The role of the nerve growth factor (NGF) carboxyl terminus in the function of NGF is not well understood. Previous work showed that deletion of residues 112–120 abolished NGF bioactivity. Several mutagenesis studies, however, have localized the binding sites of the two NGF receptors, p75 and TrkA, to other regions of the NGF molecule. To investigate the role of the NGF COOH terminus, we performed a detailed structure-function analysis of this region by deleting stepwise each of the nine COOH-terminal residues as well as constructing six point mutants. We found that point mutations within the 111–115 region, but not deletion of residues 116–120, significantly decreased NGF bioactivity, as determined by TrkA tyrosine phosphorylation and neurite outgrowth from PC12 cells. Mutation of the absolutely conserved Leu112 led to severely disrupted p75 binding on MG87-TrkA fibroblasts. This suggests that the p75 binding surface is more extended than previously believed and includes not only charged residues within loops 1 and 5 but also spatially discontinuous, uncharged residues in a region where the NH2 and COOH termini are in close proximity. Unexpectedly, deletion of COOH-terminal residues beyond Ala116 led to significantly decreased stability. These results demonstrate that residues 111–115, but not residues 116–120, are important for both the structural stability and biological activity of NGF.

The neurotrophins are a family of survival and differentiation factors that exert a profound influence on development and maintenance of the nervous system (1). This feature makes neurotrophins promising candidates as therapeutic agents for the treatment of neurological disorders (2, 3). The crystal structure of the prototype neurotrophin, NGF, revealed that it belongs to the cystine knot superfamilies of structurally related growth factors (4). The well conserved “core” of the NGF molecule consists of two pairs of anti-parallel β-strands that form an elongated dimer interface and are held together by three disulfide bridges uniquely arranged in a characteristic cystine knot motif. The β-strands are connected to the “north” of the protomer by three β-hairpin loops, whereas the NH2 and COOH termini appear to be located closer to the “south” of the protomers (see Fig. 7 for details). The loops and termini were not well resolved by x-ray crystallography and are thus likely to be highly flexible (5, 6). Moreover, these domains are regions of highest sequence divergence among neurotrophins. There is ample evidence for an involvement of these neurotrophin regions in specific receptor binding (7).

Two NGF receptors have been identified: the p75 neurotrophin receptor and the tyrosine kinase TrkA (8–10). Although TrkA is able to elicit NGF responses by itself in some systems, p75 can co-operate with TrkA to increase NGF binding to and activation of TrkA at low ligand concentration (11, 12). Mutagenesis studies have previously demonstrated that the binding of NGF to p75 is dominated by three charged residues at positions 32, 34, and 95 in the loops at the north of the molecule because a triple mutant with alanine at these positions resulted in a loss of p75 binding (27). The NH2 terminus as well as residues 31, 40–49, and 91–97 have been identified as determinants of NGF binding to TrkA (13–17).

The importance of the COOH terminus in NGF structure and function remains controversial. Previous work in this laboratory showed that a deletion mutant lacking 9 COOH-terminal residues was completely inactive (18) and a chimeric molecule in which the 10 COOH-terminal residues of NGF were substituted by the homologous sequence of BDNF had decreased bioactivity as measured by neurite outgrowth from PC12 cells, which co-express TrkA and p75 NGF receptors (19). Other investigators found that a similar chimera between rat NGF and rat BDNF had a 10% reduction in binding to TrkA-expressing fibroblasts and normal bioactivity (14). The NGF COOH terminus contains a potential proteolytic processing site, and it remains uncertain whether cleavage at this site modifies NGF bioactivity. Forms of recombinant mouse NGF expressed in insect cells, which differed from submandibular gland-derived NGF by the presence of the COOH-terminal Arg-Gly dipeptide, displayed altered bioactivity (20). However, recombinant human NGF isoforms with two or three COOH-terminal residues proteolytically removed, and thus designed to resemble mouse submandibular gland preparations (which contain a certain percentage of isoforms lacking the NH2-terminal eight amino acids and/or the COOH-terminal two residues), exerted wild type bioactivity (21). In addition, on the basis of molecular modeling (22) it has been suggested that an interaction between the NH2 and COOH termini may contribute to TrkA binding. In this study we have used a series of point and deletion mutants to investigate the NGF COOH terminus, with the goal of clarifying its role in NGF structure, receptor binding, and biological activity.
EXPERIMENTAL PROCEDURES

Cell Lines—COS-7 cells, A875 cells, and PC12 cells were maintained in Dulbecco’s modified Eagle’s medium containing 6% bovine calf serum and 6% horse serum. MG87 cells expressing human TrkA (Regeneron Pharmaceuticals, Tarrytown, NY) were grown in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum.

