Cloning and Characterization of the Human Activity-dependent Neuroprotective Protein*

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We have recently cloned the mouse activity-dependent neuroprotective protein (ADNP). Here, we disclose the cloning of human ADNP (hADNP) from a fetal brain cDNA library. Comparative sequence analysis of these two ADNP orthologs indicated 90% identity at the mRNA level. Several single nucleotide polymorphic sites were noticed. The deduced protein structure contained nine zinc fingers, a proline-rich region, a nuclear bipartite localization signal, and a homeobox domain profile, suggesting a transcription factor function. Further comparative analysis identified an ADNP paralog (33% identity and 46% similarity), indicating that these genes belong to a novel protein family with a nine-zinc finger motif followed by a homeobox domain. The hADNP gene structure spans ~40 kilobases and includes five exons and four introns with alternative splicing of an untranslated second exon. The hADNP gene was mapped to chromosome 20q12-13.2, a region associated with aggressive tumor growth, frequently amplified in many neoplasias, including breast, bladder, ovarian, pancreatic, and colon cancers. hADNP mRNA is abundantly expressed in distinct normal tissues, and high expression levels were encountered in malignant cells. Down-regulation of ADNP by antisense oligodeoxynucleotides up-regulated the tumor suppressor p53 and reduced the viability of intestinal cancer cells by 90%. Thus, ADNP is implicated in maintaining cell survival, perhaps through modulation of p53.

Mouse activity-dependent neuroprotective protein (mADNP),1 a novel vasoactive intestinal peptide (VIP)-responsive gene, was recently cloned. The relative enrichment of mADNP transcripts in the cerebellum, cortex, hippocampus, medulla, and midbrain and the increases found in the presence of VIP, an established neuroprotective substance (2), implied a potential function in brain metabolism. Specifcally, mADNP mRNA increased 2–3-fold in astroglial cells incubated for 3 h in the presence of nanomolar amounts of VIP (1). Another tissue containing increased mADNP transcripts is the mouse testis, a highly proliferative tissue, suggesting the involvement of ADNP in cell division.

As deregulation of oncogenes has been associated with neuregeneation (3), pathways that regulate neuronal survival may impinge upon cancer proliferation. VIP regulates both neuronal survival and cell division (2). A system whereby labeled VIP is suggested as a tumor marker has been proposed, localizing in vivo tumors of patients with gastrointestinal neuroendocrine cancers as well as pancreatic and colon adenocarcinomas (4). Other studies have identified a very high incidence of VIP receptor binding in breast, ovarian, endometrial, prostate, bladder, lung, esophageal, colon, and pancreatic tumors as well as in neuroendocrine and brain tumors (5).

However, the VIP effect on cancer growth depends on the specific tumor and may be stimulatory (6, 7) or inhibitory (8). In view of the high incidence of tumors containing VIP receptors, a potential intervention in tumor growth may employ a gene downstream of VIP action that is directly associated with stimulation of cell proliferation and survival.

This report mapped the human ADNP (hADNP) gene (GenBank®/EBI accession number AF250860) to a chromosomal region amplified in cancer, and ADNP mRNA expression was found to increase in proliferative tissues. Inhibition of ADNP protein expression by antisense oligodeoxynucleotides resulted in marked reduction in metabolic activity in the target cells coupled with increases in the tumor suppressor p53 (3). Furthermore, a paralogous protein was discovered, suggesting a novel protein family containing zinc fingers and a homeobox domain.

**EXPERIMENTAL PROCEDURES

RNA Preparation—Neuroblastoma cells (6) were incubated in the presence of 25 nM VIP in phosphate-buffered saline (PBS) for 3 h. Total RNA was prepared using RNAzol B solution (Tel-Test, Inc., Friendswood, TX). A similar extraction method was used for tumor tissues, obtained fresh, post-surgery, and frozen immediately on liquid nitrogen.

