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Neurotrophin-6 is a new member of the nerve growth factor family

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DURING vertebrate development, many neurons depend for survival and differentiation on their target cells[1–3]. The best documented mediator of such a retrograde trophic action is the neurotrophin nerve growth factor (NGF)[1]. NGF and the other known members of the neurotrophin family, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are conserved as distinct genes over large evolutionary distances[4–6]. Here we report the cloning of neurotrophin-6 (NT-6), a new member of this family from the teleost fish *Xiphophorus*. NT-6 distinguishes itself from the other known neurotrophins in that it is not found as a soluble protein in the medium of producing cells. The addition of heparin (but not chondroitin) effects the release of NT-6 from cell surface and extracellular matrix molecules. Recombinant purified NT-6 has a spectrum of actions similar to NGF on chick sympathetic and sensory neurons, albeit with a lower potency. NT-6 is expressed in the embryonic valvulla cerebelli; expression persists in some adult tissues. The interaction of NT-6 with heparin-binding molecules may modulate its action in the nervous system.

Neurotrophin-6 was cloned from a genomic library of the platyfish *Xiphophorus maculatus* during our attempts to clone the fish NGF gene[4]. Overlapping genomic clones (Fig. 1*a*) were identified that hybridized to the mouse NGF probe under low-stringency hybridization conditions. DNA sequencing of the hybridizing region predicted a single long open reading frame of 858 base pairs encoding a polypeptide related to the known neurotrophins. Sequence alignments (Table 1) to the other known neurotrophin sequences in fish, including the complete NGF and BDNF sequences of *Xiphophorus*[4], a partial NT-3 sequence of salmon[6], and the sequences of neurotrophins from other vertebrate species suggested that it did not represent a homologous molecule in *Xiphophorus* of any known neurotrophin but rather a new member of the neurotrophin gene family. Accordingly, this factor was termed neurotrophin-6 (NT-6).

TABLE 1 Amino-acid identity in per cent of fish NT-6 with other members of the nerve growth factor protein family

NGF, fish	61	NT-3, fish	52
NGF, human	56	NT-3, human	46
BDNF, fish	48	NT-4, frog	39
BDNF, human	48	NT-4/5, human	38

Note that for salmon NT-3 only 44 amino acids are known.

The deduced structure of the NT-6 precursor of 286 amino acids (predicted M_r of 31,424) is in accordance with the features of all known neurotrophins (Fig. 1*b*). (1) A hydrophobic domain at the N terminus with the characteristics of a signal peptide (amino acids 1–19). (2) A pro-region (amino acids 20–142) containing basic motifs (R-X-K/R-R at residues 63–66 and 139–142), necessary for proteolytic cleavage of precursor proteins into their mature, secreted forms[7]. (3) An amino-acid sequence at the carboxy half of the precursor starting at lysine 143 which encodes the mature NT-6 of 143 residues (M_r 15,968, p*I* 10.8; see also below the data on the recombinant protein). (4) Thirty-four residues, including the six cysteines conserved in all neurotrophins known so far, are also conserved in NT-6 suggesting that NT-6 shares a common tertiary structure with other neurotrophins; these residues are thought to be involved in the correct folding of the neurotrophins on the basis of the X-ray diffraction structure of NGF[8]. However, a distinguishing feature of NT-6 within the neurotrophin family is the presence of a 22 residue insert between the second and third conserved cysteine containing domain (Fig. 1*b*). This segment contains six basic and eight bend-inducing glycine[9] residues.

Analysis of NT-6 gene expression during embryonic development by northern blotting revealed a transcript of 1.4 kilobases (kb) from organogenesis onwards, in 8-day-old fish and continuing expression of this transcript in adult brain (Fig. 2*a*). NT-6 was also expressed in adult gill, liver and eye with weak expression in skin, spleen, heart and skeletal muscle (Fig. 2*a*). *In situ* hybridization in consecutive serial sections of embryos revealed expression in the valvula cerebelli, a rostral protrusion of the teleostean cerebellum under the midbrain tectum[10] (Fig. 2*b–d*).