Construction of Deletion Mutants—Mutated cDNAs were constructed by polymerase chain reaction in PKS-mNGF (18) using a T7-primer as common 5′-primer and specific 3′-primers introducing a new EcoRI site downstream of the stop codon. Polymerase chain reaction products were purified by a Qiex kit (Qiagen Inc., Chatsworth, CA) and digested with PflMl/EcoRI, and the 3′-fragment was subcloned into the previously described pBJ-5-mNGF (18). The constructs were verified by sequencing using the dideoxy method.

Expression in COS-7 Cells—COS-7 cells at ~50% confluence on 10-cm dishes were transfected with 15 μg of plasmid DNA by the DEAE-dextran-chloroquine method as described previously (18). Conditioned media (containing 1% fetal calf serum) were collected 72 h later and cleared by high speed centrifugation. To produce conditioned media with high concentrations of wt NGF and mutants, supernatants were concentrated using Centricron 10 columns (Amicon Inc., Beverly, MA) according to the manufacturer’s instructions.

SDS-PAGE and Western Blotting—After concentration on Centricron 10 columns and determination of NGF concentration by ELISA, samples were subjected to 15% SDS-PAGE. Western blotting was performed as described previously (23) using polyclonal rabbit anti-NGF antisera (Sigma) and horseradish peroxidase-conjugated goat anti-rabbit antibodies (Sigma). Blots were developed using the Renaissance Western Chemiluminescence Reagent (NEN Life Science Products). The test for proteolytic degradation was performed as follows: equal amounts of wt NGF and mutant Δ114–120 were incubated for 3 days at 37 °C versus 4 °C and afterward subjected to the above-described anti-NGF Western blot analysis.

Stability Assay—Conditioned media from transfected COS-7 cells expressing wt NGF and mutants were incubated at 4 °C versus 37 °C for 3 days and subjected, in triplicate, to a previously described ELISA (23) using the monoclonal MCo-1 as capture and a polyclonal anti-NGF antisera (Sigma) as detection antibody. This ELISA recognizes only native NGF and not reduced or denatured NGF (data not shown). The percentage of recovery for the mutants was defined as the 37 °C ELISA concentration divided by the 4 °C ELISA concentration multiplied by 100. The 272/21 ELISA was performed according to the manufacturer’s instructions (Boehringer Mannheim).

TrkA Autophosphorylation—80% confluent PC12 cells were washed twice with Dulbecco’s modified Eagle’s medium and subsequently incubated with wt NGF or mutants, adjusted to equal concentrations of 2, 10, or 50 ng/ml in a total volume of 6 ml. After an incubation of 5 min at 37 °C, supernatants were removed, and plates were placed on ice and rinsed with ice-cold phosphate-buffered saline. After removal of the phosphate-buffered saline, cells were lysed in RIPA buffer and subjected to immunoprecipitation using D203 anti-pan Trk antibody (a gift of Dr. D. Kaplan, Montreal Neurologic Institute, Montreal, Canada) and Protein A/G Plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were pelleted, washed three times, subjected to 7.5% SDS-PAGE, and transferred to nitrocellulose membranes. Blots were blocked and then incubated with PY20, an anti-phosphotyrosine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by peroxidase-conjugated donkey anti-mouse antibody (Jackson Laboratories, Bar Harbor, ME). Reactive bands were detected by chemiluminescence as described above.