The abbreviations used are: mADNP, mouse activity-dependent neuroprotective protein; VIP, vasoactive intestinal peptide; hADNP,

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1 The abbreviations used are: mADNP, mouse activity-dependent neuroprotective protein; VIP, vasoactive intestinal peptide; hADNP,
FIG. 1. The hADNP cDNA and gene. Shown is the hADNP sequence (based on clone H7). The left side of the sequence shows nucleotides, and the right side shows amino acid numbers. The beginnings of the exons are labeled and indicated by downward-pointing arrows. Alternative polyadenylation sites are numbered and indicated by upward-pointing arrows: clone H4 (1); clones H6 and H2 (2); clone H10 (3); and clones H3, H5, and H7 (4). The calculated molecular mass of the protein was 12,356.2 Da, and the theoretical pI was 6.97. Antisense oligonucleotides are underlined and labeled (AS-1, AS-8, AS-9, AS-7, and AS-6). Zinc finger domains are shown in boldface with dotted underlining. The second, sixth, and seventh dotted zinc finger domains are designated as trusted by Pfam (protein families database of alignments; alignments can be trusted—certain or potential). The bipartite nuclear localization signal is shown in boldface with dotted/dashed underlining. The homeobox domain is shown in boldface with double underlining. The proline-rich region is shown in boldface with dashed underlining. The partial glutaredoxin (thiotransferase) active site is shown in boldface with double-dotted and dashed underlining. The leucine-rich nuclear export sequence is as follows:

\[ ... ]
**Cloning and Characterization of hADNP**

**TABLE I**

<table>
<thead>
<tr>
<th>Base No.</th>
<th>Polymorphism</th>
<th>Sequences found in comparison with H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2421</td>
<td>C/T</td>
<td>H7 (11)</td>
</tr>
<tr>
<td>2913</td>
<td>C/T</td>
<td>H7 (11)</td>
</tr>
<tr>
<td>3672</td>
<td>A/G</td>
<td>H7/H3 (11); dJ914P20.0299 contig.</td>
</tr>
<tr>
<td>3704</td>
<td>C/A</td>
<td>H7/dJ914P20.0299 contig.</td>
</tr>
</tbody>
</table>

*Exon sequences are in uppercase; introns are in lowercase.

**cDNA Isolation and Sequencing—Oligodeoxynucleotide primers were synthesized in accordance with the mADNP cDNA sequence (GenBank™/EBI accession numbers AF068198 and NM_009682) (1). These primers (ACCTGCAAGCAAAACAACTAT and GCTCGTTACAGATTGGTAC, sense and antisense, respectively, for the mADNP cDNA) were thereafter used for reverse transcriptase-polymerase chain reaction using human neural stem RNA, including murine mammary leukemia virus reverse transcriptase (Life Technologies, Inc.) and AmpliTaq DNA polymerase (PerkinElmer Life Sciences). The resulting polymerase chain reaction product was sequenced automatically (Applied Biosystems sequencers) at the Weizmann Institute of Science Core Facility. The chromosomal localization of hADNP was determined by FISH with a genomic human contig (GenBank™/EBI accession number dj914P20.0299) and genomic clone H7. The chromosomal location of hADNP was determined by FISH with a genomic human contig (GenBank™/EBI accession number dj914P20.0299) and genomic clone H7. The chromosomal location of hADNP was determined by FISH with a genomic human contig (GenBank™/EBI accession number dj914P20.0299) and genomic clone H7. The chromosomal location of hADNP was determined by FISH with a genomic human contig (GenBank™/EBI accession number dj914P20.0299) and genomic clone H7.

**Northern Blot Hybridization—RNA (10–12 μg) was subjected to electrophoresis followed by Northern blot hybridization on 0.45-μm Nitran filters (Schleicher & Schuell, Dassel, Germany). For probe labeling, the cDNA library derived from human whole fetal brain (female-pooled, Caucasian, 19–23 weeks of gestation, cloned unidirectionally into the Uni-ZAP™ XR vector (Stratagene, La Jolla, CA)).

