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Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease

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In previous studies, we discovered a growth inhibitory factor (GIF) that was abundant in normal human brain, but greatly reduced in Alzheimer's disease (AD) brain. Molecular cloning of a full-length cDNA for human GIF revealed that the GIF had striking homology to metallothioneins. Furthermore, it was determined that the GIF gene was on chromosome 16, as are the metallothionein genes. GIF, in contrast to metallothioneins, was found to be expressed exclusively in the nervous system. The GIF protein produced by *Escherichia coli* harboring the GIF cDNA in a prokaryotic expression vector inhibited the growth of neonatal rat cortical neurons. These results indicate that GIF is a new member of the metallothionein family with distinct tissue-specific expression and functions. Northern blot analysis revealed that expression of the GIF mRNA is drastically decreased in AD brains. The result raises the possibility that down-regulation of the GIF gene in AD brain plays an important role in the pathogenesis of AD.

Key words: Alzheimer's disease/cDNA/down-regulation/growth inhibitory factor/molecular cloning

Introduction

Alzheimer's disease (AD) is the most common dementia in man (Mann *et al.*, 1988). In terms of neuropathological findings, it is characterized by the presence of numerous senile plaques and neurofibrillary tangles throughout the cerebral cortex (Hirano and Zimmerman, 1962; Kidd, 1964; Schoenberg *et al.*, 1987; Yamaguchi *et al.*, 1988; Wisniewski *et al.*, 1989). The major protein in senile plaques has been identified as a 39–42 amino acid polypeptide referred to as the β/A4 protein (Selkoe *et al.*, 1986; Masters *et al.*, 1985). The major component of neurofibrillary tangles has also been identified as tau, a microtubule-associated phosphoprotein (Kondo *et al.*, 1988; Wischik *et al.*, 1988). The molecular mechanisms which lead to neuronal loss and to the accumulation of senile plaques followed by neurofibrillary tangles in AD, however, remain unexplained.

An interesting hypothesis proposed by Appel is that a neurotrophic factor that supports survival of neurons in the hippocampus and cerebral cortex might be deficient in AD (Appel, 1981). Along that line, we conducted a series of experiments searching for a neurotrophic factor that is decreased in AD (Uchida *et al.*, 1988; Uchida and Tomonaga, 1989). What was found in AD brain extract was, however, an apparently increased neurotrophic activity (Uchida *et al.*, 1988; Uchida and Tomonaga, 1989). Although the observation was contradictory to Appel's hypothesis, we interpreted the increased neurotrophic activity as being concordant with the appearance of massive somatodendritic sprouting of cortical neurons in AD; this was confirmed by careful immunohistochemical observations using antibodies raised against tau (Braak and Braak, 1988; Ihara, 1988). The increased neurotrophic activity found in AD brain extract raised the possibility that massive sprouting of cortical neurons leads to exhaustion and eventually to cell death (Uchida and Tomonaga, 1989; Uchida *et al.*, 1991). With subsequent characterization of the increased neurotrophic activity, we found that the apparently increased neurotrophic activity in fact results from loss of a growth inhibitory factor (GIF) that suppresses the neurotrophic activity present in the normal human brain (Uchida *et al.*, 1991). The GIF was purified to homogeneity, and was found to be a small 68 amino acid small protein with dramatically high homology to metallothioneins (Uchida *et al.*, 1991).

Immunohistological observation using antibody raised against a synthetic peptide derived from a unique sequence of GIF has demonstrated that the GIF is expressed primarily in astrocytes in gray matter, and furthermore, that the amount of GIF is dramatically decreased in AD brain, which is consistent with previous observation of decreased growth inhibitory activity in AD brain extracts (Uchida *et al.*, 1991).

