Evidence for the Presence of Two Amino-Terminal Isoforms of Neurofibromin, a Gene Product Responsible for Neurofibromatosis Type 1

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SUZUKI, H., TAKAHASHI, K. and SHIBAHARA, S. *Evidence for the Presence of Two Amino-Terminal Isoforms of Neurofibromin, a Gene Product Responsible for Neurofibromatosis Type 1.* Tohoku J. Exp. Med., 1995, 175 (4), 225-233 — Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder, primarily affecting cells of neural crest origin, and is characterized by *café-au-lait* skin spots, multiple neurofibromas, and higher incidence of malignancy. A gene linked to NF1 encodes neurofibromin, an established function of which is to stimulate intrinsic GTPase activity of *ras* protein. The neurofibromin gene gives rise to multiple transcripts generated by alternative splicing, that encode various neurofibromin isoforms. In this study, we have cloned a cDNA encoding a newly identified species of a putative amino-terminal isoform which lacks a large portion of neurofibromin, including the domain related to GTPase-activating protein. This clone carries the insert of 2.7 kb, coding for a protein of 593 amino acid residues, tentatively termed N-isoform 11, whose amino-terminal 574 residues are identical to those of authentic neurofibromin encoded by the eleven exons located at the 5' portion of the gene. Previously, we cloned a cDNA coding for a similar isoform of 551 amino acid residues, termed N-isoform 10, whose amino-terminal portion is encoded by the first ten exons. The molecular weights of these two deduced N-isoforms are consistent with the values determined by in vitro translation of the mRNA transcribed from each N-isoform cDNA. The presence of the amino-terminal isoform(s) suggests the physiological significance of the amino-terminal portion of neurofibromin. — neurofibromatosis type 1; tumor suppressor gene; *ras* GTPase-activating protein

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Abbreviations: NF1, neurofibromatosis type 1; GAP, GTPase-activating protein; kb, kilobase pairs; bp, base pairs.

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder, primarily affecting cells of neural crest origin, and is characterized by *café-au-lait* skin spots, multiple neurofibromas, and higher incidence of malignancy (Mulvihill et al. 1990). A gene linked to NF1 has been identified by positional cloning (Cawthon et al. 1990; Wallace et al. 1990; Xu et al. 1990a) and postulated to act as a tumor suppressor gene (Basu et al. 1992; DeClue et al. 1992; Li et al. 1992). A gene product responsible for NF1 is a protein of 2818 amino acids (Marchuk et al. 1991), termed neurofibromin. A central portion of neurofibromin is a domain structurally related to GTPase-activating protein (GAP), which was subsequently shown to stimulate intrinsic GTPase activity of *ras* protein (Ballesster et al. 1990; Martin et al. 1990; Xu et al. 1990b; Hattori et al. 1992; Al-Alawi et al. 1993; Bollag et al. 1993). We postulate that neurofibromin may possess a certain function other than the GAP activity, because neurofibromin was shown to inhibit *ras*-dependent growth by a mechanism independent of its GAP function (Johnson et al. 1994) and was also shown to activate the melanocyte-specific gene promoter (Suzuki Hi. et al. 1994). In addition, we cloned a human cDNA which encodes a neurofibromin isoform lacking the domain related to GAP, termed an amino-terminal isoform of neurofibromin (N-isoform) (Suzuki et al. 1992), and showed that the mRNA coding for this isoform is ubiquitously expressed in various human tissues and tumors (Takahashi et al. 1994, 1995). In the present study, we have cloned a cDNA encoding a new type of a putative N-isoform from a human kidney cDNA library and deduced its complete structure.

MATERIALS AND METHODS

Molecular cloning and sequence analysis of cDNA

A human kidney cDNA library was constructed in the Okayama-Berg expression vector (Okayama and Berg 1983) as described previously (Yamauchi et al. 1994). A cDNA probe used for screening the library was prepared by reverse transcriptase-polymerase chain reaction using synthetic oligonucleotide primers designed to cover the amino terminus of neurofibromin (Suzuki et al. 1992). Total plasmid DNA of a human kidney cDNA library was linearized with *Sal*I, electrophoresed on an agarose gel, transferred to a nylon membrane filter (Zeta-probe membrane) (Bio-Rad, Hercules, CA, USA), and hybridized with the cDNA probe labeled with [α - 32 P] dCTP (3,000 Ci/mmol) (Amersham, Buckinghamshire, UK) by the random priming method (Feinberg and Vogelstein 1983). Because a single hybridizable band of about 6 kb was detected, the cDNA fragments at the position of about 6 kb were recovered from the agarose gel, self-ligated, and used to transform *E. coli* DH5 α . By screening such a size-fractionated cDNA library enriched with the target clones, a cDNA clone, designated pHN-1, was isolated (see Fig. 1). The nucleotide sequence was determined by the dideoxy chain-termination method (Sanger et al. 1977) using Sequenase (USB, Cleveland, OH, USA) and BcaBEST sequencing kit (Takara,

Kyoto).