**Cell Culture and Inhibition of Growth by Antisense Oligodeoxynucleotides**—The human colon cancer cell line HT29 (10) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, and 1% Pen-Strep-Nystatin (Biological Industries, Beit Haemek, Israel). The adherent cells were split when a subconfluent monolayer was formed following treatment with 0.25 μM of trypsin and 0.02% EDTA and naturalization with serum-containing medium. For growth inhibition experiments, subconfluent adherent cells were washed with PBS, treated with trypsin as described above, and resuspended in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum to a final concentration of 50,000 cells/ml. 100-μl aliquots were seeded into individual wells of 96-well microtiter plates (Nunclon, Nunc Brand Products, Roskilde, Denmark). Each plate had a blank column and the appropriate controls.

**RESULTS**

**hADNP Structure**—To isolate and characterize hADNP, the human ortholog of mADNP (1), a cDNA library derived from human fetal brain (19–23 weeks of gestation) was screened, and eight clones were isolated. The complete sequences of two cDNA clones (clones H7 and H3) indicated 90% identity to mADNP at the mRNA level. Fig. 1 shows the sequence of hADNP (clone H7) with additional deduced upstream expressed sequence tags (AW453069, AW452644, AW139427, and AW17331) (11), human genomic contig sequences containing ADNP (dJ914P20 contig ID 02099) and genomic clone sulfonyl fluoride, and 1 mM benzamidine. Protein supernatants were collected following sonication by centrifugation (16,000 × g, 20 min, 4 °C). 5 μg of the soluble proteins were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to nitrocellulose filters. Membranes were treated with 10% milk + PBS/Tween (0.2%) for 1 h and incubated overnight at 4 °C in 2% milk + PBS/Tween (0.2%) and the appropriate antibody. After incubation with peroxidase-conjugated secondary antibodies (Roche Molecular Biochemicals), signals were revealed by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech).

**Antibody Preparation**—The following commercial antibodies were used: mouse monoclonal IgG anti-human p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-β-actin antibodies (Sigma, Rehovot), peroxidase-conjugated goat anti-mouse Ig (AfiniPure, Jackson Immunoresearch Laboratories, Inc., West Grove, PA), and horseradish peroxidase-linked donkey anti-rabbit Ig (Amersham Pharmacia Biotech). Anti-ADNP antibody was generated against a synthetic peptide (BAAPEKMPGTWSDESSQEDARSSKPAKE) fused to keyhole limpet hemocyanin through the N-terminal cysteine moiety. In a parallel experiment, the carrier protein was bovine serum albumin. Affinity chromatography was performed on the peptide attached to Sepharose as described above (1).
Table I shows the exon-intron junctions of the five exons of the gene. The estimated gene size is 40,647 base pairs. A CpG island that stretches over 1135 bases as predicted by GRAIL was observed around exon 1 (69% GC). As particularly CG-rich dinucleotides have been previously associated with promoter regions, we tested this sequence using promoter prediction programs TSSW and TSSG. Results gave low scoring promoter (TSSW at base 106 with LDF 5.69 (LDF 5 = statistical promoter score 4.00 indicates a potential promoter); TSSG gave no promoter). Alternative splicing of the second exon has been observed in expressed sequence tags (AI827420 and AW007743). Only the three 3'-exons are protein-coding. The proximal gene upstream of the ADNP gene is DPM1 (dolichyl-phosphate mannosyltransferase polypeptide 1 catalytic subunit) separated by 3438 base pairs.

At the protein structure level (Fig. 1), nine potential zinc finger motifs that are identical between hADNP and mADNP (1) were identified. These zinc finger domains (12), a proline-rich region (12), a nuclear bipartite localization signal (13), and a partial homeobox domain profile (14) suggest nuclear localization (12–14). Furthermore, a glutaredoxin active site (15) as well as a leucine-rich nuclear export sequence (16) were found. One striking difference between mouse and human was a polyglutamic acid stretch of nine residues in mouse (1) shortened to one residue in human (position 931) (Fig. 1).

