The EMBO Journal vol.9 no.5 pp.1477 - 1483, 1990

Structure - function studies of nerve growth factor: functional importance of highly conserved amino acid residues

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Communicated by H.Jörnvall

Selected amino acid residues in chicken nerve growth factor (NGF) were replaced by site-directed mutagenesis. Mutated NGF sequences were transiently expressed in COS cells and the yield of NGF protein in conditioned medium was quantified by Western blotting. Binding of each mutant to NGF receptors on PC12 cells was evaluated in a competition assay. The biological activity was determined by measuring stimulation of neurite outgrowth from chick sympathetic ganglia. The residues homologous to the proposed receptor binding site of insulin (Ser18, Met19, Val21, Asp23) were substituted by Ala. Replacement of Ser18, Met19 and Asp23 did not affect NGF activity. Modification of Val21 notably reduced both receptor binding and biological activity, suggesting that this residue is important to retain a fully active NGF. The highly conserved Tyr51 and Arg99 were converted into Phe and Lys respectively, without changing the biological properties of the molecule. However, binding and biological activity were greatly impaired after the simultaneous replacement of both Arg99 and Arg102 by Gly. The three conserved Trp residues at positions 20, 75 and 98 were substituted by Phe. The Trp mutated proteins retained 15-60% of receptor binding and 40-80% of biological activity, indicating that the Trp residues are not essential for NGF activity. However, replacement of Trp20 significantly reduced the amount of NGF in the medium, suggesting that this residue may be important for protein stability.

Key words: NGF activity/NGF stability/receptor binding/site directed mutagenesis/transient expression

Introduction

Nerve growth factor (NGF) is a target-derived polypeptide, essential for development and maintenance of peripheral sympathetic and neural crest-derived sensory neurons as well as basal forebrain cholinergic neurons in the brain (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Whittemore and Seiger, 1987; Thoenen *et al.*, 1987; Ebendal, 1989a). The effects of NGF are mediated by interaction of the factor with specific receptors present on the surface of NGF responsive neurons (Banerjee *et al.*, 1973; Herrup and Shooter, 1973; Taniuchi *et al.*, 1986; Richardson *et al.*, 1986; Hefti *et al.*, 1986). The profound and selective loss of NGF sensitive basal forebrain cholinergic neurons in Alzheimer's disease (Whitehouse *et al.*, 1982; Bartus *et al.*, 1982; Price *et al.*, 1982), has led to the proposal that treatment with NGF may be therapeutically beneficial for patients with this disease (Hefti, 1983).

The primary structure of NGF from mouse submaxillary gland has been determined by amino acid sequence analysis (Angeletti and Bradshaw, 1971; Angeletti *et al.*, 1973). The NGF protein consists of two non-covalently linked 118 residue identical polypeptide chains, each containing three internal disulphide bonds. Analysis of a mouse NGF cDNA clone has shown that the mature NGF protein is generated by proteolytic cleavage from a 305 residue precursor, termed pre-proNGF (Scott *et al.*, 1983). The NGF gene has also been isolated from several other species, and nucleotide sequence analysis has shown that the mature NGF protein is evolutionarily highly conserved (Ullrich *et al.*, 1983; Meier *et al.*, 1986; Ebendal *et al.*, 1986; Selby *et al.*, 1987; Whittemore *et al.*, 1988; Schwarz *et al.*, 1989).

A structural similarity between NGF, insulin and insulin-like hormones has been suggested based on sequence homologies between the two molecules (Frazier *et al.*, 1972). The region around the conserved cysteine residue (B20) in insulin, insulin-like growth factors I and II, relaxin and NGF has been implicated in the receptor binding site of insulin (Pullen *et al.*, 1976). This region includes Gly23, Phe24, Phe25 and Tyr26 in insulin which correspond to Ser19, Val20, Trp21 and Val22 in the mouse NGF. The tryptophan residue at position 21, as well as the other two tryptophan residues in the NGF protein at positions 76 and 99, respectively, are all evolutionarily conserved and can be converted to oxindole derivatives by *N*-bromosuccinimide at different rates. Low concentrations of this reagent result only in modification of Trp21, indicating that this residue is on the outside of the molecule, freely available to the solvent (Frazier *et al.*, 1973). It was initially reported that oxidation of the rapidly reactive Trp21 did not affect the biological activity of NGF in a bioassay using sensory ganglia, while modification of both Trp21 and Trp99 markedly reduced the activity of the molecule (Frazier *et al.*, 1973), although not the binding to NGF receptors (Merrell *et al.*, 1975). However, Cohen *et al.* (1980) using a similar procedure, concluded that Trp21 may, in fact, be a critical residue both for receptor binding and biological activity, since the Trp21 modified molecule only retained 3-5% of the activity of native NGF, and binding to NGF receptors was decreased almost 100-fold compared to native NGF.