Recombinant NT-6 was obtained from a rabbit kidney cell line (RK_{13}) infected with a vaccinia virus expression vector that contained the fish NT-6 gene inserted in its genome (Fig. 3*a*), and from insect cells infected with a baculovirus expression vector containing the NT-6 gene, respectively. Purified NT-6 from both preparations showed survival activity on embryonic chick neurons prepared from different ganglia. NT-6 promotes the survival of sympathetic and sensory dorsal root ganglion (DRG) neurons to the same extent as NGF, albeit with a much lower specific activity (Fig. 3*b*). It had no survival effect on ciliary and nodose neurons (Fig. 3*b*), which also do not respond to NGF. Thus, the spectrum of responsive neurons is similar to that of NGF. A monoclonal antibody that inhibited the activity of NGF showed no inhibition of the survival activity of NT-6 (Fig. 3*c*). The high concentrations of NT-6 needed (50 ng ml^{-1} for half-maximal survival) to obtain neuronal survival with chick

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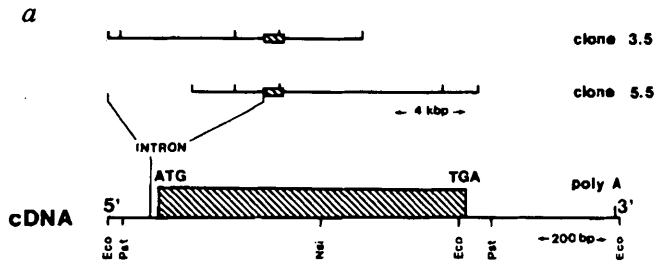
neurons probably reflect low conservation of NT-6 during evolution. Different levels of conservation for different members of the neurotrophin family have been noted: whereas the potency of fish BDNF is as high as that of mouse BDNF (60 pg ml^{-1} for half-maximal survival), the amount of fish NGF needed for half-maximal survival of chick neurons is 100 times higher[4].

During our initial experiments to express NT-6 we observed that, unlike the other neurotrophins it could not be detected in the medium after a 1 day production period (Fig. 3*d*, lane 1).



b

```
NT-6,  fish                                                    M R S S L L V L L L L I G V Q A V L N M G G G L A R N P G A A N H S A G

NT-6,  fish          Q Q E T A A A R G Q L S Q D Q T S Y Q Q H R T T H H R T K R T Q S A A S N M Q N R T P V I G P S P A G S S

NT-6,  fish          P D P S S P V V D P K L F S K R H Y R P S P R V V F S E V I P S H D V L D G E G Y D F E R V R G L R V R R

NT-6,  fish            ↓K A V S H T M H R G E Y S V C D S I N T W V N - - - K T R A T D M S G N E V T V L S H V T V N N K - V
NGF,   fish              Q P Q H R G V Y S V C E S V S V W V G - - N K T K A T D I S G K E V T V L P Y V N I N N V - K
NGF,   human         S S S H P I F H R G E F S V C D S V S V W V G - - D K T T A T D I K G K E V M V L G E V N I N N S - V
BDNF,  fish            H S D P S R R G E L S V C D S I S Q W V T A V D K K T A I D M S G Q T V T V M E K V P V P N G - Q
BDNF,  human           H S D P A R R G E L S V C D S I S E W V T A A D K K T A V D M S G G T V T V L E K V P V S K G - Q
NT-3,  human         Y A E H K S H R G E Y S V C D S E S L W V T - - D K S S A I D I R G H Q V T V L G E I K T G N S - P
NT-4/5, human      G V S E T A P A S R R G E L A V C D A V S G W V T - - D R R T A V D L R G R E V E V L G E V P A A G G S P

                                          + +                 +       +     +     +
NT-6,  fish          K K Q L F Y E T T C R S P T H R S S G I V I G G R S G G R G G K Q G S K T G N S G C R G I D S R Y W N S H
NGF,   fish          K K Q Y F F E T T C H S P P - - - - - - - - - - - - - - - - - - S G G S R C L G I D A R H W N S H
NGF,   human         F K Q Y F F E T K C R D P N - - - - - - - - - - - - - - - - - - P V D S G C R G I D S K H W N S Y
BDNF,  fish          L K Q Y F Y E T K C N P M G - - - - - - - - - - - - - - - - - - Y T K D G C R G I D K R H Y T S Q
BDNF,  human         L K Q Y F Y E T K C N P M G - - - - - - - - - - - - - - - - - - Y T K E G C R G I D K R H W N S Q
NT-3,  human         V K Q Y F Y E T R C K E A R - - - - - - - - - - - - - - - - - - P V K N G C R G I D D K H W N S Q
NT-4/5, human        L R Q Y F F E T R C K A D N A E E G G P G - - - - - - - - - - - - - - - A G G G G C R G V D R R H W V S E

NT-6,  fish          C T N T D I Y V S A L T V F K E Q - T A W R F I R I N A A C V C V L S R N S W S R R P G H
NGF,   fish          C T N S H T F V R A L T S S E N Q - V A W R L I R I N V A C V C V L S R K S W Q H
NGF,   human         C T T T H T F V K A L T M D G K Q - A A W R F I R I D T A C V C V L S R K A V R R A
BDNF,  fish          C R T T Q S Y V R A L T M D S K K K I G W R F I R I D T S C V C T L T I K R G R
BDNF,  human         C R T T Q S Y V R A L T M D S K K R I G W R F I R I D T S C V C T L T I K R G R
NT-3,  human         C K T S Q T Y V R A L T S E N N K L V G W R W I R I D T S C V C A L S R K I G R T
NT-4/5, human        C K A K Q S Y V R A L T A D A Q G R V G W R W I R I D T A C V C T L L S R T G R A
```

