Original

A Novel PHAPI-related 35-kD Protein Highly Expressed in the Developing Brain

Tokutada SATO¹⁾, Tamio HAGIWARA²⁾, Kazuko AOKI³⁾, Hiroshi KURAISHI¹⁾,
Yutaka NISHIGAKI¹⁾, Kei TATENO¹⁾, Takayoshi TANAKA¹⁾, Fumiyo TAKEDA²⁾
and Minoru TAKEDA¹⁾

Abstract : We have purified a 35-kD protein which was expressed mainly in the fetal and early postnatal rat brain cytosol fraction. Partial amino acid sequence analysis of the purified 35-kD protein showed identity with the sequence of putative human leukocyte antigen (HLA) class II associated protein I (PHAPI). To clone cDNAs encoding the 35-kD protein, reverse transcription-polymerase chain reaction (RT-PCR) was performed using primers designed on the basis of the reported PHAPI cDNA nucleotide sequence. We obtained three partial cDNA clones whose nucleotide sequences showed high similarity to PHAPI. The deduced amino acid sequences of these cDNA clones also showed similarity to PHAPI. The region of acidic amino acids at the C-terminal end of the protein suggested that this molecule may function as a transcription factor. This 35-kD PHAPI family protein may therefore function in the determination or differentiation of fetal and early postnatal brain.

Key words : MHC, HLA-DR, brain, development, acidic protein

Introduction

A number of proteins have been isolated that are specifically expressed in the developing CNS, including vitamin D-dependent 28-kD calcium-binding protein (Calbindin-D)¹⁾, elongation factor-1 α (EF-1)²⁾, cortex-enriched protein tyrosine phosphatases (CPTPs)³⁾ and D type cyclins⁴⁾. We have previously identified a 30-kD⁵⁾, 40-kD⁶⁾ and 33-kD⁷⁾ protein that were only expressed in fetal and early postnatal rat brain cytosol fractions, suggesting that these proteins may play important roles in neuronal development. In this study, we have purified a 35-kD protein expressed predominantly in the developing brain cytosol fraction, which showed amino acid and nucleotide sequence similarity to putative human leukocyte antigen (HLA) class II associated protein I (PHAPI).

¹⁾ Department of Biochemistry, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.

²⁾ Institute of Molecular Oncology, Showa University School of Medicine.

³⁾ Department of Medical Biology, Showa University School of Medicine.

Materials and Methods

Material

Pregnant and adult female Wistar rats of various ages were purchased from Saitama Experimental Animals Supply Co., Ltd (Saitama, Japan). Anion exchange column, TSK DEAE-5PW (7.5 mm inner diameter $\times$ 75 mm) and reverse phase column, Shodax C18-5B (4.6 mm inner diameter $\times$ 250 mm) were purchased from Tosoh Corporation (Tokyo) and Showa Denko K. K. (Tokyo), respectively. Other chemicals were obtained from commercial sources.

Preparation and Purification of the 35-kD Protein

Rat brains were removed under anesthesia with ether. The preparation of cytosol fraction from whole brains and the isolation of proteins were carried out as described previously⁵⁻⁷⁾. Briefly, the cytosol fraction was subjected to a high performance liquid chromatography (HPLC) using a TSK DEAE-5PW column. After fractionation by ion-exchange chromatography, proteins in each fraction were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Laemmli's buffer system⁸⁾. The fractions were subjected to a preparative SDS-PAGE to separate large amounts of the protein.

Partial Amino Acid Sequencing of the 35-kD Protein

All procedures, except the acromobacter protease I (API)-digestion, were carried out at room temperature. Forty micrograms of the purified 35-kD protein was digested in 0.5 ml of 0.1 M Tris-HCl (pH 9.0) containing 2 M urea with 0.5 mg of API for 24 hours at 37 °C. The digested peptides were separated by Shodex C18-5B column equilibrated with 0.05% trifluoroacetic acid. The elution was performed with a 90 ml linear gradient of acetonitrile from 0 % to 60% in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. Peptides were detected by measuring absorbance at 215 nm. The amino acid sequences of the separated peptides were analysed with a Milligen/Biosearch ProSequencer model 6625 (PerSeptive Biosystems, Framingham, MA). The homology search was performed with the data bank of Software Development Co (NBRF protein database).