Neurite Outgrowth on PC12 Cells—PC12 cells (5 × 10⁴/well) were seeded out in six-well Costar plastic plates with 2.5 ml of medium. After allowing cell adherence to the culture dish overnight, cells were washed, and 2-fold serial dilutions of conditioned media containing wt NGF or mutants in Dulbecco’s modified Eagle’s medium (containing 1% fetal calf serum) were added. Neurite outgrowth (percentage of cells with neurites > 2 × cell diameter in a representative visual field) was scored after 3 days.

Binding Assays—Binding of wt NGF and mutants to TrkA-expressing fibroblasts (MG87-TrkA) or p75-expressing A875 human melanoma cells was measured in a modification of the PC12 receptor binding assay (18). Purified 2.5 S NGF (Harlan Bioproducts for Science, Indianapolis, IN) was iodinated by the lactoperoxidase method to a specific activity of ~1.5 × 10⁹ cpm/μg. For each data point, 125I-NGF (1 ng) was incubated in triplicate with increasing amounts of concentrated wt NGF or mutants and 10⁵ A875 cells or 5 × 10⁵ MG87-TrkA cells in a total volume of 100 μl on ice for 2 h with gentle vortexing every 10 min. After centrifugation through a 0.15 M sucrose gradient, cell pellets were counted in a γ counter (Beckman, Palo Alto, CA). “Vector control” binding was determined by adding increasing amounts of concentrated medium from COS cells transfected with pBJ-5 vector alone. At low concentrations, no displacement of 125I-NGF was observed, although about 25% displacement was observed at the highest concentration. The percentage of 125I-NGF binding was calculated as follows: (mean cpm obtained for each recombinant competitor/mean cpm obtained in the presence of an equivalent amount of vector-transfected medium) × 100.

Molecular Graphics—The crystal structure of purified mouse des- octaβNGF (6) was displayed using Insight II, version 95.0 (Biosym, San Diego, CA). Coordinates of the C2 space group structure were downloaded from the Brookhaven protein data base. Hydrogen atoms were omitted, as well as the third protomer, which is also packed into the C2 unit cell. Amino acid residues with relevance to this report were highlighted in color, and the van der Waals’ surface of the polypeptides was calculated.

RESULTS

Construction and Expression of COOH-terminal NGF Mutants—Using polymerase chain reaction-based mutagenesis, a series of COOH-terminal mutants of murine NGF were constructed (Fig. 1). Eight stepwise deletions covering the region between residues 113–120 of mature NGF, as well as five point mutants covering the stretch between residues 111–115 and a double point mutant in which residues Arg¹¹⁸ and Arg¹¹⁹ were exchanged to serines (R118S/R119S). We also made use of previously described constructs from our laboratory such as deletion mutant Δ112–120 (18) as well as several chimeric NGF/BDNF proteins designated as CH1 to CH12 (19). CH12 contains the BDNF COOH terminus on an NGF backbone and therefore lacks residues 119–120 and contains five additional amino acid substitutions (V111T, S113T, R114I, A116R, and

![FIG. 1. Schematic representation of COOH-terminal NGF mutants. Amino acids deleted are indicated as Δ n–n. Relative expression levels of a typical COS cell transfection as determined by MCo-1 based ELISA are shown on the right. Note the disulfide bridge from Cys⁶⁰ to Cys¹¹⁹.](image-url)
The mutated cDNAs were sequenced, subcloned into the mammalian expression vector pBJ-5 (24), and expressed by transient transfection of COS-7 cells. Conditioned media were subjected to a NGF-specific ELISA based on the monoclonal NGF antiserum. The top panel shows point mutants, and the bottom panel shows deletion mutants of NGF. The arrow denotes wt NGF.

Fig. 2. Western blot analysis of NGF mutants. Conditioned media of transiently transfected COS cells were concentrated with Centri-10 columns, and equal amounts of mock transfected cells as well as wt NGF and mutants were subjected to SDS-PAGE and Western analysis as described under “Experimental Procedures” using a polyclonal antibody for the ELISA. We considered two possible explanations for the decrease in recognition by the monoclonal MCβ-1 used as capture antibody and a polyclonal anti-NGF antiserum (Sigma) as detection antibody. This ELISA only recognizes native but not reduced nor denatured NGF (data not shown). As shown in Fig. 1, expression levels were generally in the range of 200–400 pg/ml, but surprisingly, mutants Δ114–120 and Δ115–120 consistently appeared to be expressed at lower levels as determined by the monoclonal based ELISA, whereas mutants Δ112–120 and Δ113–120 were not detectable at all. The same result was obtained using a commercially available NGF-ELISA based on a different monoclonal antibody, 27/21 (data not shown).