With the above background, the major question is why and how the expression of GIF decreases in AD brain. As a first step towards a better understanding of the molecular mechanisms of the decreased expression of GIF, we have undertaken molecular cloning of human GIF cDNA. Here we describe molecular cloning of a full-length cDNA for human GIF and the expression of the growth inhibitory activity of the recombinant GIF produced by *Escherichia coli*. We categorize GIF as a new metallothionein-related molecule with distinct tissue-specific expression and discuss its implications in the pathogenesis of AD.

Results

Molecular cloning of human GIF cDNA

Because the amino acid sequence of GIF is highly homologous to those of metallothioneins, we were worried that metallothionein cDNAs would be isolated instead of human GIF cDNA during the molecular cloning process. In order to avoid this problem, we thought that it was most

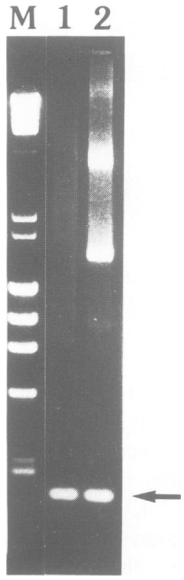

Fig. 1. Agarose gel electrophoresis of PCR products. 1 μg of plasmid DNA (lane 1) or phage DNA (lane 2) prepared from human brain cDNA libraries (Kobayashi *et al.*, 1990, 1991) was amplified by PCR using a primer pair of 488 and 487 at an annealing temperature of 60°C. The PCR products were electrophoresed through a 1.4% agarose gel. M: size markers (λ *Hin*dIII digest and φX174 *Hae*III digest).

important to isolate a GIF cDNA fragment, if not full-length GIF cDNAs. Once we had a partial GIF cDNA in hand, we would be able to screen human brain cDNA libraries for full-length GIF cDNAs under highly stringent hybridization conditions. The best solution was therefore to isolate cDNA fragments by PCR using oligonucleotide primers that were specific enough to amplify GIF cDNA but not metallothionein cDNAs. On the basis of this strategy, we devised a pair of oligonucleotides with one oligonucleotide from the amino-terminal region and the other from the 3′ carboxy-terminal region of GIF. Taking advantage of a unique region with one amino acid insertion (Thr) and an amino acid substitution (Ser to Pro) at the amino-terminus of human GIF, we synthesized an oligonucleotide that is unique to GIF (#488, 5′-ATGGATCCCGAGACCTGCCC). The other primer (#487; 5′-CTGGCAGCAGCTGCACTTCTC) was synthesized on the basis of the amino acid sequence at the carboxy-terminus of the human GIF. As the high homology of human GIF to metallothionein suggested that both genes may have been derived from a common ancestor, we assumed that the codon usage preference of GIF cDNA would be similar to that of metallothionein cDNAs (Karin and Richards, 1982; Schmidt *et al.*, 1985; Heguy *et al.*, 1986; Varshney *et al.*, 1986; Foster *et al.*, 1988). We therefore designed unique oligonucleotide primers by selecting the most likely codon

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                                                                    Met
GIF        CCAGTTGCTTGGAGAAGCCCGTTCACCGCCTCCAGCTGCTGCTCTCCTCGAC ATG   55
MT2A       CAGCGAACCCGCGTGCAACCTGTCCCGACTCTAGCCGCCTCTTCAGCTCGCC ATG
                                                                    Met

           Asp Pro Glu Thr Cys Pro Cys Pro Ser Gly Gly Ser Cys Thr Cys
GIF        GAC CCT GAG ACC TGC CCC TGC CCT TCT GGT GGC TCC TGC ACC TGC 100
MT2A       GAT CCC AAC --- TGC TCC TGC GCC GCC GGT GAC TCC TGC ACC TGC
           Asp Pro Asn --- Cys Ser Cys Ala Ala Gly Asp Ser Cys Thr Cys