Other residues in the NGF molecule have also been modified, followed by measurement of NGF activity. Iodination or nitration of the two tyrosine residues of NGF indicated that both of these residues are exposed on the surface of NGF but their integrity is not required for biological activity (Frazier *et al.*, 1973). Similarly, most if not all lysine residues are non-essential for biological activity, since they can be modified by treatment with dimethylsuberimidate (Pulliam *et al.*, 1975) or by acetylation

or succinylation (Bradshaw *et al.*, 1977) without loss of NGF activity. Complete modification of the arginine residues by cyclohexanedione results in inactivation, although kinetic analysis of the reaction suggests that the loss in biological activity is most likely due to gross structural changes rather than modification of specific active sites (Bradshaw *et al.*, 1977).

Knowledge of which amino acid residues are important for binding of NGF to its receptor and essential for the biological activity of the factor will provide insights into the molecular mechanisms whereby NGF exerts its neurotrophic effects. This information could also provide NGF agonists and antagonists that may have both research and clinical applications. To reach this goal we have begun to replace selective amino acid residues in the chicken NGF using site-directed mutagenesis. The mutated NGF sequences were then cloned in a mammalian expression vector followed by transient expression in COS cells and measurement of NGF activity present in conditioned medium from transfected cells (Hallböök *et al.*, 1988). In addition, binding of mutated NGF molecules to receptors on PC12 cells was examined, and the antigenic characteristics of mutated NGF molecules were studied by immunoprecipitation using antibodies against purified mouse NGF. In this study we report that the substitution of Ala for the wild-type Val21, included in the region homologous to the receptor binding site of insulin, significantly reduces the biological activity of the molecule, indicating that this residue is important for retaining a fully active NGF. (Since the mature chicken NGF lacks one amino acid in the amino terminus compared to mouse NGF, Val21 corresponds to Val22 in mouse NGF). The conservative replacement of Arg99 by Lys did not affect the biological properties of the molecule although both binding and biological activity were drastically reduced by the simultaneous replacement of both Arg99 and Arg102 by Gly without changing its main antigenic properties. Our data also show that none of the conserved Trp residues are essential for binding of NGF to its receptor or to retain biological activity, although the replacement of Trp20 by Phe may greatly decrease the stability of the protein.

Results

Production of wild-type and mutated recombinant NGF

The structural and functional importance of highly conserved residues within the chicken NGF polypeptide was determined by the systematic replacement of selected amino acids using site-directed mutagenesis (Figure 1). In most cases the mutant residues were chosen for having similar chemical characteristics to the replaced amino acids in the native sequence, thereby causing minimal conformational distortions. In addition, non-conservative changes were introduced to test further the structural importance of a particular residue. The mutated NGFs were transiently expressed in COS cells using the plasmid pXM (Yang *et al.*, 1986). The amount of each mutant polypeptide accumulated in conditioned medium was assessed by Western immunoblotting. This determination allowed us to correct for differences in the levels of protein, which varied over a 12-fold range. This variation was not related to the relative biological activity of the mutants and may reflect differences in protein synthesis, stability or secretion of individual polypeptides in COS cells.

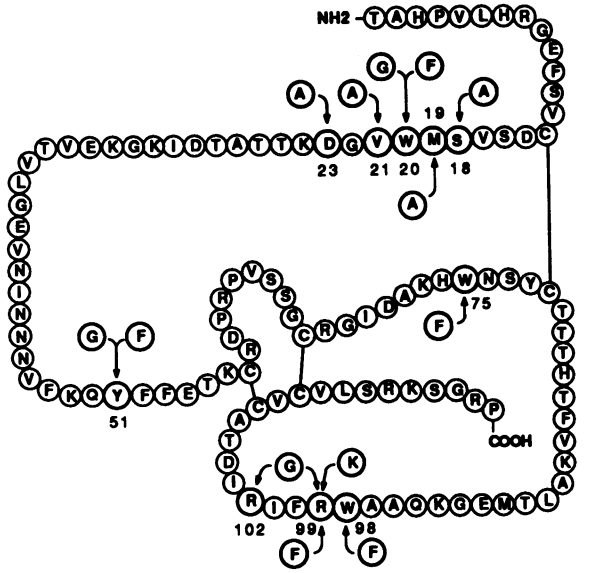

Fig. 1. Schematic representation of the amino substitutions analysed. Solid lines indicate disulphide bridges. Mutated residues are numbered from the amino terminus of the mature chicken NGF protein.