COOH-terminal Deletions Led to Conformational Alterations and Decreased Stability—In contrast to the ELISA results, mutants Δ112–120 and Δ113–120 were detected at levels comparable with those of the other mutants by the less sensitive Western blot analysis, in which aliquots of the same conditioned COS supernatants were detected by the anti-NGF polyclonal antiserum (Fig. 2). Because this antiserum was also used as detection antibody for the MCβ-1 based ELISA, we concluded that mutants Δ112–120 up to Δ115–120 might have a decreased recognition by the monoclonal MCβ-1 used as capture antibody for the ELISA. We considered two possible explanations: the COOH terminus could represent the epitope for MCβ-1, or deletions at the COOH terminus might induce conformational changes that alter an epitope located in a different part of the NGF structure.

To test the first possibility, we took advantage of previously described NGF/BDNF chimeras to investigate the MCβ-1 epitope. In these chimeras, short stretches of approximately 10 amino acids are systematically exchanged from BDNF into the homologous NGF backbone substituting the corresponding NGF sequences (19). Because MCβ-1 does not cross-react with BDNF (data not shown), it is unlikely to recognize a chimera in which the epitope within NGF is replaced by the corresponding region of BDNF. Chimeric proteins were expressed in COS-7 cells, and supernatants were subjected ELISA analysis. The COOH-terminal chimeras CH10, CH11, and CH12, as well as NH2-terminal chimeras CH1 and CH2 were recognized well by ELISA, providing evidence that MCβ-1 does not recognize the COOH terminus nor the NH2 terminus. Moreover, this suggested that the larger deletion mutants were not recognized by MCβ-1 (nor the 27/21 monoclonal) due to a broader conformational disruption or destabilization.

To investigate if COOH-terminal deletions destabilized NGF, we assayed the stability of all NGF mutants used in this study using the following assay. Conditioned COS cell media containing wt NGF or mutant proteins were incubated for 3 days at 37 °C versus 4 °C and afterward subjected to MCβ-1-ELISA. As shown in Fig. 3A, most of the mutants as well as wt NGF were stable for 3 days at 37 °C. Deletions beyond residue 116, however, led to a time-dependent decrease in NGF recovery (Fig. 3B). This decrease in MCβ-1 ELISA recognition was not accompanied by obvious proteolytic events, as shown in Fig. 3C, where wt NGF and Δ114–120 were subjected to Western blot analysis after a 3-day incubation period at 37 °C versus 4 °C. No lower molecular weight bands were observed in the samples incubated at 37 °C. We therefore limited our subsequent analysis of the mutants to those that were stable for at least 3 days.

Receptor Binding and Bioactivity of COOH-terminal Mutants—Next, we determined the activity of the COOH-terminal mutants using a standard PC12 neurite outgrowth assay. Serial dilutions of wt NGF and mutants were applied to the cells, and neurite outgrowth was scored after 3 days. As seen in Fig. 4, deletion mutant Δ116–120 showed no appreciable decrease in neurite outgrowth compared with wt NGF, suggesting that residues 116–120 are not essential for bioactivity. Consistent with these findings, R118S/R119S also had normal levels of bioactivity. Deletion mutants extending beyond residue 116 (Δ115–120, Δ114–120, and so on) had reduced bioactivity (data not shown), but for reasons described above, we could not determine if this was due to decreased stability, impaired activation of receptors, or both. Several point mutants showed significantly reduced bioactivity. L112A showed approximately a 6-fold reduction, V111T, R114S, and K115S showed a 4-fold reduction, and S113T had only a slightly reduced activity.