Reverse Transcriptase PCR

Poly A⁺ RNA was purified from nine brains of E17 rat fetuses using a commercial mRNA purification kit (Pharmacia Biotech LKB Biotechnology, Piscataway, NJ) based on an oligo-dT method. Approximately 240 ng of purified mRNA was incubated in 20 μ l of mixture containing 100 pmol random hexamer (Takara, Kyoto), RT-buffer (final concentration: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 28 U RNasin (Promega, Madison, WI), 0.01 M dithiothreitol (Gibco BRL Products, Gaithersburg, MD), 1 mM dNTP (Wako Pure Chemical Industries, Ltd., Osaka), and 200 U Superscript II reverse transcriptase (Gibco BRL Products) for 1 h at 42 °C. For PCR, primers were synthesized on the basis of the published human PHAPI cDNA sequences⁹⁾. Two sense primers (primer A: 5'-ATGGAGATGGGCAGACGGA) and primer B: 5'-CAATTG-CGAGGTAACCAACC) and two antisense primers (primer C: 5'-CATTTTCTCGGTAG-TCGTTC and primer D: 5'-AGTCATCATCTTCTCCCTCAT) corresponding to the

Fig. 1. Temporal expression of the 35-kD protein in developing rat brain. The 35-kD protein is indicated by an arrow.

nucleotide sequence from 104–122, 466–485, 486–506 and 831–850, respectively, were prepared. PCR was performed in a volume of $100\ \mu\text{l}$ containing Taq DNA polymerase buffer (final concentration: 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2), 200 μM of each dNTP (Takara), 100 pmol of each primer, 2.2 U Taq DNA polymerase (Takara) and 0.484 μg Taq Start Antibody (CLONTECH Laboratories Inc., Palo Alto, CA). The reactions were performed on the Program Temp Control System PC-700 (ASTEC, Carlsbad, CA) using a program with initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation for 1 min at 94°C , annealing for 1 min at 42°C and extension for 2 min at 72°C . After the last cycle, final extension was performed for 10 min at 72°C . Ten μl of each RT-PCR product was resolved by electrophoresis on a 2.5% Amplisize Agarose (Bio-Rad, Hercules, CA) gel containing 0.04 M Tris-acetate, 0.001 M EDTA and 0.05 $\mu\text{g/ml}$ ethidium bromide. The cDNA was extracted with Tris-EDTA buffer (pH 8.0) from the gel using the Sephaglas Band Prep. Kit (Nycomed Amersham, Little Chalfont, UK), according to the instruction manual. The purified cDNA was ligated into the Sma I site of pBluescript II (SK+). The final construct was used to transform *Escherichia coli* XL-1 blue, and the plasmid was isolated by the alkaline-SDS preparation and purified by 20% polyethylenglycol/2.5 M NaCl¹⁰. The cDNA was sequenced with a DNA Sequencing System Model 373A (Applied Biosystems, Foster City, CA).

Results

Isolation of the 35-kD protein from the cytosol of fetal brain

We have previously compared brain cytosol protein maps at different stages to detect proteins transiently expressed in restricted periods. More than 30 proteins were specifically

Fig. 2. Confirmation of the isolation of the 35-kD protein by SDS-PAGE. A: pooled fraction (45-50) from ion-exchange chromatography by TSK DEAE-5PW in E14. B: the isolated 35-kD protein. Molecular weight markers are indicated on the left side.

Fig. 3. Peptide map analysis of the 35-kD protein by RP-HPLC on a Shodex C18-5B column after digestion by the lysylendopeptidase (AP1). The peak marked by Peak 1 could be sequenced.

Fig. 4. Comparison of amino acid sequence between the portion that could be sequenced in the 35-kD protein and the corresponding portion in PHAPI.

Fig. 5. Agarose gel electrophoresis of RT-PCR products. M: markers, 1: products of the 5'-terminal portion using primers A and C, 2: products of the 3'-terminal portion using primers B and D. The bands of the length corresponding to the each portion of PHAPI are indicated by arrows.

detected in the sample from E14 fetal brain⁵⁾. The protein described in this report, one of these proteins, was detected in HPLC fraction 49 and its molecular weight was estimated to be 35-kD on SDS-PAGE. The 35-kD protein was detected in the embryonic day 14–21 (E14–E21) prenatal stage and expression was reduced by postnatal 14 day (Fig. 1). The 35-kD protein was isolated from the pooled fraction by SDS-PAGE (Fig. 2).