           Ala Asp Ser Cys Lys Cys Glu Gly Cys Lys Cys Thr Ser Cys Lys
GIF        GCG GAC TCC TGC AAG TGC GAG GGA TGC AAA TGC ACC TCC TGC AAG 145
MT2A       GCC GGC TCC TGC AAA TGC AAA GAG TGC AAA TGC ACC TCC TGC AAG
           Ala Gly Ser Cys Lys Cys Lys Glu Cys Lys Cys Thr Ser Cys Lys

           Lys Ser Cys Cys Ser Cys Cys Pro Ala Glu Cys Glu Lys Cys Ala
GIF        AAG AGC TGC TGC TCC TGC TGC CCT GCG GAG TGT GAG AAG TGT GCC 190
MT2A       AAA AGC TGC TGC TCC TGC TGC CCT GTG GGC TGT GCC AAG TGT GCC
           Lys Ser Cys Cys Ser Cys Cys Pro Val Gly Cys Ala Lys Cys Ala

           Lys Asp Cys Val Cys Lys Gly Gly Glu Ala Ala Glu Ala Glu Ala
GIF        AAG GAC TGT GTG TGC AAA GGC GGA GAG GCA GCT GAG GCA GAA GCA 235
MT2A       CAG GGC TGC ATC TGC AAA GGG GCG --- --- --- --- --- --- TCG
           Gln Gly Cys Ile Cys Lys Gly Ala --- --- --- --- --- --- Ser

           Glu Lys Cys Ser Cys Cys Gln ***
GIF        GAG AAG TGC AGC TGC TGC CAG TGA GAAGGCACCCCTCCGTGTGGAGCACGT 286
MT2A       GAC AAG TGC AGC TGC TGC GCC TGA GACAGCCCCGCTCCCAGATGTAAAGAA
           Asp Lys Cys Ser Cys Cys Ala ***

GIF        GGAGATAGTGCCAGGTGGCTCAGTGCCACCTATGCCTGTGTGAAGTGTGGCTGGTGTCC 345
MT2A       CGCGACTTCCACAAACCTGGATTTTTTATGTACAACCCTGACCGTGACCGTTTGCTATA

GIF        --CCTTCCCCTGCTGACCTTGGAGGAATGACAATAAATCCCATGAACAGCATG(A)n
MT2A       TTCCTTTTTTCTATAAAATAATGTGAATGATAATAAAACAGCTTTGACTTGA
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Fig. 2. The nucleotide sequence and the predicted amino acid sequence of the human GIF cDNA (pTKGIF6). The nucleotide sequence of pTKGIF6 and the deduced amino acid sequence are shown in upper lines. The nucleotide sequence and the amino acid sequence of MT2A are also shown in lower lines for comparison. Colons indicate the nucleotides that are identical in GIF and MT2A cDNAs. Asterisks mark the termination codon. The nucleotide sequence data reported here have been deposited in the EMBL/DDBJ/GenBank databases under the accession number D13365.

for each amino acid rather than producing oligonucleotides with a high degeneracy for each amino acid, as is the usual method for creating oligonucleotide probes for cDNA cloning.

As the template cDNA for the PCR amplification, we used total plasmid DNA or phage DNA prepared from human brain cDNA libraries, which were constructed in our laboratory (Kobayashi *et al.*, 1990, 1991).

As there might be mismatches in the oligonucleotide primers because of the selection of unique codons, we tried PCR amplification at various annealing temperatures including 40, 45, 50, 55 and 60°C. In fact, a discrete DNA fragment of ~200 bp was amplified in all of these temperature ranges (Figure 1). The PCR products were subsequently subcloned into a plasmid vector, pBluescriptKS(+); nucleotide sequence analysis confirmed that the cDNA contains nucleotide sequences coding for human GIF, but not for metallothioneins.

Using the GIF cDNA fragment as a probe, we screened the human brain cDNA library under highly stringent hybridization conditions to avoid cross-hybridization to metallothionein cDNAs. Two dozen cDNA clones were isolated, and nucleotide sequence analyses confirmed that the cDNA did code for human GIF. Of the GIF cDNA clones, pTKGIF6, which contained the longest cDNA insert, was analyzed in detail.