The binding of NGF to TrkA induces receptor homodimerization and transphosphorylation, which activates downstream signaling events (26). To further characterize the activity of selected mutants we analyzed their ability to activate TrkA receptors in PC12 cells. COS cell-conditioned supernatants containing wt NGF or mutants were applied to PC12 cells in three different concentrations ranging from 2 to 50 ng/ml. Cells were lysed, and TrkA was immunoprecipitated and subjected to Western blot analysis with anti-phosphotyrosine antibodies (Fig. 5). Consistent with the PC12 neurite outgrowth data, R118S/R119S and Δ116–120 (not shown) had wild type activity. R114S, K115S, and CH12 showed significant reductions in TrkA activation, whereas L112A and V111T were the most strongly impaired.

Because PC12 cells express both the TrkA and p75 neurotrophin receptors, impaired neurite outgrowth and TrkA phosphorylation may reflect decreased binding to either TrkA or p75. To measure the ability of selected mutants to interact with each receptor individually, we performed binding competition experiments on p75-expressing A875 cells or TrkA-expressing fibroblasts (Fig. 6). Consistent with the above mentioned results, R118S/R119S had wild type binding to both TrkA and p75. L112A and CH12 both showed small but reproducible
reductions in TrkA binding. By contrast, the binding of L112A to p75 was reduced by at least 50-fold; the exact IC50 could not be determined because 100 nM of L112A was able to displace only 25% of radiolabeled NGF tracer. The binding of CH12 to p75 was also impaired but to a lesser extent than that of L112A, with an approximately 15-fold increase in the IC50.

**DISCUSSION**

In this study we have used site-directed mutagenesis to analyze the role of the COOH terminus in NGF bioactivity as well as its binding to the NGF receptors p75 and TrkA. We focused on this region for several reasons. The COOH terminus contains a potential cleavage site that has been proposed to modify NGF bioactivity (20). Furthermore, although previous mutagenesis studies have highlighted spatially distinct regions of the NGF structure as being critical for NGF-receptor interactions (14), our findings demonstrate that part of the COOH terminus plays a role in NGF receptor

**FIG. 3. Stability assay of mutants.** A, conditioned media of COS cells transiently transfected with wt NGF or mutants were incubated for 3 days at 37 °C or 4 °C, and the concentration of NGF was determined by a conformationally sensitive ELISA using the MCβ-1 monoclonal antibody. Each sample was measured in triplicate. The percentage of recovery for each protein was defined as the concentration of NGF (after 37 °C incubation) divided by the concentration of NGF (after 4 °C incubation) multiplied by 100. The error bars indicate S.D. B, conditioned media of COS cells transiently transfected with wt NGF or mutants Δ114–120, Δ115–120, and Δ116–120 were subjected to the above described assay; aliquots were removed after 0, 3, 6, 12, 36, and 72 h and stored at 4 °C until ELISA measurement in triplicate were performed. ●, wt NGF; ○, Δ116–120; ×, Δ115–120; ■, Δ114–120. C, conditioned media from COS cells transfected with wt NGF and mutant Δ114–120 were concentrated, incubated for 3 days at 37 °C versus 4 °C, and subsequently subjected to Western blotting using a polyclonal NGF antiserum from Sigma. No obvious proteolysis was evident. This experiment was performed twice.

only 25% of radiolabeled NGF tracer. The binding of CH12 to p75 was also impaired but to a lesser extent than that of L112A, with an approximately 15-fold increase in the IC50.

**FIG. 4. Neurite outgrowth on PC12 cells.** Conditioned media of COS cells transfected with wt NGF and mutants were subjected to ELISA, and their concentrations were determined in triplicate. Serial dilutions in 1% fetal calf serum were added to PC12 cells in 6-well plates, and neurite outgrowth was scored after 3 days by counting of ~200 cells per data point. Cells with neurites > 2 cell diameters were considered positive, and the percentage of neurite outgrowth was expressed as a quotient of positive cells/total number of cells multiplied by 100. This assay was carried out four times with comparable results; a representative assay is shown. ●, wt NGF; ○, V111T; ×, L112A; ■, S113T; □, R114S; ▲, K115S; ◄, R116S/R119S; ◆, Δ116–120.

