1, rNGF2, rNGF3, respectively, according to their peak numbers in Mono S FPLC.

Three Forms of rNGF Display Distinct Biological Activities—Three forms of rNGF were collected from Mono S FPLC, quantitated by the micro-BCA method and used in defined medium PC12 bioassay. No significant difference in the morphology was observed when PC12 cells were treated with mNGF or each form of rNGF; however, a significant difference in the biological potency of each form of rNGF to stimulate neurite extension was detected by the quantitative bioassay. rNGF1 exhibited the same dose response curve and ED50 value of 20 pm as mNGF in the bioassay, whereas rNGF2 and rNGF3 showed lower specific biological activities by 7- and 30-fold, respectively (Fig. 2A). However, in serum-containing PC12 bioassay, rNGF1 exhibited the same dose response curve and ED50 value of 30 pm as mNGF, whereas rNGF2 and rNGF3 showed lower specific biological activities by only 1.5- and 3-fold, respectively.

To test the biological activity of the three forms of rNGF with primary neurons, we examined the ability of each form of rNGF to stimulate neurite extension in a defined medium bioassay of dissociated dorsal root ganglia (DRG) neurons from neonatal rat. At 10 nm concentration, each form of rNGF and mNGF was equally active in promoting neurite outgrowth of DRG neurons. At 1 nm concentration, although rNGF1, rNGF2, and mNGF remained fully active, rNGF3 was less potent. At 100 pm concentration, although rNGF1 and mNGF remained fully effective, rNGF2 became less active and rNGF3 fell to the control level (Fig. 2B). No significant difference in the morphology of DRG neurons treated with mNGF or each form of rNGF could be observed. Thus, in two independent assay systems (PC12 and DRG), rNGF1 appeared equally active as mNGF, but rNGF2 was less potent and rNGF3 was the least active form.

Three Forms of rNGF Differ in Their High Affinity Binding—To determine the cause of the different biological potency observed in three forms of rNGF, equilibrium displacement binding studies were carried out. PC12 cells were incubated with 50 pm rNGF in the presence of 10 pm to 10 nm concentrations of competitive ligands (three forms of rNGF and mNGF) at 37°C for 60 min, and total binding was measured (data not shown). Fitting of the displacement binding data by the LIGAND program (Munson, 1983) yielded a single class of binding with association constants of 4.0 ± 1.0 × 106, 1.2 ± 0.2 × 106, 1.6 ± 0.2 × 105, 5.8 ± 0.7 × 104 M−1 for mNGF, rNGF1, rNGF2, rNGF3, respectively. Thus the differences, although evident, in low affinity binding of three forms of rNGF and mNGF are not correlated with the observed difference in their biological activities. The particular
To study the structural basis for the differences observed in the effect of internalization (Kasaian and Neet, 1988), the competition binding remained when compared with $^{125}$I-NGF binding of high affinity binding by three forms of rNGF in the presence of 1 nM of rNGF and mNGF, about 23 and 21% was chased with a 4000-fold excess of cold mNGF at 0.5 °C of slow dissociation binding remained when compared with low affinity binding by mNGF was noted. To measure the ability of three forms of rNGF and mNGF to compete with high affinity binding, PC12 cells were incubated with 100 pM of $^{125}$I-NGF in the presence of a 1 nM concentration of competitive ligands (three forms of rNGF and mNGF) at 37 °C for 45 min, and then the binding reaction was chased with a 4000-fold excess of cold mNGF at 0.5 °C for 30 min (Landreth and Shooter, 1980). In the presence of 1 nM of rNGF1 and mNGF, about 33 and 37% slow dissociation binding remained when compared with $^{125}$I-NGF binding in the absence of a competitive ligand; about 41 and 70% of slow dissociation binding remained in the presence of rNGF2 and rNGF3, respectively (Fig. 3A). Since the measurement of slow dissociation binding at 37 °C could be complicated by the effect of internalization (Kasaian and Neet, 1988), the competition of high affinity binding by three forms of rNGF and mNGF was measured after binding at 0.5 °C as well. In the presence of 1 nM of rNGF1 and mNGF, about 23 and 21% of slow dissociation binding remained when compared with $^{125}$I-NGF binding in the absence of a competitive ligand; about 60 and 95% of slow dissociation binding remained in the presence of rNGF2 and rNGF3, respectively (Fig. 3B). The ability of mNGF and three forms of rNGF to compete with high affinity binding is fully consistent with the observed differences in their biological activities. Thus, we conclude that the difference observed in the biological activities of the three forms of rNGF are caused by their difference in high affinity binding to NGF receptors.

