Characterization and Crystallization of Recombinant Human Neurotrophin-4*

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Neurotrophin-4 (NT-4) is the most recently discovered member of the neurotrophin family. We have expressed, refolded, and purified recombinant human NT-4 from Escherichia coli and compared it with recombinant human NT-4 secreted into the culture medium of baculovirus-infected insect cells. Both preparations were characterized and determined to be indistinguishable according to several biochemical criteria. Recombinant NT-4 from E. coli was crystallized in a form suitable for x-ray analysis, and characterization of these crystals indicated that NT-4 was present as a dimer within the asymmetric unit. NT-4 was active in promoting the survival of rat Trk receptor-expressing fibroblasts, but was inactive on embryonic chicken sensory neurons, unlike the other members of the neurotrophin family and in contrast to the reported activities of partially purified NT-4.

The neurotrophin family of growth factors includes nerve growth factor (NGF)1, brain-derived neurotrophic factor (BDNF)2, neurotrophin-3 (NT-3)3-8, and neurotrophin-4 (NT-4)9-11. These factors are all expressed as precursors that are subsequently processed and secreted as mature proteins. Members of the neurotrophin family share about 50-55% sequence identity and are among the most highly conserved proteins across species lines (10).

The cellular responses initiated by neurotrophins are mediated by their interaction with the Trk family of tyrosine kinase receptors. The members of this family (Trk or TrkA, TrkB, and TrkC) are 120-160-kDa transmembrane glycoproteins that are expressed widely in the nervous system. Each neurotrophin preferentially binds and activates one of the Trk family members; NGF binds to TrkA, BDNF and NT-4 bind to TrkB, and NT-3 binds to TrkC, and to a lesser extent to TrkB (12-14). In addition to the Trk family of receptors, the low affinity NGF receptor (p75NGF) binds all members of the neurotrophin family, but its role in mediating neurotrophin activity is less clear; it appears to be incapable of mediating a response to neurotrophin binding, and it is not required for signal transduction by the Trks (12, 13).

Human NT-4 cDNA was cloned by virtue of its homology to the Xenopus NT-4 gene (9, 10). The human NT-4 gene was transiently expressed in COS cells (9) or a human embryonic kidney cell line (11). Partially purified supernatants from these cells were reported to stimulate neurite growth from embryonic chicken dorsal root ganglia (DRG) (9, 11), sympathetic neurons and differentiation of PC12 cells (11).

Thus far, expression and purification of active recombinant neurotrophins from Escherichia coli has proven to be difficult. Attempts to express, refold, and purify the neurotrophins, either as mature proteins, or as fusion proteins, have resulted in poor yields or low specific activity (15-18). This paper describes the expression, purification, and biochemical characterization of human NT-4 from recombinant E. coli and compares these properties to NT-4 purified from protein secreted into the cell culture medium of recombinant baculovirus-infected insect cells. Finally, the growth and characterization of crystals of the E. coli-derived NT-4 are reported.

The recently determined crystal structure of NGF defined a new protein fold that has since been identified in both the transforming growth factor-β and platelet-derived growth factor structures (19, 20). Determining the crystal structure of NT-4, the most divergent neurotrophin sequence so far identified, will further the understanding of the structural features of these molecules that are critical for their different binding specificities toward their cognate Trk receptors.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Human NT-4—A synthetic gene was constructed that encodes the 130 amino acids of mature human NT-4, starting with the glycine at position 81 of the prepro region (9, 11) immediately following an initiating methionine. The gene was placed under control of the phage T7 0.1 promoter and ribosome binding site (21) in pRQ227 and expressed in RFJ42, a lacIβ derivative of strain W3110 that is lysogenic for AD8, a λ phage that carries the T7 RNA polymerase gene under control of the lacUV5 promoter (22). Cultures of RFJ42 containing pRG227 were grown to late log phase in LB medium (23) at 37 °C, then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown overnight at 37 °C. Cells were harvested, washed, and lysed in 100 mM Tris-HCl, pH 8.0, 50 mM EDTA. Inclusion bodies were collected by centrifugation and solubilized in 6 M guanidine HCl, 50 mM Tris-HCl, pH 8.5, 5 mM EDTA. NT-4 accounted for 60-80% of protein in the inclusion bodies. The NT-4 was refolded out of inclusion bodies under denaturing redox conditions (24, 25) and purified to near homogeneity by cation exchange chromatography through Fosors 5 in 50 mM HEPES, pH 8.0, followed by fractionation on Sephacryl S-100 equilibrated in phosphate-buffered saline.