Samples of each mutant protein were assayed for their binding to NGF receptors on PC12 cells and for their ability to stimulate neurite outgrowth from E9 chick sympathetic ganglia. Competitive binding assays were performed using concentrations of [^{125}I]NGF (~1.5 nM) at which ~80% of the [^{125}I]NGF is bound to the low affinity site in the absence of competitor (Sutter *et al.*, 1979). In addition, the antigenic properties of the different mutants were studied by immunoprecipitation of *in vivo* labelled proteins from conditioned medium followed by SDS–PAGE.

The three conserved tryptophan residues are not essential for biological activity

The three tryptophan residues at positions 20, 75 and 98 in chicken NGF are all conserved within the family of NGF-like polypeptides (Schwarz *et al.*, 1989), including brain derived neurotrophic factor (Leibrock *et al.*, 1989), and may be essential for biological activity (Cohen *et al.*, 1980). To define the functional importance of these residues, we replaced them with phenylalanine, a residue that also has an aromatic side chain but lacks the indole ring. Predictions on the secondary structure and hydrophilicity of the mutants by computer analysis did not show any major differences when compared to those based on the wild-type sequence. However, large differences were found in the yield of production and the W20F mutant accumulated only 15% as much NGF protein as the wild-type (Table I). Both W20F and W98F mutants showed a somewhat reduced activity in the sympathetic ganglion bioassay (Table I and Figure 2) which correlated with a decreased binding affinity to NGF receptors on PC12 cells (Table I and Figure 3) [mutants are abbreviated by the wild-type residue (one letter amino acid code) followed by its codon number and the mutant residue]. The modification of Trp75 did not significantly affect the activity of the molecule (Table I). All three mutant proteins were detected by immunoprecipitation with mouse NGF antibodies, which indicates that the replacement by phenylalanine did not significantly change their antigenic

Table I. Relative receptor binding, specific biological activity, immunoprecipitation and yield of mutant NGF proteins

Mutant protein[a]	Receptor binding[b]	Biological activity[c]	Immuno-precipitation	Yield[d]	Effect[e]
		% of wild-type			
wt	100	100	100	100	
S18A	44	59	13	34	antigenicity
S18G	N.D.	53	16	42	antigenicity
M19A	107	84	52	103	non-essential
W20F	17	63	15	15	stability
W20G	B.D.	B.D.	B.D.	B.D.	synthesis?
V21A	27	22	10	30	activity
D23A	137	118	13	8	stability
Y51F	100	95	38	43	non-essential
Y51G	B.D.	B.D.	B.D.	B.D.	synthesis?
W75F	63	84	160	103	non-essential
W98F	48	42	37	48	antigenicity
R99K	108	130	116	97	non-essential
R99/102G	3	8	118	35	structure
R99F	B.D.	B.D.	B.D.	B.D.	synthesis?

[a]Mutants are abbreviated by the wild-type (wt) residue (single amino acid designation), followed by its codon number and the mutant residue.
[b]Data from two experiments varied by ±20% of the average values reported here.
[c]Standard deviation was at or generally below ±20% of the average values reported here.
[d]The relative yield values were calculated from the amount of NGF protein detected by immunoblotting.
[e]This column summarizes the proposed effects of the various NGF mutations as suggested by the data shown in the preceding columns. B.D. (below detection) indicates that the level of mutant protein was below the detection limit of the immunoblotting estimated to be 2% of wild-type N.D. (not determined).

characteristics. Trp20 was also replaced by Gly, a residue which disrupts α and β conformations, to probe for the structural importance of the aromatic side chain. However, this mutant did not accumulate in the medium concentrations at high enough to be detected by Western blotting.