The second cDNA clone (H3) was identical to clone H7 except for several polymorphic regions (Table II) and utilization of a different polyadenylation site (Fig. 1). Moreover, clone H3 contained a frameshift mutation (an additional A nucleotide at position 3393) (Fig. 1), with a premature termination codon at position 3408 (Fig. 1). Unexpectedly, the H3 cDNA contained an additional protein-coding sequence downstream of a short

FIG. 2. The ADNP gene is conserved among species. Comparative studies identified a new family member, KIAA0863. Dashed lines are zinc finger domains; the solid line is a presumptive homeobox domain region.
poly(A) stretch, encoding the human immunodeficiency virus Tat 
TBP1 protein (transactivator-binding protein 1) (17, 18).

Comparative analysis utilizing BLAST identified part of rat 
ADNP (GenBank™/EBI accession number AAF40431) (90% 
identity) (Fig. 2). Further analysis revealed 33% identity and 
46% similarity to the paralogous brain protein KIAA0863 (Gen-
Bank™/EBI accession number AB020670) (19). This protein re-
vealed similar nine-zinc finger domains and a similar homeobox 
domain as found in ADNP, suggesting a new gene family (Fig. 2).

hADNP Expression—Northern blot hybridization utilizing 
mADNP (1) and hADNP identified one major mRNA band (5.5 
kilobases) (Fig. 3A). This mRNA showed increased expression 
in the heart, skeletal muscle, kidney, and placenta. As ADNP 
was originally cloned from embryonic brain tissue (see above 
and also Ref. 1), further analysis of different brain regions was 
performed (Fig. 3B). The results indicated increased expression 
in the cerebellum and cortex (Fig. 3B). Serial analysis of gene 
expression was also performed. The results obtained suggested 
increased expression in tumor tissues, adenocarcinoma (breast 
and ovaries), medulloblastoma (brain), and glioblastoma 
(brain) and colon cancer. In normal tissues, ADNP sequences 
were found in microvascular endothelial cells and in brain 
(mostly white matter). Serial analysis of gene expression of the 
related KIAA0863 (cDNA isolated from human brain; Gen-
Bank™/EBI accession number AB020670) revealed increased 
expression in tumors (colon and prostate) and in brain white 
matter as well as in the kidney and testis.

Chromosomal Localization—20 metaphase cells from a normal 
male were examined by FISH. All of these metaphase cells 
showed signal on one or both chromatids of chromosome 20 in 
the region 20q12–13.2; 40% of this signal was at 20q12, 32% 
was at 20q13.1, and 28% was at 20q13.2 (Fig. 4). Similar 
results were obtained utilizing public data bases, localizing the 
gene to chromosome 20q13.2 (with identity to the ordered 
markers G30243 and W45435 in linkage to the Genome Data 
Base locus D20S831) and to 20q13.13–13.2 utilizing a human 
contig sequence containing the hADNP gene. KIAA0863 was 
localized to human chromosome 18 using public data bases.

hADNP and Cancer—Since serial analysis of gene expres-
sion identified increased ADNP expression in cancer cell lines 
and since the chromosomal region 20q12–13 is amplified in a 
wide variety of tumors (19–23), we investigated the association 
of hADNP with cancer growth. Three lines of experimental 

FIG. 3. Patterns of expression of the hADNP mRNA. A, master 
blot (human 12-lane multiple tissue Northern blot 7780-1, CLON-
TECH, Palo Alto, CA). Lane 1, brain; lane 2, heart; lane 3, skeletal 
muscle; lane 4, colon; lane 5, thymus; lane 6, spleen; lane 7, kidney; lane 
8, liver; lane 9, small intestine; lane 10, placenta; lane 11, lung; lane 12, 
peripheral blood leukocytes. B, hADNP mRNA in brain tissues. The 
human brain RNA master blot (7755-1) was purchased from CLON-
TECH. Hybridization was performed as described under “Experimental 
Procedures.” Lane 1, cerebellum; lane 2, cerebral cortex; lane 3, me-
dulla; lane 4, spinal cord; lane 5, occipital lobe; lane 6, frontal lobe; lane 
7, temporal lobe; lane 8, putamen. kb, kilobases.