Three Forms of rNGF Are Structurally Indistinguishable—To study the structural basis for the differences observed in biological activity and high affinity binding, the three forms of rNGF were characterized biochemically. In SDS-PAGE stained with Coomassie Blue, as well as by Western blot, each form of rNGF displayed a 13-kilodalton single band with equal intensities (Fig. 4A). Although each form of rNGF runs at the same place in SDS-PAGE, they run slightly slower than mNGF. Since the N termini of rNGFs are properly processed to that of mature mNGF, the C-terminal processing of rNGF was examined in nonequilibrium isoelectric focusing gel. This method has also been used to analyze individual, denatured polypeptides of the proteolytically processed forms of mature mNGF (Mobley et al., 1976). In nonequilibrium isoelectric focusing gel, each form of rNGF runs at the same place; however, they run at a more positively charged position than that of mNGF (Fig. 4B). Since the full-length NGF gene predicts an additional Arg-Gly dipeptide at the C terminus of mature NGF, the three forms of rNGF probably contain the C-terminal Arg-Gly dipeptide that is proteolytically removed from mature mNGF.

The C-terminal Dipeptide Modulates the Conformation and Biological Activities of rNGF—Since the three forms of rNGF, in contrast to mNGF, contain the C-terminal Arg-Gly dipeptide, removal of the C-terminal dipeptide from each form of rNGF by γ-NGF treatment was investigated. Each form of rNGF and mNGF was treated with a catalytic amount of γ-NGF at 37 °C for 2 h, and half of the sample was analyzed by IEF. The position of each form of rNGF shifted to that of mNGF after treatment with a catalytic amount of γ-NGF, whereas the position of mNGF itself did not change (Fig. 5A). This result strongly indicated that the C-terminal dipeptides of rNGFs were completely removed by γ-NGF treatment. To
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Fig. 4. SDS-PAGE and IEF of mNGF and rNGF. 200 ng of mNGF and each rNGF were run on 16% SDS-PAGE (A) or IEF (B), electroblotted onto nitrocellulose membrane, probed with rabbit anti-NGF antiserum, and detected with chemiluminescence. Pre-stained molecular weight standards (A) and the pH of the ampholyte (B) are indicated.

To test whether the elution profile of rNGF in Mono S FPLC was altered after γ-NGF treatment, the immunopurified rNGF containing all three forms was incubated with a catalytic amount of γ-NGF to cleave the C-terminal dipeptide and applied to Mono S FPLC. The rNGF after γ-NGF treatment was eluted in a single, sharp peak at 0.6 M NaCl (Fig. 5C), the same position in the gradient where mNGF was eluted. Thus, only a single chromatographic form of rNGF exists after γ-NGF treatment.