FIG. 1 Structure and sequence of the fish NT-6. *a*, Structure and restriction map depicting the inserts of overlapping genomic clones from *X. maculatus* and a coding length complementary DNA clone from *X. helleri*; the *Eco*RI sites in the genomic clones are indicated. Hatched rectangle, open reading frame of the predicted protein; position of the intron is marked. *b*, Deduced amino-acid sequence of *Xiphophorus* NT-6; the genomic and cDNA sequences encode identical proteins. The precursor protein of 286 residues is proteolytically cleaved (arrow) to yield a secreted protein of 143 residues; the two multi-basic motifs for proteolytic cleavage are underlined. The mature factor is aligned to the neurotrophins from *Xiphophorus* and human; conserved amino acids are highlighted by shading. Note the strongly positively charged (+) insert in NT-6. Experimental evidence for the presence of this domain in the mRNA and therefore the translational product of the genomic clone was obtained by the isolation of a complementary DNA clone from adult fish brain mRNA.

METHODS. A fish (*Xiphophorus maculatus*, origin Rio Usumacinta, Mexico) genomic library was screened with a radiolabelled 360 bp DNA probe corresponding to mature mouse NGF as described[4]. Hybridizing *Eco*RI and *Xba*I subfragments of two plaque-purified clones (3.5 and 5.5) were subjected to manual DNA sequencing and analysed as described[4]. A cDNA library from fish brain was prepared by reverse transcription with oligo(dT)-priming of poly(A)$^+$ mRNA isolated from *X. helleri* (origin Rio Lancetilla). Recombinant clones (500,000) of the library were screened with the ^{32}P-labelled 389 bp *Nsi*I–*Eco*RI fragment derived from the fish genomic clone; one NT-6 cDNA clone was obtained. The NT-6 sequences appear in the Genbank/EMBL sequence databases under the accession numbers L36325 and L36942.

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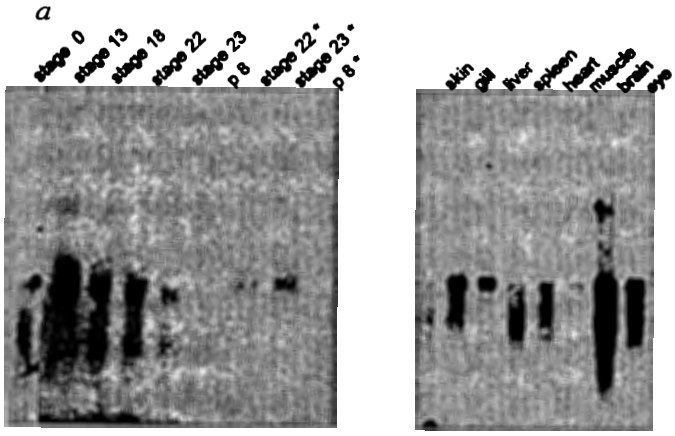

FIG. 2 Expression of NT-6 transcripts in embryonic development and adult tissues of fish. *a*, Northern blot analysis of total RNA (20 μg per lane) or poly(A)$^+$ RNA (1 μg per lane, shown by an asterisk) from different stages of *Xiphophorus* embryos, from 8-day-old fish (p8) and from adult tissues probed with a NT-6 riboprobe. Data represent one of four (developmental stages) and one of two experiments (adult tissues), respectively. Localization of NT-6 mRNA was by *in situ* hybridization histochemistry. Bright-field (*b*) and dark-field (*c*) micrographs showing a parasagital section through the brain of a stage-21 platyfish embryo[23] hybridized with a ^{35}S-labelled NT-6 antisense riboprobe. Silver grains are concentrated in the valvula cerebelli (VC). Control hybridization (*d*) of the adjacent section hybridized with the sense probe revealed no specific signal. The bright staining seen in the pigment epithelial layer arises from pigment molecules under dark-field illumination and does not represent silver grains. All sections were counterstained with toluidine blue. Scale bar, 100 μm.