To express human NT-4 in Spodoptera frugiperda SF21 cells with recombinant baculovirus, a DNA clone containing the sequence of NT-4 fused to the prepro region of NT-3 was inserted into the BamHI site of pBlueBac2 (Invitrogen). This fusion construct was utilized due to its superior secretion and processing properties compared with an unmodified cDNA clone of NT-4 (9). Recombinant plasmid was co-transfected with viral DNA (BaculoGold, Pharmingen) into S. frugiperda SF21 cells, and recombinant virus was purified and amplified as described (26). Purified NT-4 was obtained from SF21 cultures infected with at
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least five plaque-forming units/cell and grown for 3 days in serum-free medium (SF-900 II, Life Technologies, Inc.). The tissue culture medium was clarified of cells and debris by centrifugation, and the supernatant was adjusted to pH 8.0 with NaOH. NT-4 was purified from the conditioned media to near homogeneity in two steps by cation exchange chromatography through Poros HSF equilibrated in 50 mM bicarbonate, pH 8.0, and followed by fractionation on Sephatry S-100 HR/HiLoad 16/60 in phosphate-buffered saline.

Analytical Methods—Mass determinations by electrospray ionization mass spectrometry were done on a Finnigan Mat TSQ-700 triple quadrupole mass spectrometer with an Analytica electrospray interface. The N-terminal amino acid sequence of NT-4 preparations, purified by C4 reversed phase chromatography, were determined using a Protein Instruments gas phase protein sequenator. Protein concentration of purified NT-4 was determined by absorbance at 280 nm, where A280 = 18.5.

Circular Dichroism Spectroscopy—Estimation of the secondary structure of NT-4 in solution was performed by circular dichroism spectroscopy. All measurements were made at 25 °C using a Jasco model J-710 spectropolarimeter with a 0.1-cm path length cell. The NT-4 was used at a concentration of 0.15 mg/ml in 20 mM potassium phosphate buffer, pH 7.3. Each spectrum was recorded as an average of 20 scans with the averaged spectrum of the buffer subtracted out.

To analyze the CD spectrum of NT-4 after reduction, NT-4 (1.57 mg protein/ml) was incubated at 5°C for 15 min in 6.0 M guanidine HCl, 0.1 M Tris-HCl, pH 8.5, with or without 0.1 M dithiothreitol. NT-4 was then isolated from the reaction mixture by reverse phase high performance liquid chromatography, as described, then dialyzed against 20 mM phosphate buffer, pH 3.0.

Crystallization, X-ray Diffraction Measurements, and Calculations—The sparse matrix screen of Jancarick and Kim (27) was used to identify suitable crystallization conditions. A grid search around these conditions was used to improve the size and quality of the crystals. A grid search around these conditions was used to improve the size and quality of the crystals. Diffraction data were collected from a single crystal with dimensions 0.07 x 0.07 x 1.5 mm (Fig. 5A). The crystal was aligned with the *c* axis parallel to the capillary tube and the oscillation axis. Data were measured with Fuji imaging plates at the X4A HHMI beamline (Brookhaven National Laboratory, New York) and subsequently scanned using a Fuji BAS-2000 scanner. Twenty imaging plates of 2.9° oscillations (0.3° overlap) were recorded using an *x* ray wavelength of 0.98 Å and with the beamline hutch maintained at 12 °C. The Lorentz polarization corrected intensities were profile fitted using DENZO (written and kindly provided by Z. Otwinowski). The data were merged to a unique data set using ROTAVATA and AGROVATA programs from the CCP4 package (SERC, United Kingdom) Collaborative Computing Project No. 4, A Suite of Programs for Protein Crystallography, Daresbury Laboratory, Warrington WA4 4AD, Great Britain, 1979). The symmetry R value was used to monitor the quality of the reduced data and is defined as 

\[ R = \frac{1}{N} \sum |I_{obs} - I_{calc}| \]

where \( I_{obs} \) is the weighted mean of all measurements of intensity I.

Self- and cross rotation function calculations were performed using the self and cross rotation function method as implemented in MERLOT (28) and a Patterson search method as implemented in XPLOR (29). Data with Bragg spacings between 8 and 4 Å and structure factor amplitudes greater than 0.2 were used for these calculations. The maximum Patterson vector was 25 Å. For the cross rotation function, a model of NT-4 was built based on the coordinates of murine NGF (19) and modified for the NT-4 sequence, including only highly conserved side chains and excluding two loop regions where insertions are present in the human NT-4 sequence.

Biological Assays—M887 fibroblasts expressing rat TrkB receptor (MG87trkB) were plated onto Primaria tissue culture plates (Falcon) at a density of 2,500 cells/ml in the presence or absence of neurotrophic serum-free medium. In the absence of serum factors or TrkB ligands, MG87trkB fibroblasts die over the course of 4 days in culture. However, the cells can be rescued in a dose-dependent manner by TrkB ligands (30). Human BDNF (Amgen) and NT-4 test samples were added to serum-deprived MG87trkB cells and incubated for 4 days. Cell viability was then scored by cytoplasmic structure determined by MTT staining (31, 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described (14, 30, 31). Data are expressed as mean OD (550–570) ± S.D. of triplicate wells, with the plate background (medium with no cells added) subtracted from each point.