To test the biological activity of each form of rNGF after γ-NGF treatment, each form of rNGF treated with or without γ-NGF was used in defined medium PC12 bioassay. mNGF and rNGF1 treated with γ-NGF did not change their specific biological activities significantly, but rNGF2 and rNGF3 treated with γ-NGF increased their biological potency to the same level as that of mNGF and rNGF1 (Fig. 5B). Such changes in the specific biological activities of rNGF2 and rNGF3 are unlikely to be a result of a stabilizing effect by γ-NGF, since only a catalytic amount of γ-NGF was used and no significant differences in the biological activities of mNGF

Fig. 5. The three forms of rNGF are converted into a single form by γ-NGF treatment. 400 ng of mNGF and each rNGF were incubated at 37 °C for 2 h in the presence or absence of 10 ng of γ-NGF. Half of each sample was run on IEF (A), and the other half was used in defined medium PC12 bioassay (B). A. IEF of mNGF and rNGF with or without γ-NGF treatment. B, dose response of mNGF and each form of rNGF with (●) or without (○) γ-NGF treatment in defined medium PC12 bioassay. A dilution series of 1 nM to 1 pm of mNGF and of each rNGF was used. C, Mono S FPLC of rNGF after γ-NGF treatment. 20 μg of immunopurified rNGF was treated with 500 ng of γ-NGF at 37 °C for 2 h. IEF gel indicated that the C-terminal dipeptide of rNGF was proteolytically removed. The sample was diluted with an equal volume of distilled water and loaded into the Mono S FPLC column.
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and rNGF1 were observed after γ-NGF treatment. The fact that three forms of rNGF after γ-NGF treatment converted to a single form in Mono S FPLC and shared the equal specific biological activity strongly suggested that individual forms of rNGF possess no intrinsic differences in their compositions and/or structures. Thus, the three forms of rNGF indicate three stable protein conformations, and such conformations are generated by the presence of the C-terminal dipeptide.

If three forms of rNGF indeed represent three different protein conformations, they are likely to yield a thermodynamically stable form of rNGF upon protein denaturation and renaturation. Since fluorescent measurement indicated that mNGF was denatured in the presence of 4 M GdnHCl and completely renatured by dilution, the biological activities of rNGFs in defined medium PC12 bioassay were measured after GdnHCl treatment. After GdnHCl treatment, the specific biological activity of rNGF1 decreased by 3-5-fold, whereas mNGF remained fully active (Fig. 6). The rNGF1 was also compared with the γ-NGF-treated, Mono S FPLC-purified rNGF in the bioassay and was found to be 5-fold less active after GdnHCl treatment. Thus, rNGF1, although equally active as mNGF before GdnHCl treatment, was converted to a more stable, yet less active conformation upon GdnHCl treatment. The reason that the three forms of rNGF did not yield an equally active form in the bioassay upon GdnHCl treatment is not clearly understood. Perhaps the rNGF with a C-terminal dipeptide requires a longer period of GdnHCl incubation/dilution to achieve a complete denaturation/renaturation. However, diminished biological activities of mNGF and rNGF upon prolonged incubation at room temperature were observed, a result likely due to their notorious stickiness.

Circular Dichroism and Fluorescence Study of the Three Forms of rNGF—How the C-terminal dipeptide regulates the protein conformation is not clear at present. Since NGF exists as a dimer, it is possible that three different protein conformations of rNGF may originate from two distinct conformations of the C-terminal dipeptide. Such distinct orientations of the C-terminal dipeptide in rNGF could result in three dimeric protein conformations that differ either at a global or at a local level. To determine whether a gross conformational change has occurred in three forms of rNGF, each form and mNGF were subjected to CD and fluorescence study. The fluorescence absorption and emission spectrum, as well as polarization parameters, did not differ significantly among three forms of rNGF and mNGF (not shown). The CD spectrum of three forms of rNGF and mNGF exhibited little difference throughout the entire scanning wavelength (Fig. 7). Thus little, if any, detectable gross conformational difference exists among the three forms of rNGF. The lack of gross conformational changes in three forms of rNGF indicates that the C-terminal dipeptide may be directly responsible for the differences observed in Mono S FPLC, in binding activity, and in biological activity among the three forms.