**FIG. 5. Tyrosine phosphorylation of TrkA on PC12 cells.** Conditioned media of COS cells transfected with wt NGF and mutants were subjected to ELISA, and their concentrations were determined in triplicate. Concentrations of 2, 10, and 50 ng/ml were subsequently applied to PC12 cells. TrkA was immunoprecipitated from the cell lysates using the pan-Trk antibody D203; after SDS-PAGE, tyrosine phosphorylated TrkA was detected by Western blotting using the anti-phosphotyrosine monoclonal PY20.

In this study we have used site-directed mutagenesis to analyze the role of the COOH terminus in NGF bioactivity as well as its binding to the NGF receptors p75 and TrkA. We focused on this region for several reasons. The COOH terminus contains a potential cleavage site that has been proposed to modify NGF bioactivity (20). Furthermore, although previous mutagenesis studies have highlighted spatially distinct regions of the NGF structure as being critical for NGF-receptor interactions (14), our findings demonstrate that part of the COOH terminus plays a role in NGF receptor
Interestingly, additional contact points for p75 were suggested by the alteration of Trp-21, an uncharged residue located at the northern part of the COOH terminus of NGF, but also uncharged residues in spatially discontinuous regions of the COOH terminus located at the south end in close proximity to the NH2 terminus of the other protomer (Fig. 7). This could indicate some degree of conformational freedom in the region where the NGF NH2 and COOH termini are in close proximity. Even subtle changes, like the conservative substitutions of Val111 and Leu112 with alanine might locally alter the configuration of the area where the NGF termini overlap (22). Given that alanine is less bulky than valine and leucine, the observed effects are unlikely to be due to the introduction of steric hindrance. Because L112A is comparable with wt NGF in terms of stability (Fig. 3A) and retains considerable Trk binding (Fig. 6A), its impaired p75 binding is more likely to be the result of a local modification of the p75 binding surface rather than a global conformational disruption. Alternatively, carboxyl-terminal mutations could disrupt some interaction between the NH2 and COOH termini, which is important for receptor binding. We cannot, however, rule out the theoretical possibility that mutation relieves the termini from constrained conformation, thereby allowing them to interfere with interactions between p75 and loops 1 and 5 of NGF (27). Leu112 is strictly conserved in all known neurotrophins, suggesting that it may play a similarly critical role in other neurotrophins as well.

Previous studies highlighted the importance of basic residues in neurotrophins for binding to p75 (Lys32, Lys34, and Lys36 of NGF; Lys35, Lys36, and Arg97 of BDNF; Arg31, His33, Arg68, Arg114, and Lys115 of NT-3; Arg34 and Arg36 of NT-4/5) (27, 28, 30). Because Leu112 is surrounded mostly by uncharged amino acid residues, nonionic interactions are also likely to play a role in the binding of NGF to p75. Taken together with previous studies, our data suggest that p75, like TrkA (14), binds to an extended surface of NGF. This surface appears to include not only positively charged residues at the north end of NGF, but also uncharged residues in spatially discontinuous region of the COOH terminus located at the south end in close proximity to the NH2 terminus of the other protomer (Fig. 7). An extended interface between p75 and northern plus southern contact points on NGF would be generally compatible with a hypothetical molecular model of the NGF:p75 complex (31).

A chimeric protein consisting of the BDNF COOH terminus with an NGF backbone, CH12, differs from wt NGF in that it contains five different residues and lacks residues 119–120 (19). We find that CH12 has a 15-fold decreased ability to interact with the CH12:p75 complex (31).