METHODS. Total RNA from fish embryos was isolated by guanidinium thiocyanate lysis followed by caesium trifluoroacetate centrifugation[24]; poly(A)$^+$ RNA was isolated using oligotex latex beads (Quiagen). Electrophoresis and blotting of RNA and hybridization of blots were as described in ref. 25. Filters were hybridized with a ^{32}P-labelled RNA-probe in 50% formamide at 65 °C for 12–24 h and subsequently washed at a final stringency of 0.2 × SSC, 1% SDS at 80 °C for 1 h before autoradiography. The single-stranded (antisense) riboprobe was synthesized with RNA polymerase from a linearized plasmid template that contained the 1 kb *Eco*RI subfragment of the cDNA (Fig. 1*a*); this template served also to produce a sense RNA probe for *in situ* hybridization. *In situ* hybridizations were done on 7-μm sections of stage-21 *Xiphophorus* embryos that had been cut from paraffin-embedded specimens. Glass slides containing adjacent serial sections were hybridized with either sense or antisense NT-6 riboprobes using the *in situ* hybridization protocol described in ref. 26. Riboprobes were labelled with ^{35}S-UTP and used without alkaline hydrolysis.

The cell lysate, however, contained a 36K antigen (Fig. 3*d*, lane 7) that was not present in cells infected with wild-type virus (lane 8) and that could be eliminated by preincubation of the antiserum used to detect the protein on western blots with the corresponding peptide antigen (lane 9). Thus, this protein is probably the NT-6 precursor.

The absence of NT-6 in the conditioned medium might be due to its sequestration at the cell surface and/or matrix. Proteoglycans have been found to be the binding sites of many growth factors with different heparin binding domains[11]. The sequence of NT-6 contains a motif 'inserted' between the second and third cysteine residues with the characteristics of a heparin-binding domain formed by the positively charged amino acids (Fig. 1*b*). Interestingly, this basic domain of NT-6 would be expected to be on the protein surface on the basis of the X-ray structure of NGF[8] and therefore would probably be available for interaction with anionic charges of heparin. This hypothesis was supported by the fact that the addition of heparin to the expressing cells resulted in the appearance of a band of 21K in the medium (Fig. 3*d*, lanes 2 and 3). This form of NT-6 represents the mature molecule (Fig. 3*a*) which we propose is bound to the cell surface and/or extracellular matrix. Addition of chondroitin sulphate, another glycosaminoglycan, did not release NT-6, showing the specificity of heparin (Fig. 3*d*, lane 4). Further support of the heparin-binding capacity of NT-6 was obtained by heparin agarose chromatography. NT-6 eluted at 700 mM sodium chloride (data not shown); similar salt concentrations (600–1,100 mM) are required for the elution from heparin of some other heparin-binding molecules such as heparin-binding epidermal growth factor and the membrane-associated forms of platelet-derived growth factor[12–15].

The most likely explanation for these observations is the binding of NT-6 to proteoglycans of the cell surface and/or matrix. Whether heparin protects secreted soluble NT-6 from proteolytic degradation must be investigated. It also remains to be established whether NT-6 requires the presence of heparin or

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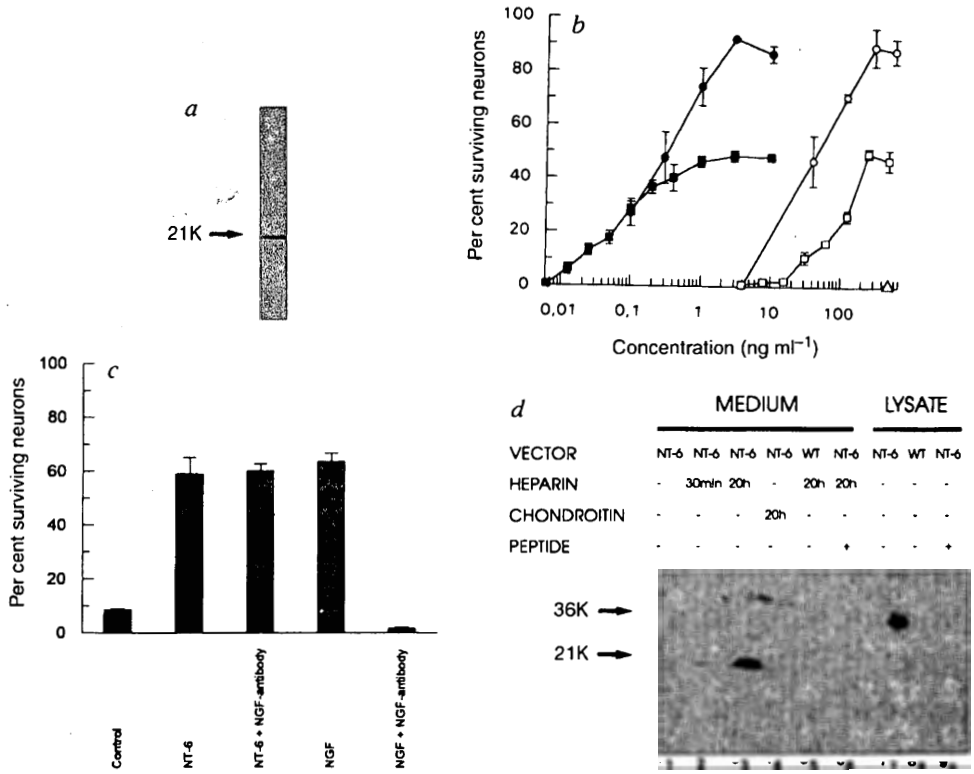