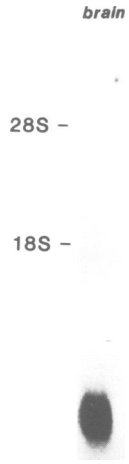

Fig. 3. Northern blot analysis of human brain RNA. 20 μg of total RNA extracted from human brain was electrophoresed through a denaturing 1.4% agarose−2.2 M formaldehyde gel, transferred to a nitrocellulose membrane and hybridized with a DNA fragment amplified from the 3′ noncoding region of GIF cDNA using a primer pair of 511 and 518.

GIF is a new molecule belonging to the metallothionein family

Figure 2 shows the nucleotide sequence of pTKGIF6 and the deduced amino acid sequence. For comparison, the nucleotide sequence of human MT2A (Karin and Richards, 1982) and the deduced amino acid sequence are also aligned. The pTKGIF6 cDNA is 396 bp long, and the open reading frame extends for 204 bp. The predicted amino acid sequence was completely colinear with those determined by chemical sequencing of the purified human GIF (Uchida *et al.*, 1991). The molecular weight calculated from these 68 amino acids is 6926.9 and in close agreement with the molecular weight of human GIF observed by SDS−PAGE (Uchida *et al.*, 1991). A polyadenylation signal appears 22 bp upstream of the poly(A) tail. As Northern blotting analysis of mRNAs extracted from a normal brain has shown an ~500 nucleotide (nt) transcript (Figure 3), it is most likely that we have isolated a full-length GIF cDNA.

As shown in Figure 2, the GIF molecule has striking homology to previously described mammalian metallothioneins (Karin and Richards, 1982; Schmidt *et al.*, 1985; Heguy *et al.*, 1986; Varshney *et al.*, 1986; Foster *et al.* 1988) (54% identity in nucleotide sequence and 63% identity in amino acid sequence). All cysteine residues are conserved between GIF and the mammalian metallothioneins, and the cysteine content of the GIF molecule is as high (29 mol%) as those of mammalian metallothioneins. The central domain (amino acid residues 11−39 of human GIF) shows the highest homology to MT2A. There are, however, 3 bp and 18 bp insertions in the coding sequence for the amino-