FIG. 4. Chromosomal localization of hADNP. Shown are photo-
graphs and idiogram (insert) of the hybridization sites of clone H7. A 
total of two nonspecific background dots were observed in the 20 met-
aphases tested. A similar result was obtained from hybridization of the 
probe to 10 metaphases from a second normal male (not shown). Two 
representative pictures are shown.
Cloning and Characterization of hADNP

Fig. 5. ADNP mRNA content is increased in tumors. RNA was extracted from human primary tumors (breast) and from adjacent normal tissue and subjected to Northern blot hybridization. C, control tissue; T, tumor. This is a breast cancer sample from a 48-year-old female. Shown are autoradiograms of ADNP and actin mRNAs and ethidium bromide-stained RNA.

Fig. 6. HT29 cell growth is inhibited in the presence of antisense oligodeoxynucleotides specific for ADNP mRNA. Five oligodeoxynucleotides were synthesized (see Fig. 1) and utilized to inhibit cancer growth. A representative figure is shown. Bar 1, control; bar 2, antisense oligodeoxynucleotide 1; bar 3, sense oligodeoxynucleotide 8; bar 4, antisense oligodeoxynucleotide 8; bar 5, antisense 8 with all internucleotide bonds of the phosphorothioated type.

Fig. 7. Western blot analysis: reduction in ADNP in HT29 cells in comparison with actin and p53. Experiments were performed as described under “Experimental Procedures.” −, no antisense oligodeoxynucleotide; +, cells incubated in the presence of the antisense oligodeoxynucleotide.

oligodeoxynucleotides were chosen as complementary to the 5′-most methionines (indicated in Fig. 1). The results showed that antisense oligodeoxynucleotide 1 inhibited cell division (measured as metabolic activity) in the human intestinal cancer cell line HT29 (p < 0.001) (Fig. 6). A similar inhibition was observed with antisense oligodeoxynucleotide 8 (p < 0.001) (Fig. 6). Furthermore, antisense oligodeoxynucleotide 9 inhibited by ~37.5 ± 3%, and antisense oligodeoxynucleotide 68 also inhibited growth (by 45 ± 3%; p < 0.001). In contrast to antisense oligodeoxynucleotides 8 and 9, the sequence of antisense oligodeoxynucleotide 68 is shared by other cDNA sequences; hence, it may not be specific. Further specificity was determined with a control sense oligodeoxynucleotide complementary to antisense oligodeoxynucleotide 8 and with an antisense oligodeoxynucleotide 8 with all internucleotide bonds of the phosphorothioated type (Fig. 6). In addition, antisense oligodeoxynucleotides 7 and 67 did not inhibit growth.

3) To determine that the antisense oligodeoxynucleotides indeed inhibited ADNP expression, Western blot analyses were performed with actin and the tumor suppressor p53 as internal standards. The results show that ADNP (114,000 Da) was decreased by ~3-fold in comparison with actin (densitometric scan results: 1.11 ± 0.23 versus 0.31 ± 0.11, respectively; p < 0.023; n = 3), whereas p53 levels showed an apparent increase (1.04 ± 0.04 versus 2.41 ± 0.41; p < 0.029; n = 3) (Fig. 7).

DISCUSSION

This report characterizes the hADNP gene, encoding an mRNA that is abundantly expressed in distinct normal tissues and that may be alternatively spliced. The 5′-untranslated region of the mRNA is GC-rich, as has been recently shown for several other genes (e.g. Refs. 24–26). hADNP was found to contain zinc fingers and a homeobox domain profile. Furthermore, a family including at least two genes of significant homologies is described.