Determination of partial amino acid sequence of the 35-kD protein

The purified 35-kD protein was digested with lysylendopeptidase API, and peptide fragments were separated on a Shodax C18-5B reverse phase column. The peptide map is shown in Fig. 3. The amino acid sequence of peak 1 (shown in Fig. 3) was determined by automated amino acid sequencer. The partial sequence was analyzed by computer homology search for comparison with known proteins. The peptide fragment derived from the purified 35-kD protein was found to have identity with residues 65–89 of PHAPI (Fig. 4).

PHAPI (N)	1:	ATGGAGATGGGCAGACGGAT	ATTATAGAGCTGCGGAACAGGACGCCCTCTGATGTGAA	60
PCR Product (N-1)	1:	ATGGAGATGGGCAGACGGAT	ATTATAGAGCTGCGGAACAGGACGCCCTCTGATGTGAA	60
primer A				
PHAPI (N)	61:	GAACTCTCTCTGGTCAACATCGGTGCAATGAAGGCAAAATCGAAGGCCATCATATGA	119	
PCR Product (N-1)	61:	GAACTCTCTCTGGTCAACATCGGTGCAATGAAGGCAAAATCGAAGGCCATCATATGA	119	
PHAPI (N)	120:	ATTGAAGAACTGGAATTC	TAAGTCAATCAACGTAGGCTTCACTCATCTCTAAC	177
PCR Product (N-1)	120:	ATTGAAGAACTGGAATTC	TAAGTCAATCAACGTAGGCTTCACTCATCTCTAAC	177
PHAPI (N)	178:	TACCAAAAGTTAAACAAAC	AAGAAGCTTGAAATGAAGCAACACAGATCTCAGGGCT	237
PCR Product (N-1)	178:	TACCAAAAGTTAAACAAAC	AAGAAGCTTGAAATGAAGCAACACAGATCTCAGGGCT	237
PHAPI (N)	238:	CTGGAAGTATTGGCAGTAAATGTCGGAACCTTACCATCTAAATTTAAGTGGCAACA	296	
PCR Product (N-1)	238:	CTGGAAGTATTGGCAGTAAATGTCGGAACCTTACCATCTAAATTTAAGTGGCAACA	296	
PHAPI (N)	297:	AATTAAGATCTCAGCACAATAGAGCTCTGAATAAGTTAGAAATCTCAAGAGCTTAGA	356	
PCR Product (N-1)	297:	AATTAAGATCTCAGCACAATAGAGCTCTGAATAAGTTAGAAATCTCAAGAGCTTAGA	356	
PHAPI (N)	357:	CCCTTTCAATTCGAGGTACCAACCTTGACGACTACCGAGAAAAATC	403	
PCR Product (N-1)	357:	CCCTTTCAATTCGAGGTACCAACCTTGACGACTACCGAGAAAAATC	403	
primer C				