In vitro transcription/translation assays

The BamHI/BamHI fragment containing the entire cDNA insert of pHN-1 or the SmaI/XhoI fragment (–236/vector) of pHN-3, a previously isolated N-isoform cDNA (Suzuki et al. 1992), was subcloned into pBluescript II vector. The BamHI and the XhoI sites are located near the cloning sites of the Okayama-Berg vector. Each subclone thus obtained was linearized with an appropriate enzyme (BamHI or XhoI) and was used as a template for mRNA synthesis. Capped mRNA was synthesized with mCAPTM mRNA Capping Kit (Stratagene, La Jolla, CA, USA). Synthesized mRNAs (1 or 3 μ g) were translated in vitro using rabbit reticulocyte lysate (Promega, Madison, WI, USA) with [³⁵S]methionine (1,200 Ci/mmol) (NEN, Wilmington, DE, USA), and the [³⁵S]-labeled peptides were analyzed on a 4–20% gradient polyacrylamide gel (Multigel 4/20) (Daiichi Pure Chemicals, Tokyo).

RESULTS AND DISCUSSION

A cDNA clone, pHN-1, was isolated from a human kidney cDNA library and carries the insert DNA of about 2.7 kb. The initiating methionine codon (ATG, nucleotide residues 1–3) was assigned by comparing the nucleotide sequence of its 5'-portion with the published sequence of neurofibromin cDNA (Marchuk et al. 1991) (Fig. 1). A codon for termination (TGA) is present at positions 1780 to 1782, and a putative polyadenylation signal, AATAAA, is located 14 nucleotides upstream from a poly(A)-tail. Thus, pHN-1 contains the open reading frame of 1779 nucleotides, coding for 593 amino acid residues with a molecular weight of 67,542. The 19 amino acid residues at its carboxyl terminus are not present in the authentic neurofibromin as detailed below (see Fig. 2).

The nucleotide sequence of pHN-1 (from the initiation codon to the residue 1721) is identical to the published sequence, except for one base at position 702 (Fig. 1). The same base change at position 702 was present in pHN-3, a cDNA clone coding for another N-isoform, previously isolated from a human placenta cDNA library derived from another Japanese individual (Suzuki et al. 1992). Thus, both pHN-1 and pHN-3 contain the A residue at position 702, while the published cDNA contains the G residue (Xu et al. 1990a; Marchuk et al. 1991). This base change does not lead to an amino acid substitution and may represent polymorphism which is popular among Japanese. It is noteworthy that this base change disrupts the RsaI site (GTAC) present in other cDNA sequence.

Based on the exon/intron junctions of the neurofibromin gene (Viskochil et al. 1993), the nucleotide residue at position 1721 of pHN-1 was identified as the 3' end of exon 11, and the nucleotide sequence downstream from the position 1721 is entirely different from that of a neurofibromin cDNA (Fig. 1). Computer search revealed that the 3' region of pHN-1 (positions 1722 to 2556), excluding the