To assay NT-4 on chicken DBG explants, DBG from thoracic, lumber, and sacral levels were dissected from embryonic day 8 (E8) chicken embryos and embedded in collagen gels (five ganglia/dish) as described (32). Explants were incubated with neurotrophins for 20–24 h, then the density of neurite growth was scored using an arbitrary scale of 0–5.

RESULTS AND DISCUSSION

Biochemical Characterization of Recombinant NT-4—To compare recombinant NT-4 purified from *E. coli* or from insect cells, we purified protein to greater than 95% purity from both sources and studied their properties with regard to several biochemical parameters. NT-4 from either preparation migrated on a reducing sodium dodecyl sulfate, 15% polyacrylamide gel as a single band with *M*ₐ of about 15,000 (Fig. 1A). Determination of the N-terminal amino acid sequence confirmed that the purified protein from insect cells was processed to serum-deprived MG87trkB cells and incubated for 4 days. Cell viability was then scored by cytoplasmic structure determined by MTT staining (31, 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described (14, 30, 31). Data are expressed as mean OD (550–570) ± S.D. of triplicate wells, with the plate background (medium with no cells added) subtracted from each point.

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By analogy to NGF, the structure of NT-4 is expected to include three disulfide bonds. Fig. 2B shows that the CD spectrum of NT-4 was significantly different after denaturation and reduction of the disulfide bonds, followed by dialysis. In con-
of insect cells were purified, the CD spectra, in 20 mM phosphate buffer, pH 3.0, were determined for purified recombinant NT-4 from a crystal structure of NGF. In contrast, denaturation without reduction, followed by dialysis resulted in the same CD spectrum as control NT-4 crystals and crystals of NGF, suggesting a secondary structure for its native conformation. Interestingly, the CD spectrum of NT-4 resembled that observed for other members of the neurotrophin family. Deconvolution analysis of the spectrum showed a predominance of β-sheet (59%) and no α-helix, with the remainder of the protein existing as random structure. This analysis suggested a secondary structure for NT-4 similar to the x-ray crystal structure of NGF.

Analysis of Crystals of Recombinant NT-4—Recombinant human NT-4 from E. coli, containing both methionine and desmethionine forms, was concentrated to approximately 8 mg/ml in 20 mM sodium acetate, pH 4.5. Crystals were grown at 20 °C from 0.2 M zinc acetate, 0.1 M sodium cacodylate, pH 6.5, and 18% polyethylene glycol 8000. The crystals grew after 3-4 days. Screenless precession photographs indicated the crystals belonged to the tetragonal space group P4₁2₁2₁ (or its enantiomorph P4₂2₂) with unit cell dimensions of 75.3 x 75.3 x 107.8 Å. Solvent considerations were consistent with an NT-4 dimer being present within the asymmetric unit (56.3% solvent content). A relationship between the unit cell lengths of these NT-4 crystals and crystals of NGF was evident. In particular, the ab diagonal of the NGF crystal, along which the molecular dyad lies, has nearly the same length as the a and b cell edges of the NT-4 crystal. Together with a knowledge of the molecular dimensions of an NGF dimer (60 x 30 x 30 Å) (19), this suggested a possible orientation of the NT-4 dimer where the molecular 2-fold axes lie perpendicular to the c axis and are aligned close to both the a and b axes.

An essentially complete data set was measured from a single NT-4 crystal. A total of 17,192 measurements were reduced to 6869 unique reflections for data with Bragg spacings between 12.0 and 2.8 Å. These data are 86% complete (64% in the shell from 2.87 to 2.80 Å spacings). The overall symmetry R value for intensities was 9.4% (29.7% between 2.87 and 2.80 Å, with a mean intensity/σ(intensity) of 2.5 in this outermost shell).

The largest non-origin peak in the self-rotation function was at (15.4°, 0.3°, 179.8°) polar coordinates. This peak is at the edge of the crystal symmetry and have a peak height of 2.8σ, 23% of the origin peak height (Fig. 3B). This would indicate that a noncrystallographic 2-fold axis lies close to the a and b axes and perpendicular to c, as would be predicted from the similarity with the NGF unit cell. A cross-rotation function calculated using a model of NT-4 based on the NGF dimer backbone atoms and selected conserved side chains, consistently gave two large peaks at (94.0°, 105.1°, 92.4°) and (90.1°, 111.8°, 63.1°) polar coordinates (data not shown). Both peaks were related by the 2-fold axis at (15.4°, 0.3°, 179.8°), confirming the position of the non-crystallographic dyad axis of NT-4 consistent with the interpretation of the self-rotation function. These calculations confirmed that NT-4 was a dimer, similar to the other members of the neurotrophin family, and was in agreement with the biochemical characterization of recombinant NT-4; the apparent native M₉ for NT-4 was estimated to be 24,000 by gel fil-