Model of the Three Forms of rNGF—Based on the CD and fluorescence studies, we postulate that the three forms of rNGF differ primarily in the orientation of the C-terminal dipeptide, with both C-terminal dipeptides buried inside the rNGF1 dimer and both C-terminal dipeptides exposed outside in the rNGF3 dimer (Fig. 8). The rNGF2 represents a heterodimer between rNGF1 and rNGF3. Such arrangements of the C-terminal dipeptides explain the different behaviors of the three forms of rNGF in Mono S FPLC. In the model, the two C-terminal dipeptides of rNGF1 dimer cannot contact the monobeads of Mono S FPLC, whereas the two C-terminal dipeptides of rNGF3 dimer can make contact. From the GdnHCl denaturation experiments, rNGF1 appears to be in a thermodynamically unstable form. The hypothesized model also explains the different binding and biological activities among the three forms of rNGF, if the orientation of the C-terminal dipeptide in rNGF3 dimer interferes with the high affinity binding, either through steric hindrance or through unfavorable interactions, the orientation of the C-terminal dipeptides in rNGF1 dimer does not interfere, and the heterodimer rNGF2 is partially hindered.

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DISCUSSION

We present evidence that the three conformations of rNGF stably existed upon purification from insect cells. First, three forms of rNGF behave differently and reproducibly in high resolution cation exchange chromatography and have distinct biological and binding activities. Second, they are biochemically and structurally indistinguishable. Third, they are converted into a single form after removal of their C-terminal Arg-Gly dipeptides. Finally, their biological activities are altered upon protein denaturation/renaturation. These results also demonstrate the advantage (and necessity) of characterization of recombinant NGF that has been highly purified from the insect cell system, in contrast to determination of properties of activity expressed in mammalian cell culture media (e.g. Ibanez et al., 1990, 1991; Suter et al., 1992).

NGF purified from mouse submaxillary gland has been shown to contain a small fraction of a glycosylated form (Murphy et al., 1989). The three forms of rNGF are unlikely to differ in the extent to which glycosylation occurred, since they share the same size in SDS-PAGE, exist as a single peak in Mono S FPLC and do not have distinct, reproducible peaks in Mono S FPLC, and have equal biological potency after the removal of the C-terminal dipeptide. The same facts also argue against the possibility of other differential post-translational modifications. The rNGF forms are unlikely to be a heterodimer between rNGF1 and rNGF3 subunits.

How rNGF folds into three stable conformations in the insect cell environment is not known. Three protein conformations could potentially arise from differential interaction with other proteins in the insect cells. Several lines of evidence indicated that such interaction may exist in the insect cell environment. First, when partially purified rNGF from the CM52 column was applied to Mono S FPLC, only a broad peak around 0.3 M NaCl gradient was observed. Second, rNGF was not able to bind to the immunosaffinity column efficiently at neutral pH, yet was able to achieve a quantitative binding at high pH. Third, rNGF was not separated from other high molecular proteins on Superose 12 FPLC at neutral or low pH, yet isolated well at high pH. The association of NGF produced in fibroblast cells with other proteins was also observed by other groups (Simonski and Murphy, 1987). Whether such interactions are specific remains to be determined.

The fact that three forms of rNGF do not change their gross conformation significantly yet possess distinct high affinity binding suggest that C-terminal residues may be directly involved in high affinity binding of NGF to NGF receptor. Thus, we suggest that the C-terminal residues represent an interesting and fruitful region for further mutational studies.

We have also tested the biological activity of human recombinant NGF expressed in insect cells in defined medium PC12 bioassay and found that human recombinant NGF is about 10-fold less active than mNGF but becomes fully active upon γ-NGF peptidase treatment. This result is in contrast to the reports that human recombinant NGF is more active than the 2.5 S NGF (Barnett et al., 1991; Knuelsen et al., 1990; Buxser et al., 1991). We have noted that human recombinant NGF becomes equally active to mNGF in the lengthier, serum-containing bioassay with PC12 cells, but bovine serum albumin added to the defined medium has no effect. Thus, the increase in biological activity of human recombinant NGF in serum medium bioassay is likely due to the presence of specific factors that modify or modulate the not fully processed human recombinant NGF. Interestingly, purified human recombinant NGF was shown to be less potent in inducing choline acetyltransferase activity than 2.5 S NGF when infused to the rat brain at a low dose (Barnett et al., 1991).