CCCTCTTCCCGCCCGAGGCGCCGCCACCTTCCCTCCGCCGCCCGCGCGGGAGGAC	-1
ATGGCCGCGCAGGCGCGGTGGATGGGTCCAGGCCGTGGTCAGCGCTTCGACGAGCAGCTTCCAATAAAAAACAGGACAGCAGACACA	90
M A A H R P V E W V Q A V V S R F D E Q L P I K T G Q Q N T	30
CATACCAAAGTCAGTACTGAGCACAACAAGGAATGTCTAATCAATATTTCCAAATACAAGTTTCTTGGTTATAAGCGGCCCTCACTACT	180
H T K V S T E H N K E C L I N I S K Y K F S L V I S G L T T	60
ATTTAAAGAAATGTTAAACAATGAGAAATTTGGAGAAGCTGCTGAAAAAATTATATCTCTCTCAGTTGATTATATTGGATACACTG	270
I L K N V N N M R I F G E A A E K N L Y L S Q L I I L D T L	90
GAAAAATGTCTTGGTGGGCAACCAAGGACACAATGAGATTAGATGAAACGATGCTGGTCAACAGTTGCTGCCAGAAATCTGCCATTTT	360
E K C L A G Q P K D T M R L D E T M L V K Q L L P E I C H F	120
CTTCACACCTCTCGTGAAGGAAACAGCATGCAGCTGAACCTCGGAATTCGCTCTGGGGTTTTATTTCTCTCAGCTCAACAACTTC	450
L H T C R E G N Q H A A E L R N S A S G V L F S L S C N N F	150
AATGACGCTTTAGTCGATTTCTACAGGTTACAGGAATTAACGTTTGGTTCAGAAGCAATGTTGATGTTTCATGATAGAAATGTTA	540
N A V F S R I S T R L Q E L T V C S E D N V D V H D I E L L	180
CAGTATATCAATGTGGATTCTGCAAAATTAAGGACTCTGGAAGGAAACAGCATTTAAATTTAAAGCCCTAAAGAGGTTGCGCAGTTA	630
Q Y I N V D C A K L K R L L K E T A F K F K A L K K V A Q L	210
GCAGTTATAAATAGCCTGGAAGGCAATTTGGAACCTGGGTAGAAAAATTATCCAGATGAATTTACAAAACCTATACAGGATCCACAGACT	720
A V I N S L E K A F W N W V E N Y P D E F T K L Y Q I P Q T	240
GATATGGCTGAATGTGCAGAAAAGCTATTGACTTGGTGGATGGTTTTGCTGAAAGCACCACAAAGCTAAAGCAGCAGTTTGGCCACTACA	810
D M A E C A E K L F D L V D G F A E S T K R K A A V W P L Q	270
ATCATTCTCTCTTATCTGTGTCGCAAAATAATCCAGGATATATCCAAAGACGTGGTTGATGAAAAACAATGAATAAGAAAGTTATTTCTG	900
I I L L I L C P E I I Q D I S K D V V D E N N M N K K L F L	300
GACAGCTACGAAAAGCTCTTGTGCGCCATGGAGGAAGTAGGCAGCTGACAGAAAGTGCTGCAATTGCTCTGTGCAAACTGTGTAAAGCA	990
D S L R K A L A G H G G S R Q L T E S A A I A C V K C K A	330
AGTACTTACATCAATGGGAAGATAACTCTGCTATTTCCTACTTGTTCAGTCCATGGTGGTTGATCTTAAGAACTGCTTTTTAATCCA	1080
S T Y I N W E D N S V I F L L V Q S M V V D L K N L L F N P	360
AGTAAGCCATTCTCAAGAGGCGAGCTGAGCTGCAGATGTGGATCTAATGATTGACTGCTTGTCTTGTCTGCTATAGGCCCTCACAAAC	1170
S K P F S R G S Q P A D V D L M I D C L V S C F R I S P H N	390
AACCAACACTTTAAGATCTGCTGGCTCAGAAATTCACCTTCTCATATTGCTATGCTGGTAAATTCATCCATCGAATCATCACCAAT	1260
N Q H F K I C L A Q N S P S T F H Y V L V N S L H R I T N	420
TCCGCAATGGATTGGTGGCCTAAGATTGATGCTGTGATTGTGCACTCGGTGAACCTCGAAATATGTTTGGTGAACACTTCATAAAGCA	1350
S A L D W W P K I D A V Y C H S V E L R N M F G E T L H K A	450
GTGCAAGGTTGTGGAGCACCAGCAATACGAATGGCACCAGTCTTACATTTAAAGAAAAAGTAACAGCTTTAAATTTAAAGAAAA	1440
V Q G C G A H P A I R M A P S L T F K E K V T S L K F K E K	480
CCTACAGACCTGGAGACAAAGAGCTATAAGTATCTTCTCTTGTCCATGGTGAACCTAATTCATGCAGATCCAAAGCTCTTGTCTTGTAAAT	1530
P T D L E T R S Y K Y L L L S M V K L I H A D P K L L L C N	510
CCAAGAAAAACGGGCGCCGAAACCCGAGGAGTACAGCAGAAATTAATACAGGCTGCTCCAACTGGTCCCTCAGTCACACATGCCAGAG	1620
P R K Q G P E T Q G S T A E L I T G L V Q L V P Q S H M P E	540
ATTGCTCAGGAAGCAATGGAGCTCTGCTGGTCTTTCATCAGTTAGATAGCATTGATTGTGGAATCCTGATGCTCCTGTAGAAACATT	1710
I A Q E A M A E L L V L H Q L D S I D L W N P D A P V E T F	570
TGGGAGATTAGGTATATGACTTTTTATTTTTTAAATCAACTTTTAAATTTTATTTTGTATTTTGTCTGAAATATTAACCTCTGTAGTA	1800
W E I R Y M Y F Y F L N S T F K F Y F V F L S *	593
CTTAGTACATTGTAACACTTACACTTCCAAAGGTTTATGGTTTGTATTTTATTTGACTTCAAAATATTAGAAATTTCTTGTTTTAACTG	1890
TAAGAAAAAGTATACAGCAATTTAGAAAAATAAATTTTAAAGATAGTGTAAATTTTGTACCCCTAACATAAGTACTGTTGTTTGTATAT	1980
TACTTTTTTCAGATTCAATGTGGTTACTACTGTATTTTAAAGATTTTCATAGTTATAAGCCTAGAGATGATAAAATTTTGTAAACAATA	2070
CTGTTTTTTCAGTTTTTTGAACATGATCTTTTCAATAACTTTCTGTAATACCAATGCTTTTCGATGAATGAATTAATGACACCTGCT	2160
TAGAAGAAAAAATGTATGCAGAAATTTTGGTCTGCTTCTAGATTATACAAATCATTACATTTTAAATGAGCATGAAGTCACACACGG	2250
AGGAAAAATGTAATGTGTAAACCTCAAGTTGCCATTATCTTATAAGAAATGGGTGCTGAAGTTACTTGGCAGCTGAATTAACCTTACT	2340
CTAGAGTAGTGCTGCCACTAGTAATATAATGAGAACCCTATGTTAAAAACTTTTTACTTACTACATTAAAGAGTAAAAAGAGCAG	2430
GTAATAATTAATGTAATATGTTTGTTTAACCCGGTGATATATCTATAATTTCAACATGTAATCAATATAAAGTTATTAATGACAT	2520
TTCAATCTCTTTTTTAAATAGCTCTTCAAACTTT(A)n	2556