Functional importance of NGF residues homologous to the receptor binding site of insulin

Several residues around Phe25 in insulin have been implicated in receptor binding (Pullen *et al.*, 1976). Based on the postulated structural relationship between insulin and NGF (Frazier *et al.*, 1972), we investigated whether the corresponding residues in the NGF molecule could likewise be involved in receptor binding or biological activity. For this purpose, the alanine-scanning mutagenesis strategy was employed (Cunningham and Wells, 1989). Ser18, Met19, Val21 and Asp23 were systematically substituted by alanine, a residue that eliminates the side chain beyond the β-carbon yet does not alter the main conformation or impose extreme electrostatic or steric effects. The mutant NGFs with either M19A and D23A substitutions showed levels similar to wild-type in both biological activity and receptor binding, although they differed markedly in their yield of production (Table I). The S18A mutant exhibited about half the specific activity of the wild-type, which correlated with a decrease of the same magnitude in receptor binding. Interestingly, the modification of Val21 substantially reduced both binding and biological activity, suggesting the relative importance of this residue (Table I, Figures 2 and 3). Both S18A and V21A showed lower levels of immunoprecipitated protein compared to their corresponding yields measured by

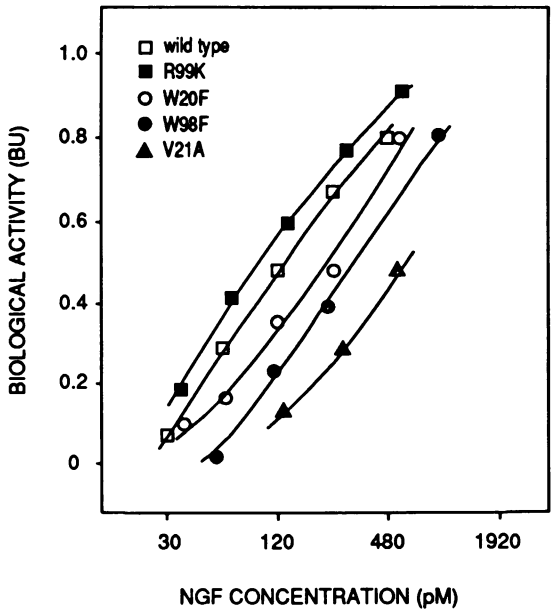

Fig. 2. Biological activity of wild-type and mutant NGF. Serial dilutions of wild-type (□), R99K (■), W20F (○) W98F (●) and V21A (▲) mutant NGF were assayed for their ability to stimulate neurite outgrowth from E9 chick sympathetic ganglia. The fibre outgrowth was scored on a semiquantitative scale in biological units (BU) by comparison to standards obtained with purified mouse NGF, for which 1 BU is equivalent to ~5 ng protein per ml (200 pM). Standard deviation was at or generally below ±20%.

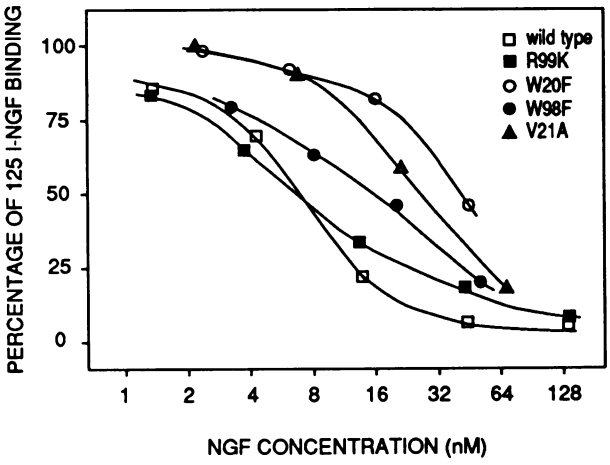

Fig. 3. Receptor binding of wild-type and mutant NGF. Serial dilutions of wild-type (□), R99K (■), W20F (○), W98F (●) and V21A (▲) mutant NGF were assayed for their ability to displace mouse [^{125}I]NGF from NGF receptors on PC12 cells. Data from two experiments using media pooled from two independent transfections varied by ±20%.

immunoblotting (Table I), indicating a possible alteration in their antigenic properties.