PHAPI (C)	1:	CAATTTCGAGGTAACCAACCTGAA	-	CTACCTACCGAC	AAA	CTGTGTTCAAC	CTCC	-	CCG	58	
PCR Product (C-1)	1:	CAATTTCGAGGTAACCAACCTGAA	TCG	-	CTACCTACCGAC	AAA	CTGTGTTCAAC	CTCC	CCG	58	
PCR Product (C-2)	1:	CAATTTCGAGGTAACCAACCTGAA	TAC	-	CTACCGAC	-	CTGTGTTCAAC	CTCC	CCG	55	
primer B											
PHAPI (C)	58:	CGAATCTCATATCTTCGA	CGCTATG	ATCTAC	ACACAAAGGAGGCCCTGACTCT	GATCT	CGATCT			117	
PCR Product (C-1)	59:	CAGGTCATCTGACTCTCGAT	CGCTATG	ATCTAC	ACACAAAGGAGGCCCTGACTCT	GATCT	CGATCT			117	
PCR Product (C-2)	56:	CAGGTCATCTGACTCTCGAT	CGCTATG	ATCTAC	-	ACAAAGGAGGCCCTGACTCT	CGATCT			111	
PHAPI (C)	118:	TGAGGCTCTACGTTGGGGCC	TGATGAT	CTCG	TAGGAC	TATGAGGA	TAGGA	CTAGTATGA		177	
PCR Product (C-1)	118:	TGAGGCTCTACGTTGG	-	-	-	GGATGACCACTAGGA	AGATGAGGAT	TAGGA	CTAGTATGA	171	
PCR Product (C-2)	112:	TGAGGCTCTACGTTGG	-	-	-	GGATGACAT	TAGGA	AGATGAGGA	CTAGGA	CTAGTATGA	165
PHAPI (C)	178:	TGAAAGCTCTCTACCTAGT	CGAAAT	CTCT	TAGGAC	TAGGA	TAGGAGGA	CTAAGCT	TGAACTGA	237	
PCR Product (C-1)	172:	TGAAAGCTCTCTACCTAGT	CGAAAT	CTCT	TAAGAGGA	TAAGGAT	TAGGAGGA	TAAGCT	CTGAACTGA	230	
PCR Product (C-2)	166:	TGAGGTCTCTCTACCTAGT	-	-	-	TAAGAGGA	TAAGGAT	TAGGAGGA	TAAGCT	CTGAACTGA	219
PHAPI (C)	238:	CGAGGACCTGAGTGCATGCG	CTGAGGAGGAT	GAAGAAGCT	CTACCA	CGCGGA	CTG			295	
PCR Product (C-1)	231:	AGAGGACCTGAGTGCATGCG	CTGAGGAGGAT	GAAGAAGCT	CTACCA	CGCGGA	CTG			288	
PCR Product (C-2)	220:	AGAGGACCTGAGTGCATG	CTG	CTG	CTGAGGAGGAT	GAAGAAGCT	CTACCA	CGCAAGGA	CTG	274	
PHAPI (C)	296:	CTATGATCTAGCAATGAAGAAGA	GCCT	-	CTGTGAAGAAC	AAAT	CGCTCAGAAC	CTGAAAA		352	
PCR Product (C-1)	289:	CTATGATCTAGCAATGAAGAAGA	CTCTCT	CTG	CTGAAGAAGAAC	-	GCATCTCAGAAC	CTGAAAA		345	
PCR Product (C-2)	275:	CTATGATCTAGCAATGAAGAAGA	-	-	CTGCTGGAAGAAGAAC	-	GCATCTCAGAAC	CTGAAAA		327	
PHAPI (C)	353:	CTGAGATCTCTGATGAGGGAGAAGAT	GATGACT							385	
PCR Product (C-1)	346:	CTGAGATCTCTGATGAGGGAGAAGAT	GATGACT							379	
PCR Product (C-2)	328:	CTGAGATCTCTGATGAGGGAGAAGAT	GATGACT							361	
primer D											

Cloning of cDNAs encoding the brain 35-kD protein.

To confirm that the 35-kD protein was a rat homologue of human PHAPI, we cloned cDNAs encoding this protein. Two sets of oligonucleotide primers derived from human PHAPI cDNA sequences were used in RT-PCR of E17 rat fetal brain poly A⁺ RNA. One primer set amplified the 5'-terminal half of the PHAPI cDNA and the other set amplified


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PHAPI : MEMGRRIHLE LRNRTPSDVK ELVLDNSRSN EGKLEGLTDE FEELEFLSTI NYGLTSIANL
N-1 : MEMGRRIHLE LRNRTPSDVK ELVLDNSRSN EGKLEGLTDE FEELEFLSTI NYGLTSIANL

PKLNKLLKLE LSNRNVSGQL EVLAIEKCPNL THLNLSGNKI KDLSTIEPLK KLENKLSLDL
PKLNKLLKLE LSENRTSGDL EVLAIEKCPNL KHLNLSGNKI KDLSTIEPLK KLENKLSLDL

FNCEVTNLND YREN
FNCEVTNLND YREN

PHAPI : NCEVTNLNDY RENVFKLLPQ LTYLDGYDRD DKEAPDSIAE GYVEGLDDEE EDEDEEDED
C-1 : NCEVTNLNAY RENVFKLLPQ VMYLDGYDRD NKEAPDSIVE GYVE*DDD* *****EDED
C-2 : NCEVTNLN-T SD-VFKELPQ *MYLDGYMRD *KEAPDSIVE GYVE*DDNE EDEE*****

EEEYDEDAQV VEDEDEDEE EEEDEEDVSG EEEDEEEGYN DGEVDDEEDE EELCEEDRQQ
EEEYDEYAQV VEDEDEDEE EEEDEEDVSG EEEDEEEGYN DGEVDDEEDE DSSSEEGSQ
EEEYDEYAQV VEDEDEDEE EEEDEEDVSG EEEDEEEGYN DGEVDDEEDE DAGEE*GNQ