We have demonstrated here that the C-terminal dipeptide is able to modulate the protein conformation and biological activity of rNGF. Modulation of the conformation and biological activity of rNGF by the C-terminal dipeptide is novel and may bear physiological relevance. The fact that the C-terminal dipeptide is also able to modulate the biological activity of human recombinant NGF indicated that the regulatory effect of the C-terminal dipeptide in NGF may be common among different species. Comparison of the predicted amino acid sequences of NGF from different sources revealed that NGF from mouse, human, bovine, rat, and guinea pig possess a C-terminal dipeptide and bear resemblance at their N termini as well, whereas NGF from chick and snake have quite different sequences at both ends (Scott et al., 1983; Ullrich et al., 1983; Meier et al., 1986; Ebendal et al., 1986; Wion et al., 1986; Selby et al., 1987; Whittemore et al., 1988; Schwarz et al., 1989). Whether the lack of the C-terminal dipeptide in chick and snake results from their evolutionary distance to other species is not known. Brain-derived neurotrophic factor and neurotrophin-3 also differ from NGF at both the C and N termini (Leibrock et al., 1989; Maisonnier et al., 1990) and do not appear to have a processing site at the C terminus.

γ-NGF has previously been shown to process the NGF precursors to a form with size and biological activity similar to mature mNGF (Berger and Shooter, 1977; Edwards et al., 1988; Jongstra-Bilen et al., 1989). However, γ-NGF has not been adequately demonstrated to be the physiological enzyme that processes NGF precursors in mouse submaxillary gland (Bradshaw, 1983), since other proteases are able to process the NGF precursors as well (Berger and Shooter, 1977; Edwards et al., 1988; Bresnahan et al., 1990). The results presented here are the first demonstration with purified components that a catalytic amount of γ-NGF is able to cleave the C-terminal dipeptide from NGF. Our result, combined with the previous finding that the C-terminal Arg of mature β-NGF is critical for γ-NGF binding in the 7 S NGF complex (Moore et al., 1974), provides strong evidence that γ-NGF is

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3 Y. Luo and K. E. Neet, unpublished data.
the processing enzyme leading to the production of mature β-NGF in the mouse submaxillary gland.

The physiologically significant form of NGF remains biochemically uncharacterized due to the extraordinarily small amount of it present in target tissues (Barde, 1989). However, physiologically significant NGF clearly does not originate from mouse submaxillary gland, since excision of the gland does not affect the nervous system in any appreciable manner (Levi-Montalcini, 1987). Studies have demonstrated that α-NGF and γ-NGF are co-localized with β-NGF within the same cells in mouse submaxillary gland (Mowry et al., 1984). However, these subunits are not detectable in several cell lines originated from target tissues where NGF is produced (Pantazis, 1983; Kim and Pantazis, 1985) nor have they been reported from nervous tissue. Other proteases, furin for example, that have more ubiquitous distribution than γ-NGF have also been shown to be able to process precursor NGF to its active form (Bresnahan et al., 1990; Barr, 1991). These results indicate that a different processing enzyme other than γ-NGF is involved in the processing event at target tissues. Moreover, if the C-terminal Arg-Gly is not removed, lower activity toward some neuronal populations may occur, such as we found with the DRG neurite extension assay. Thus, the possibility exists that the physiologically significant form of NGF may differ structurally from the β-NGF found in mouse submaxillary gland, depending upon the availability of active proteases. Such differences, as demonstrated in this study, have the potential to regulate the biological activity of NGF in vivo.

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