Fig. 1. Nucleotide sequence of cDNA coding for a putative amino-terminal iso-form of neurofibromin and its deduced amino acid sequence. The nucleotide sequence of pHN-1 is shown and is numbered in the 5' to 3' direction from the A residue of the initiating methionine codon ATG. The nucleotides on the 5' side of residue 1 are indicated by negative numbers. The deduced amino acids are shown below the nucleotide sequence and are numbered beginning with the initiating methionine. Double underlines indicate the base change at the position 702. A putative poly-adenylation signal is underlined, and a poly(A)-tail is shown as (A)n. An open and a closed triangles indicate the junction of exon 10 and exon 11 and that of exon 11 and its contiguous exon, respectively. Note that the nucleotide sequence downstream from the position 1721, the 3' end of exon 11, is entirely different from that of the neurofibromin cDNA (Xu et al. 1990a; Marchuk et al. 1991), but shows 88% identity with that of NF1-related pseudogene (Suzuki Ha. et al. 1994).

Fig. 2. Schematic representation of neurofibromin mRNA and its amino-terminal isoform mRNAs.

The 5'-terminal coding region of neurofibromin mRNA is schematically drawn, in which vertical lines indicate the relevant exon/intron junctions (Viskochil et al. 1993) and numbers indicate exons. The two types of mRNAs, coding for N-isoform 10 represented by pHN-3 and for N-isoform 11 represented by pHN-1, are also shown. In case of pHN-3, the nucleotide sequence downstream from the position 1641, the 3' end of exon 10, is different from that of the neurofibromin cDNA (Suzuki et al. 1992). Closed boxes represent the 5'-terminal protein-coding regions, each nucleotide sequence of which is identical to that of a neurofibromin cDNA, and open boxes, the unique protein-coding region of mRNA coding for either N-isoform 10 or N-isoform 11. Solid lines indicate the 3'-untranslated regions. Note that the nucleotide sequence of the regions, indicated by open boxes and solid lines, is unique for each cDNA and differs from the published sequence of the neurofibromin cDNA.