KRKREDEDEG EDDD
KRKREDEDEG EDDD
NKQELDEG EDDD

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Fig. 7. Deduced amino acid sequence of the 35-kD protein. N-1 was deduced from PCR product (N-1). C-1 and C-2 were deduced from PCR product (C-1) and (C-2). Asterisks show deletions of amino acid residues.

the 3'-terminal half. The PCR products from primers A/C and B/D were 400 bp and 380 bp in size, respectively, which were consistent with the molecular sizes predicted from human PHAPI (Fig. 5). These products were subcloned and several cDNA clones were isolated and sequenced. The sequence of the 5'-terminal clone showed 91% similarity to PHAPI. Two kinds of sequences were detected in the 3'-terminal site, which showed 84% (C-1) and 76% (C-2) similarity with PHAPI, respectively (Fig. 6). The amino acid sequences deduced from the clone sequences showed 98% identity with the PHAPI protein at the N-terminal site. Furthermore, at the C-terminal end they showed 83% (C-1) and 64% (C-2) identity with PHAPI (Fig. 7). Other PCR products were also sequenced, but showed no similarity to PHAPI in both the cDNA and the deduced amino acid sequence.

Discussion

We have recently purified, obtained cDNA clones and determined the primary structures of a number of proteins which were expressed in an age-dependent manner in rat brain cytosol fraction^{6,7)}. In this report, we describe a 35-kD protein which was expressed predominantly in the fetal and early postnatal brain. The partial amino acid sequences of the purified 35-kD protein showed similarity to PHAPI. cDNA clones for this 35-kD protein were obtained by RT-PCR of fetal rat brain RNA using primers designed on the basis of the reported nucleotide sequence of human PHAPI cDNA. The nucleotide sequences of the cDNA clones encoding the 35-KD protein showed similarity with human PHAPI cDNA sequences. Vaesen *et al.*⁹⁾ first isolated the PHAPI protein from cytosolic fraction of human lymphoblastoid H2LCL B-cell line. Based on the primary structure of PHAPI, they speculated that it was involved in the generation of intracellular signalling events that led to regulation of transcriptional activity after binding of a ligand to HLA class II molecules. However, the molecular mechanism of action of PHAPI still remains to

be clarified. The high level of sequence similarity observed between our novel 35-kD protein and human PHAPI suggests that this rat brain cytosolic protein might be a PHAPI family protein.

MHC class II molecules are expressed on the surface of antigen-presenting cells (APCs) such as lymphocytes, macrophages, dendritic cells and activated T-cells¹¹⁾. However, the function of antigen-presenting cells in the brain remains controversial. Although several brain cells, including microglia, astrocytes and endothelial cells, act as APCs *in vitro*, it is generally assumed that there is low level or no expression of MHC class II antigen in the brain¹²⁾. However, recent new immunocytochemical techniques have suggested that rat or mouse resting microglia specifically and constitutively express MHC class II antigens in certain locations such as the white matter in, for example, the corpus callosum or within the cerebellum¹³⁾. In the human nervous system, different levels of MHC class II antigen expression have been described. The resting microglia constitutively express MHC class II antigens mainly in the white matter¹⁴⁾ in addition to the MHC class II-immunoreactive perivascular cells described by Graeber *et al.*¹⁵⁾.

We purified the 35-kD protein from whole brain, and cannot therefore identify the specific region of expression. Glial cells such as astroglia and microglia, proliferate and differentiate in late prenatal and early postnatal human brain^{16,17)}. Furthermore, axons and synapses have been shown to be overproduced in the white matter, and subsequently eliminated by active microglia to reorganize the CNS^{18,19)}. Since the 35-KD protein is also highly expressed in that period, it might be derived from glial cells. Although the biological function of the 35-kD protein in the brain is unknown, a long stretch of acidic amino acids at the C-terminal end suggests that this protein may be a transcription factor.

The expression of MHC class II antigens (Ia) in the nervous system has become a recent focus of investigation because of their presumed active roles in immune-mediated diseases such as multiple sclerosis^{20,21)}. More recently, the emergence of Ia-expressing cells has also been reported in non-immunological neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease²²⁾.

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