Based on cDNA and deduced protein sequence (12–14), hADNP and KIAA0863 may represent nuclear DNA-binding proteins, putative transcription factors. The thiotransferase/glutaredoxin active site (15) found in ADNP (Fig. 1) may modulate its own DNA binding activity or that of other DNA-binding proteins in response to oxidative stress and signal transduction pathways implicated in the redox state of the cell (27). We have previously hypothesized that mADNP is a secreted protein (1). To reconcile this discrepancy, one hypothesis may involve alternate utilization of the seven putative initiator methionine residues at the N terminus of hADNP (Fig. 1), resulting in processing pathways that may yield secreted portions. An alternative hypothesis was put forward by us in a recent report suggesting the existence of a nuclear export signal within the ADNP mRNA (Fig. 1) (28). A similar sequence was discovered in the engrailed transcription factor (16) as well as in the ADNP-related protein KIAA0863.

The ADNP-containing locus, the 20q12–13.2 chromosomal region, is amplified in many tumors (19–23). In breast tumors, comparative genomic hybridization revealed ~20 regions of recurrent increased DNA sequence copy number (23, 29–31). These regions are predicted to encode dominant genes that may play a role in tumor progression or response to therapy. Three of these regions have been associated with established oncogenes: ERBB2 at 17q12, MYC at 8q24, and CCND1 and EMS1 at 11q13. Amplification at 20q13 occurs in a variety of tumor types, but up to date, does not involve a previously known oncogene (20). Another aspect of ADNP/cancer/neuroprotection interaction is the fact that ADNP and p53 expression may be interrelated, as shown here, and both proteins may influence tumor growth as well as brain function (1, 3).

The hADNP cDNA (clone H3) contained the TBP1 cDNA sequence downstream of the coding region of ADNP. Previously, the TBP1 gene was localized to chromosome 11p12–13 (18), and the TBP1 gene product was associated with the cell cycle. The finding of TBP1 downstream of hADNP either may be trivial, resulting from molecular cloning manipulations, or may indicate translocation involved with cancer abnormalities.

The discovery of ADNP (1) as a VIP-responsive gene in astroglial cells (a major component of brain white matter) is now extended to the serial analysis of gene expression finding of ADNP-encoding sequences in brain (mostly white matter) as well as in microvascular endothelial cells. VIP-binding sites have been described in astrocytes (32) as well as in endothelial cells (33). In both cases, developmental functions (33, 34) and proliferation (34–36)/survival (32, 37) functions have been hypothesized. The homeobox-containing protein ADNP may thus mediate some of the VIP developmental/survival-associated ef-

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fects involving normal growth and cancer proliferation. The abundance of ADNP mRNA in heart, skeletal muscle, kidney, and placenta may represent, in part, an astrocyte-like cell population (38) or enrichment in blood microvessels (39). Indeed, the original characterization of VIP was as a vasodilator (40); and since endothelial cells play a major role in vasodilatation, endothelial ADNP points toward a new avenue for research on potential VIP/ADNP interactions.

Our original findings related ADNP to VIP-mediated neuroprotection. Thus, ADNP mRNA increased in glial cells incubated with VIP, and a very short peptide fragment derived from ADNP (NAPVSIPQ, termed NAP) provided potent neuroprotection. Thus, ADNP mRNA increased in glial cells incubation, endothelial ADNP points toward a new avenue for research on potential VIP/ADNP interactions.

From a clinical perspective, this report provides methods of protecting (1). Given the abundant expression of ADNP, future experiments are aimed at further assessing the question of protection (1). The increased ADNP mRNA expression in the cerebellum (a structure enriched in VIP-binding sites) (41) suggests a further avenue of research dealing with tissue-specific expression and function.

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REFERENCES