The conserved Arg99 and Tyr51 are not essential for function but may play a structural role

Chemical modification of the arginine residues of mouse NGF has been shown to affect the activity of the molecule, although it was suggested that this was due to changes in conformation rather than conversion of specific active sites (Bradshaw *et al.*, 1977). We next investigated the structural

and functional role of the highly conserved Arg99 and Arg102. The conservative replacement of Arg99 by Lys did not affect the behaviour of the molecule in any of the assays performed (Table I, Figures 2 and 3). However, both binding and biological activity were greatly reduced when both Arg99 and Arg102 were simultaneously changed into Gly (Table I), probably due to conformational alterations. Interestingly, the R99/102G mutant was still efficiently immunoprecipitated by mouse NGF antibodies, indicating that the change in conformation did not affect the main antigenic epitopes of the molecule. The yield of production of this mutant was somewhat reduced, but still sufficient to be detected by immunoblotting (Table I). This was not the case with the R99F mutant, where the substitution of Arg99 by the aromatic amino acid phenylalanine dramatically reduced the production of NGF protein.

A stretch of aromatic residues around the conserved Tyr51 has been suggested as a potential site of contact with the NGF receptor (Sabesan and Harper, 1980). We therefore replaced Tyr51 with either Phe or Gly to define the importance of this residue. The Y51F mutant displayed a similar binding affinity and biological activity to the wild-type (Table I). Furthermore, the replacement did not significantly affect the main antigenic determinants of the molecule. However, substitution of Tyr51 by Gly prevented the accumulation of detectable levels of NGF protein in the medium, suggesting the structural importance of this position in determining a stable protein conformation.

Effects of the W20F substitution on the yield of production of recombinant NGF in COS cells

The levels of mutant NGF protein in conditioned medium were found to vary over a 12-fold range. Moreover, several of the mutant proteins did not accumulate in the medium at detectable levels. Notably, removal of the indole ring of Trp20 by replacement with Phe caused a 7-fold reduction in the level of NGF protein accumulated in the medium (Figure 4A). We therefore investigated the effect of this replacement, TGG (for Trp) by TTT (for Phe), on the steady-state levels of mRNA of the respective NGF constructs. Both wild-type and W20F plasmids produced similar amounts of NGF mRNA of the predicted size (1.2 kb) (Figure 4B), indicating that the differences in NGF protein production are not due to a decreased rate of transcription or stability of NGF mRNA.

Discussion

The results presented in this report are the first attempts to understand structure–function relationships of NGF using site-directed mutagenesis combined with transient expression of the mutated NGF sequences in a mammalian cell. Since no information is available on the tertiary structure of NGF, the choice of the mutated amino acids was based on inter-species homologies and earlier results obtained by chemical modification. The high sequence similarly and biological cross-reactivity displayed by NGFs from different species suggest that the results obtained in the present study can be extended to include NGFs from other species.

Quantitative immunoblotting was used to correct for differences in the amount of NGF protein accumulated in the culture medium. Since the molecules are detected in a denatured state, it is expected that they will be similarly

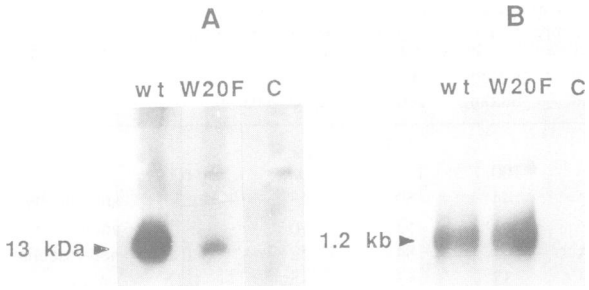

Fig. 4. Effects of the W20F substitution on the production of recombinant NGF in COS cells. (**A**) Western blot of concentrated conditioned medium from COS cells transfected with the wild-type NGF (wt), W20F mutant NGF (W20F) and control (C) plasmids constructs. 30 μl of each concentrated medium was analysed by SDS–PAGE and immunoblotting using a pool of three different rabbit antisera against mouse βNGF. (**B**) Northern blot of total RNA from COS cells transfected with the wild-type NGF (wt), W20F mutant NGF (W20F) and control (C) plasmids constructs. 2 μg of total RNA from each sample was electrophoresed and blotted onto a nitrocellulose membrane. The membrane was probed with a ^{32}P-radiolabelled chicken NGF gene fragment and washed at high stringency.