poly(A)-tail, shares about 88% nucleotide sequence identity with the segment located at the 3' side of the exon 11-like sequence of the NF1-related pseudogene (Suzuki Ha. et al. 1994). Thus, it is conceivable that the mRNA for pHN-1 is generated by alternative splicing of the neurofibromin gene transcripts at the junction of exon 11 and its contiguous intron (Fig. 2), although the nucleotide sequence of the 5' region of intron 11 was not published. In this report, the protein encoded by pHN-1 was tentatively termed N-isoform 11, which is composed of the 574 amino acid residues encoded by the eleven exons (from exon 1 to exon 11) and the 19 amino acid residues encoded by a hitherto undetermined exon. Similarly, the protein of 551 amino acid residues, encoded by a previously cloned cDNA pHN-3 (Suzuki et al. 1992), was termed N-isoform 10, because the nucleotide sequence of pHN-3 (positions 1 to 1641) is the same as that of the authentic neurofibromin cDNA and the nucleotide residue 1641 is the 3' end of exon 10 (Figs. 1 and 2). Thus, N-isoform 10 is composed of the 547 residues encoded by the ten exons (from exon 1 to exon 10) and the additional four residues (Suzuki et al. 1992). In contrast to N-isoform 10 mRNA ubiquitously expressed (Takahashi et al. 1994, 1995), we were unable to detect the expression of N-isoform 11 mRNA in human kidney and placenta RNA by Northern and S1 nuclease-mapping analyses and by reverse transcriptase-polymerase chain reaction (data

Fig. 3. In vitro translation of the cDNA-derived mRNAs coding for amino-terminal isoforms.

Each lane contains translated products of the synthesized capped mRNA in rabbit reticulocyte lysate. A closed and an open arrowheads indicate the major translation products of about 69 kDa and 62 kDa, representing N-isoform 11 and N-isoform 10, respectively. Lane M contained the size markers, ^{14}C -labeled RainbowTM protein molecular markers and lane 1, lysate without mRNA. Lanes 2 and 3, translated products from 1 and 3 μg of the capped mRNA synthesized from pHN-1. Lanes 4 and 5, translated products from 1 and 3 μg of the mRNA derived from pHN-3.

not shown), suggesting that the expression levels of N-isoform 11 mRNA are very low in these tissues.

The translational initiation site was assigned for neurofibromin (Marchuk et al. 1991) and for N-isoform 10 (Suzuki et al. 1992), because of the presence of an in-frame termination codon (TGA) at positions -159 to -157 . To confirm this assignment and to determine the molecular weight of each N-isoform encoded by pHN-1 or pHN-3, we carried out in vitro transcription/translation assays. Synthesized mRNAs (1 or 3 μg) were translated in vitro using rabbit reticulocyte lysate with $[^{35}\text{S}]$ methionine, and the $[^{35}\text{S}]$ -labeled peptides were analyzed on a 4–20% gradient polyacrylamide gel. One band, detected near the bottom in all lanes, may represent the translation product of endogenous mRNA present in the reticulocyte lysate, because lane 1 contained the translation mixtures lacking the exogenous mRNA (Fig. 3). The mRNA of pHN-1 gave rise to two major products of about 69 kDa and 64 kDa (lanes 2 and 3), and the size of a larger product is consistent with the value (67.5 kDa) calculated from the amino acid sequence of N-isoform 11. The identity of a smaller product remains to be investigated. In contrast, the mRNA of pHN-3 gave rise to a major product of about 62 kDa (lanes

4 and 5), in good agreement with the calculated value (62.3 kDa) for N-isoform 10 (Suzuki et al. 1992). These results support our assignment of the initiating methionine for each N-isoform.

Multiple species of neurofibromin isoforms include the neurofibromin containing the insertion of 21 amino acid residues in the GAP-related domain (Nishi et al. 1991; Suzuki et al. 1991), the isoform containing the insertion of 18 amino acid residues in the C-terminal region (Xu et al. 1990a; Gutmann et al. 1993), and the amino-terminal isoform(s) lacking the GAP-related domain. Each isoform may possess a certain physiological function, and altered function of such multiple isoforms may be responsible for diverse clinical manifestations of NF1.

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Note Added in Proof

While submitting this manuscript, the exon/intron organization of the human neurofibromin gene has been reported (Li et al. 1995), demonstrating that exon 10 is divided into three exons, designated 10a, 10b, and 10c in the 5' to 3' direction. Thus, the DNA insert carried by the N-isoform 10 cDNA, pHN-3, represents the mRNA encoded by the first 12 exons (from exon 1 to exon 10c) and by a novel exon which corresponds to the 5' portion of the continuous intron 10c. Based on this information, it is more reasonable to rename the pHN-3-protein N-isoform 10c.

Li, Y., O'Connell, P., Breidenbach, H.H., Cawthon, R., Stevens, J., Xu, G., Neil, S., Robertson, M., White, R. & Viskochil, D. (1995) Genomic organization of the neurofibromatosis 1 gene (NF1). *Genomics*, **25**, 9-18.