![Circular dichroism spectra for NT-4. A, the CD spectra of purified recombinant NT-4 from E. coli (—) and from baculovirus-infected insect cells (— — —). Protein concentration was 0.15 mg/ml in 20 mM phosphate buffer, pH 7.3. Measurements were made as described. B, the CD spectra, in 20 mM phosphate buffer, pH 3.0, were determined for purified NT-4 treated with guanidine HCl (—) or with guanidine HCl and dithiothreitol (— — —), then allowed to refold as described.](image1)

![Crystallization of human NT-4. A, a crystal of NT-4 used for data collection. It has dimensions of 0.07 x 0.07 x 1.5 mm. The scale bar represents 0.125 mm. B, self-rotation function of the dyad symmetry in NT-4 crystals. Results of the self-rotation function are shown according to the spherical polar convention (3b). The angles Φ and Ψ describe the orientation of the rotation axis with respect to the crystallographic axes, and θ is the rotation about this axis. The θ = 180° section is plotted as an equi-area projection. Φ is displayed radially from 0 to 90°. Ψ is shown every 45° and runs counterclockwise from the crystallographic a* axis. The self-rotation function map is contoured in intervals of 0.25 standard deviations (σ), starting at 2.25 sigmas. The peak at approximately (15°, 0°, 180°) polar coordinates, labeled A, corresponds to the molecular two-fold axis of NT-4.](image2)
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A molecular replacement analysis of these NT-4 crystals is currently underway. It is expected that this will lead to a high resolution structure of NT-4 in the near future.

Biological Activity—Supernatants from COS cells that expressed recombinant human NT-4 promoted the survival of MG87 fibroblasts that express the rat TrkB receptor (MG87trkB) (9, 14). In agreement with these results, both preparations of purified NT-4 strongly promoted the survival of MG87trkB cells with dose-response curves that were nearly superimposable to one another as well as to that of recombinant BDNF (Fig. 4). The EC_{50} values (concentration that yields one-half maximum activity) of these curves (4 ng/ml) were similar to those reported for NGF-stimulated survival of TrkA-expressing MG87 cells and NT-3 stimulated survival of TrkC-expressing MG87 cells (14). Together with the extensive biochemical similarities between the E. coli-derived and the SF21 insect cell-derived NT-4, these results established that the two NT-4 preparations were equivalent.

Previously, it was reported that supernatants from human embryonic kidney cells that expressed NT-4 support survival and neurite growth of sensory neurons from E8 chicken DRG and sympathetic ganglia at estimated concentrations of less than 10 ng/ml (11). Also, supernatants from COS cells that expressed NT-4 were reported to stimulate E8 chicken DRG but not sympathetic ganglia (9). In contrast to these results, both the E. coli and the insect cell-derived NT-4, at levels up to 100 ng/ml, did not support survival and neurite growth of sensory neurons from E8 chicken DRG (Fig. 5). Furthermore, up to 100 ng/ml of NT-4 also failed to promote survival of dissociated sensory neurons from E8 DRG, as well as survival and neurite growth of nodose and sympathetic ganglia (data not shown).

There are several explanations for the discrepancy regarding the stimulation of embryonic chicken neurons by NT-4. For example, the cell supernatants used in the previous studies may also contain some other factor that acts alone, or synergistically, with NT-4. Alternatively, the discrepancy in activity may be due to underestimation of the NT-4 concentration in the partially purified cell supernatants. In fact, at concentrations of purified NT-4 above 100 ng/ml, we observed stimulation of chicken DRG (data not shown).

The relative inability of NT-4 to stimulate embryonic chicken sensory neurons makes it unique among the neurotrophins. Although both NT-4 and BDNF are equally active on MG87trkB cells (Ref. 14 and Fig. 4), NT-4 does not stimulate embryonic chicken DRG in contrast to BDNF (Fig. 5). This difference may suggest some type of species specificity; it should be noted that the primary sequence of NT-4 diverges across species more than the other members of the neurotrophin family (9, 11). Whereas fibroblasts expressing rat TrkB are responsive to human NT-4, the TrkB receptor, or other accessory proteins such as p75LNGFR, on chicken sensory neurons may be unable to either bind NT-4 or mediate signal transduction in response to NT-4 binding.

Further identification of other NT-4-sensitive cells should provide insights into the nature of the NT-4 receptor and signaling pathway. In addition, elucidation of the crystal structure of NT-4, and its comparison with that of NGF, will provide considerable insight as to how NT-4 interacts with TrkB.

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