recognized by a pool of polyclonal antibodies against intact NGF, independent of single point mutations. On the other hand, immunoprecipitation of *in vivo* labelled proteins from conditioned medium with the same antisera is more dependent on the correct folding and tertiary structure of the molecule. Quantitative differences between the values obtained from immunoblotting and immunoprecipitation can therefore be used to assess if a given mutation affects major antigenic epitopes in the NGF protein. In the case of the S18A, V21A and W98F mutants, which showed relative immunoprecipitation values lower than the relative yields, the mutation may have altered the conformation of the native molecule in such a way that it is no longer recognized as efficiently by the antibodies. The 50% reduction in the relative value of immunoprecipitation of M19A agrees with the fact that the mutation removed one of the two methionine residues in the molecule, therefore reducing the incorporation of radiolabel by half in each *in vivo* labelled NGF polypeptide. This result also shows that the methods used to estimate the yield and efficiency of immunoprecipitation are accurate. In the case of D23A, W75F and, most strikingly, R99/102G, the relative higher values obtained by immunoprecipitation could be due to a decreased stability of the mutant molecule. In this case, the relative levels of mutant protein accumulated in the culture medium would be higher after an overnight pulse than after a 3 day incubation. A decrease of nearly 7 times in the level of NGF protein accumulated in the medium was seen after the sole elimination of the indole ring of Trp20 to give Phe. It was shown that this reduction was not due to a difference in transcription efficiency of the plasmid construct. Although a decreased rate of secretion of the mutated protein cannot be ruled out, preliminary experiments showed that the protein is not accumulated in a stable form inside the cell. The efficiency of protein synthesis cannot be ruled out either, although it seems more likely that the decreased yield is due to a lower stability of the polypeptide either inside the cell or in the culture medium. An even lower yield was obtained with the D23A mutant, which might also be the result of

decreased stability, in this case due to a non-conservative amino acid replacement.

Our results show that replacement of any of the three highly conserved tryptophan residues in NGF by phenylalanine did not markedly reduce the biological activity of NGF. Because Trp and Phe have similar chemical characteristics, minimal structural distortion was caused by the replacement, keeping the NGF molecule as close as possible to the native conformation. However, if the Trp residues were part of the active site, responsible for biological activity, one would expect the conversion into another amino acid, even with similar characteristics, would result in a drastic drop in activity. Thus, it is likely that these three Trp residues play only a structural role, as suggested by their reduced but still significant activity. The possibility that the aromatic moiety of the Trp residues is responsible for binding or biological activity cannot be ruled out, although it has been shown that the hydrophobic modification of Trp20 with hydroxy-nitrobenzyl groups did not alter the activity of NGF (Frazier *et al.*, 1973).

Alanine-scanning mutagenesis was used to evaluate the importance of NGF residues homologous to the receptor binding site of insulin. Both M19A and D23A derivatives showed wild-type levels of receptor binding and biological activity. Consistent with this result is the presence of alanine and threonine, respectively, at the equivalent positions in snake NGF (Selby *et al.*, 1987), indicating that the lack of conservation correlates with the low functional significance of these residues. Interestingly, both S18A and V21A, which are conserved in all NGFs described to date (Schwarz *et al.*, 1989) including BDNF (Leibrock *et al.*, 1989), showed reduced, although still detectable, levels of receptor binding and activity. The fact that the S18G mutant was as active as the S18A suggests a low structural constraint of this position (Table I). The V21A mutant exhibited the lowest activity of all mutants tested. However, its residual activity indicates that, although Val21 represents a functionally important amino acid, alanine may substitute for valine at this position and provide considerable levels of biological activity. Thus, the results of the alanine-scanning showed that one amino acid in this region (Val21) is to some extent important for proper NGF function. However, all other mutations show NGF receptor binding and activity comparable to wild-type, indicating that this region in the NGF protein cannot be regarded as functionally equivalent to the proposed homologous region in insulin.

The replacement of Arg99 with Lys did not alter the biological properties of the molecule. On the other hand, the double mutant R99/102G showed a markedly reduced activity, probably due to conformational disturbances. However, the mutated protein was still efficiently immunoprecipitated by NGF antibodies, suggesting that the change in conformation did not affect the main epitopes of the molecule. It is not known if the reduced activity is due to the double substitution, the disruptive effects of the mutating amino acid (Gly) or both.