FIG. 3 Expression and biological activity of NT-6. *a*, SDS–PAGE analysis of purified NT-6 (2 μg of protein loaded) stained with Coomassie brilliant blue. N-terminal sequencing of the NT-6 obtained after the final reversed phase HPLC chromatography yielded the sequence KAVSHTM?R (single-letter amino-acid code) confirming the proteolytic cleavage on the carboxyl side of the subtilisin-like serine protease PACE/furin cleavage motif[7] (Fig. 1*b*). *b*, Effect of NT-6 on the survival of sensory neurons prepared from the DRG (white squares) or nodose ganglia (white triangle) of 8-day-old chick embryos (E8) and sympathetic E10 neurons (white circles). Dose–response curves of the survival effect of NT-6 in comparison to that of mouse NGF on sympathetic (black circles) and sensory (black squares) neurons are shown. *c*, Bar graph showing the effects on the survival of sensory neurons in the presence of NT-6 (500 ng ml^{-1}) or NGF (10 ng ml^{-1}) with or without the anti-NGF antibody (clone 27-21; Boehringer-Mannheim). *d*, Heparin requirement to release NT-6 from producing cells into the medium. Western blot analysis of medium and cell lysates from cells infected with a NT-6 recombinant vaccinia virus or a wild-type virus (WT) vectors. Heparin or chondroitin (100 μg ml^{-1}) were added as indicated for a period of 20 h or for 30 min at the end of the 20 h incubation period. Medium from 2×10^5 cells and the lysate from 4×10^3 cells were loaded. The amount of precursor per cell is about 50-fold greater than the 21K mature protein and therefore the latter is not detectable on the blot. A control blot was developed (lanes 6 and 9) where the antiserum was preincubated with NT-6 peptide (1 μg ml^{-1}).

METHODS. A vaccinia virus expression vector with the NT-6 coding region inserted was constructed and used to infect the RK_{13} cell line; purification of NT-6 followed a protocol established for mouse neurotrophin-3 (ref. 27) but with an additional heparin agarose chromatography step before the final reversed-phase HPLC. Details of the expression vector and the purification are to be published in detail elsewhere. N-terminal sequencing of NT-6 was done on a gas/liquid-phase sequencer 477A from Applied Biosystems equipped with an on-line 120A phenylthiohydantoin analyser using the conditions given by the manufacturer. Proteins were separated by 0.1% SDS, 15% polyacrylamide electrophoresis[28], transferred to nitrocellulose filter membranes and the immune complexes were detected with chemiluminesence. For the detection of NT-6 protein we raised polyclonal antibodies against a peptide corresponding to the N terminus of the mature NT-6 sequence (KAVSHTMHRGEYSVC, see Fig. 1*b*). Neuronal survival assays were as described previously[29,30]. Results are the mean ± s.d. of the per cent survival for 6 wells. All experiments were done at least twice.

a specific heparan sulphate proteoglycan for optimal biological activity in addition to a signal-transducing receptor, as is the case with the fibroblast growth factors[16,17]. The binding of NT-6 to the cell surface and extracellular matrix might spatially restrict the action of this molecule and might also be the basis for an extracellular storage form as has been shown for fibroblast growth factor bound to heparan sulphate proteoglycans[18,19]. Small aquarium fish can be used as model systems combining the power of genetic approaches with experimental embryology[20–22] to study the physiological role of this new property of NT-6 in the development and function of the nervous system. □

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