We have determined the bioactivity of the mutants in PC12 cells, which express both p75 and TrkA receptors. Neurite outgrowth can occur via TrkA activation alone (27, 34). However, p75 is likely to facilitate TrkA-mediated signaling at low concentrations.
ligand concentrations (11, 12). Therefore, decreased neurite outgrowth may reflect impaired binding to TrkA, p75, or both. We find that conservative point mutations in the 111–115 region (V111T, L112A, R114S, and K115S) significantly reduced bioactivity. By contrast, deletion of the five COOH-terminal residues (D116–120) had no discernible effect. Consistent with these findings, point mutants in the 111–115 region also had a reduced ability to induce TrkA tyrosine phosphorylation in PC12 cells. Interestingly, L112A, which exhibited severely impaired p75 binding, had only a relatively modest reduction in binding to fibroblasts expressing TrkA alone but a far greater reduction in both Trk phosphorylation and neurite outgrowth in PC12 cells. Although the binding, Trk phosphorylation, and neurite outgrowth results cannot be directly compared quantitatively, the L112A data suggest that the loss in p75 binding leads to a reduction in Trk activation and bioactivity in PC12 cells, as suggested by earlier studies (11).

**COOH-terminal Deletions That Include Residues 111–115 Destabilize NGF** —For COOH-terminal mutants containing deletions of more than five residues (mutants Δ112–120 up to Δ115–120), we observed a striking discrepancy in the amount of NGF detectable by Western blotting, which uses a polyclonal antiserum to detect reduced, denatured protein, and a two-site ELISA, which utilizes a monoclonal antibody (MCβ-1) that recognizes native but not denatured NGF. For example, conditioned media from cells transfected with Δ113–120 contained near wild type levels of NGF as detected by Western blotting but no detectable NGF by ELISA (the detection limit of the Western blot is ~5 ng/ml; the detection limit of the ELISA is ~0.025 ng/ml). Two possible explanations for the discrepancy are that 1) the deleted region of the COOH terminus forms part of the MCβ-1 epitope, or 2) the deletions cause conformational perturbations in regions not contained within the epitope. We consider the first explanation unlikely for several reasons. First, experiments using a second monoclonal antibody (27/21) yielded similar results. Furthermore, MCβ-1 does not cross-react with BDNF but does recognize CH12, a chimera in which the NGF COOH terminus is replaced by the corresponding region of BDNF. Experiments using additional chimeras indicate that the epitope for MCβ-1 does not lie between residues 92–120 nor at the NH2 terminus (residues 1–22). Therefore we consider it likely that the observed discrepancy is caused by significant perturbations in the neurotrophin structure.

The crystal structure of NGF revealed that the NGF core contains a cystine knot motif as well as an extended dimer interface, both of which are presumably critical for the struc-
Role of the COOH Terminal of NGF

...stability, but this does not guarantee that the COOH terminus serves a similar role in other neurotrophins as well.

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Bioactivity—Although our data provide evidence that the 111–115 region is important for both NGF stability and bioactivity, we do not find residues 116–120 to be essential for either function. Both R118S/R119S, the mutant in which the dibasic prolyteolytic processing site is replaced, and Δ116–120 were essentially indistinguishable from wt NGF in terms of stability, TrkA phosphorylation, and pC12 neurite outgrowth. Furthermore, R118S/R119S and wt NGF were comparable in TrkA and p75 binding. We cannot, however, exclude the possibility that this region of the COOH terminus serves other functions not tested in this study, such as directing intracellular targeting or adhesion to the extracellular matrix. It is worth noting, for example, that the β chain of PDGF, a cystine knot family member structurally related to NGF, contains a stretch of basic residues at the carboxyl terminus that has been identified as a cell surface retention signal (35). In an earlier study, we found that the Δ117–120 mutant undergoes regulated secretion like wild type NGF when transiently expressed in neuroendocrine cells (36), indicating that at least this part of the COOH terminus does not serve to direct NGF to the regulated secretory pathway.

In conclusion, we have used site-directed mutagenesis to investigate the NGF COOH terminus. Mutations of residues within the 111–115 region, most notably L112A, dramatically impaired the binding of NGF to p75 and, to a lesser extent, TrkA. The p75 binding surface is therefore more extended than previously known, involving not only a cluster of positively charged residues in loops 1 and 5 but also nonionic interactions in a noncontiguous part of the molecule. Unexpectedly, the same region of the COOH terminus also plays a role in stabilizing the NGF structure. The remainder of the COOH terminus, residues 116–120, was found to be nonessential for stability and bioactivity. Further investigations will be needed to determine if the COOH terminus serves a similar role in other neurotrophins as well.