It is commonly believed that evolutionary conservation of amino acids is due to the fact that conserved residues are essential for function. In the present study, most of the positions examined are completely conserved in NGFs from seven different species from snake to man (Schwarz *et al.*, 1989). After mutation, however, many of them showed significant and, in some cases, indistinguishable biological activity as compared to wild-type. Similar results have also been obtained when highly conserved amino acids were replaced in other molecules such as cytochrome *c* (Pielak *et al.*, 1985), interferons α and β (Valenzuela *et al.*, 1985) and tumor necrosis factor (Van Ostade *et al.*, 1988). If strict amino acid conservation at the positions examined is not essential for binding of NGF to its receptor or biological activity, what is the significance of the constancy of these amino acids in the different NGF molecules? The mutations could lead to inadequate expression of the NGF gene by affecting transcription, translation or post-translational events such as secretion or protein stability. The latter possibility may apply to the D23A and W20F mutants described in this study. On the other hand, for some of the mutants described here, for instance R99K, a restricted set of double and simultaneous mutations must occur in the corresponding codon in order to create a conservative amino acid change, whereas a single base mutation would generate a completely unrelated residue. This would, in most cases, select against a NGF protein generated after a single point mutation in a given codon triplet.

The analysis presented here begins to unravel the importance of some structural and/or functional elements in the NGF protein. More insight into the structures responsible for receptor binding and biological activity of NGF will be gained by the systematic application of site-directed mutagenesis to additional regions of the NGF molecule.

Materials and methods

Bacterial strains, cells and plasmids

The *Escherichia coli* K12 strains MC1061 (Huynh *et al.*, 1985) and MV1190 (Biolabs, Boston, MA) were used to propagate and maintain pXM and pBS DNAs, respectively. CJ236 (Kunkel *et al.*, 1987) was used to prepare uracil DNA for site-directed mutagenesis. COS cells (Gluzman, 1981) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). PC12 cells (Greene and Tischler, 1976) were grown in DMEM supplemented with 5% FCS and 10% horse serum. pXM (Yang *et al.*, 1986) was used as expression vector for all NGF genes. An *Eco*RI fragment containing the pre-proNGF coding sequence from the chicken NGF gene (Ebendal *et al.*, 1986) was cloned in pBS-KS M13$^+$ (Stratagene, La Jolla, CA) and used as template for site-directed mutagenesis. pCH110 (Pharmacia, Uppsala, Sweden), an eukaryotic expression plasmid carrying a β-galactosidase gene, was used to control transfection efficiency.

Site-directed mutagenesis, recombinant DNA and DNA sequencing

Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (1987). pBS was used, instead of M13, for single strand template production. Oligonucleotides, 24–30 nucleotides long, corresponding to NGF sequences incorporating the desired mutation, were made complementary to the template DNA and used as primers in the mutagenesis reactions. Three to four plasmid clones from each reaction were sequenced using the dideoxynucleotide method (Sanger *et al.*, 1977). Where possible, the entire mature NGF coding sequence was examined. DNA inserts containing correct mutations were subsequently cloned in pXM for protein expression. A construct with the chicken NGF fragment in the opposite orientation was also isolated and used as a negative control. Mutants are abbreviated by the wild-type residue (one letter amino acid code), followed by its codon number and the mutant residue.

Production and quantitation of recombinant NGF

COS cells grown to ~70% confluency were transfected with 20 μg plasmid DNA per 10 cm dish using the DEAE dextran–chloroquine protocol (Luthman and Magnusson, 1983). A plasmid expressing a β-galactosidase gene (pCH110) was transfected in parallel dishes, and β-galactosidase activity was measured in cytoplasmic extracts as a control of transfection efficiency. Transfected cells were grown for 16 h in complete medium and then for 3 days in medium with 1% serum. The medium was then collected, dialysed against 50 mM NH_4HCO_3 and freeze-dried. Aliquots of 10–50 μl of 50

times concentrated crude medium were analyzed by SDS–PAGE and immunoblotting (Pettman *et al.*, 1988). The primary antibody, at a 1:300 dilution, was a pool of three different rabbit antisera against purified mouse βNGF described elsewhere (Ebendal *et al.*, 1989). Biotinylated goat anti-rabbit IgG (Vector) was used as the secondary antibody at a concentration of 7.5 μg/ml. Immunoreactive proteins were detected using [^{125}I]streptavidin (Amersham, UK) at ~2.5 × 10^4 c.p.m./ml. Dried blots were exposed to Kodak XAR5 films with intensifying screens for 48 h at −80°C. Autoradiograms were scanned in a Shimadzu densitometer and the concentration of wild-type and mutant NGF protein was determined by comparing the area of the signal in the autoradiogram corresponding to the processed monomer form of each protein with a standard curve generated with purified mouse βNGF. Internal calibration standards were included in each gel. Yields of wild-type and recombinant NGF in the culture medium ranged from 100 to 300 ng/ml.

RNA preparation and Northern blots

Total RNA was extracted as previously described (Ebendal *et al.*, 1986). Two μg of total RNA was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to nitrocellulose membranes. The membranes were then hybridized with a ^{32}P-radiolabelled chicken NGF gene fragment (Ebendal *et al.*, 1986) and washed at high stringency.

Immunoprecipitation of recombinant NGF

Medium from transfected COS cells, grown over the third night after transfection in the presence of [^{35}S]methionine, was immunoprecipitated with a pool of three different rabbit antisera against mouse βNGF for 16 h at 4°C. Immunocomplexes were collected using streptococcal cells (Calbiochem, San Diego, CA), washed 5–6 times, boiled in SDS–PAGE sample buffer and electrophoresed in 13% polyacrylamide gels. The gels were then treated with Enhance (NEN, Boston, MA), dried and exposed to Kodak XAR5 films with intensifying screens for 48 h at −80°C. Autoradiograms were scanned as described above. Results were expressed as the area of the signal in the autoradiogram corresponding to each mutant protein relative to that of the wild-type NGF, and reflect the average value of two independent experiments.

Binding assay of recombinant NGF to PC12 cells

Mouse NGF was labeled with ^{125}I by the chloramine-T method (Hunter and Greenwood, 1962) to an average activity of 1.6 × 10^7 c.p.m./μg. Steady-state binding of each mutant NGF was measured in a competition assay against [^{125}I]NGF. Serial dilutions of concentrated medium pooled from two independent transfections were incubated in duplicate with 2 × 10^6 cells and 2 ng [^{125}I]NGF in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 0.1% glucose in a total volume of 100 μl at 37°C. After 30 min incubation, 1 ml of ice-cold buffer was added. The samples were then centrifuged for 5 min in 10 000 *g* and the cell pellets were counted. Binding of [^{125}I]NGF in the absence of competitor resulted in ~10 000 c.p.m. Control experiments using medium from mock transfected COS cells showed that other proteins present in the conditioned medium had no effect on the binding of wild-type NGF to PC12 cells. Non-specific binding was measured in a parallel incubation to which at least a 1000-fold excess of unlabelled NGF was added. All results were corrected for this non-specific binding, which was always < 10% of total binding. Percent of binding was calculated as the c.p.m. obtained with each competitor divided by the c.p.m. obtained when concentrated medium from mock transfected cells was used as competitor times 100. The concentration of each mutant and wild-type NGF that gave 50% binding was determined, and relative binding was calculated using the relationship: ($[\text{mutant}]_{50}$/$[\text{wild type}]_{50}$) × 100, where $[\text{mutant}]_{50}$ and $[\text{wild type}]_{50}$ are the concentrations of mutant and wild-type NGF, respectively, that gave 50% binding in the competition binding assay.

Biological assay of recombinant NGF

The biological activity of wild-type and mutated recombinant NGF was measured by the ability of conditioned medium from transfected COS cells to stimulate neurite outgrowth from explanted sympathetic ganglia from 9-day-old chicken embryos (Ebendal, 1984; Ebendal, 1989b). The fibre outgrowth was scored on a semiquantitative scale in biological units (BU) by comparison to standards obtained with purified mouse βNGF, for which 1 BU is equivalent to ~5 ng/ml (200 pM). Serial dilutions of conditioned medium from at least six independent transfections were assayed, and the concentration of each mutant and wild-type NGF that gave 0.3 BU in this scale was determined. The relative activity of each sample was calculated using the relationship: ($[\text{mutant}]_{0.3}$/$[\text{wild type}]_{0.3}$) × 100, where $[\text{mutant}]_{0.3}$ and $[\text{wild type}]_{0.3}$ are the concentrations of mutant and wild-type NGF, respectively, that gave 0.3 BU in the sympathetic ganglion bioassay.

Acknowledgements

We thank Dr Martin Schalling for help with the oligonucleotide synthesis. Technical asssistance was given by Mrs. Annika Kylberg, Mrs. Stine Söderström and Mrs. Mona Gullmert. Support was obtained from the Swedish Natural Science Research Council and The Swedish board for Technical Development.

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Received on 22 December, 1989; revised on 6 Feburary, 1990