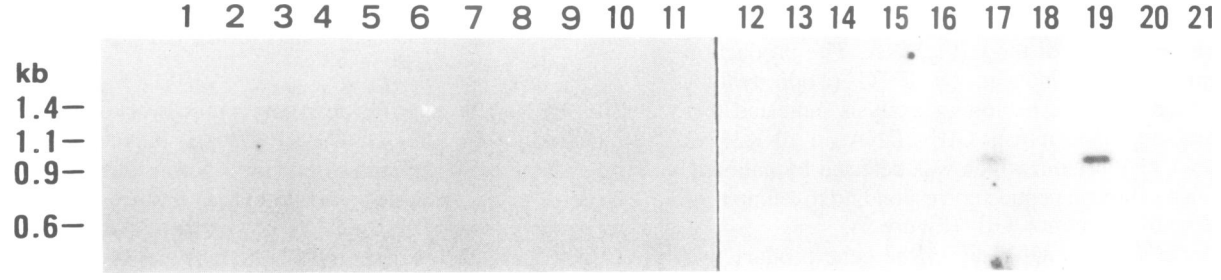

Fig. 4. Determination of chromosome localization of human GIF gene by Southern blot hybridization to a panel of somatic cell hybrids. A panel of somatic cell hybrids, NIGMS Human Rodent Somatic Cell Hybrid Mapping Panel # 1, was obtained from the Coriell Institute for Medical Science Research, Camden, NJ, USA. 7 μg of genomic DNA of each cell line was digested with *Pvu*II, run through a 0.8% agarose gel, transferred to a nitrocellulose membrane and hybridized with the 3′ noncoding region of pTKGIF6, which was generated by PCR amplification of pTKGIF6 using the primer pair of 511 and 518. The filter was washed to a final stringency of 0.1×SSC−0.1% SDS at 65°C. Only a 0.9 kb band was observed in lanes 17 (GM/NA10567 carrying only chromosome 16 as human material) and 19 (NAIMR91, a human cell line). Lane 1, GM/NA09925; lane 2, GM/NA09926; lane 3, GM/NA09927; lane 4, GM/NA09928; lane 5, GM/NA09929; lane 6, GM/NA09930A; lane 7, GM/NA09931; lane 8, GM/NA09932; lane 9, GM/NA09933; lane 10, GM/NA09934; lane 11, GM/NA09935A; lane 12, GM/NA09936; lane 13, GM/NA09937; lane 14, GM/NA09938; lane 15, GM/NA09940; lane 16, GM/NA10324; lane 17, GM/NA10567; lane 18, GM/NA10611; lane 19, NAIMR91 (human cell line IMR91); lane 20, NA10908 (Chinese hamster ovary line UV-135); and lane 21, NA00347A (mouse line B-82). For details of the hybrid cell lines, see Table I.

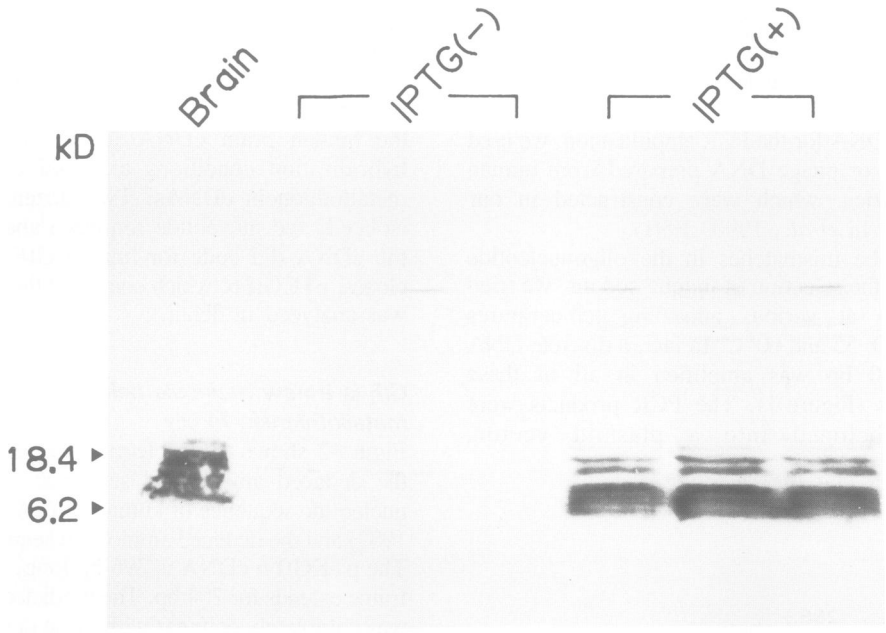

Fig. 5. Western blot analysis of proteins produced by *E.coli* harboring human GIF cDNA in a prokaryotic expression vector. Human GIF cDNA was inserted into a prokaryotic expression vector, pKK233-2, and propagated through *E.coli* JM105. Three individual colonies were picked up and grown in the presence [IPTG(+)] or absence [IPTG(−)] of 1 mM IPTG. 20 μg of *E.coli* lysate was run through an SDS−7−20% linear gradient polyacrylamide gel, electroblotted onto a nitrocellulose membrane and stained with anti-human GIF antibody, which was raised against a synthetic peptide synthesized from amino acid residues 61−68 of human GIF.

terminal and carboxy-terminal domains in comparison with MT2A, respectively. Such insertions have never been observed in vertebrate metallothioneins (Hamer, 1986).

To determine the chromosomal localization of the human GIF gene, a somatic cell hybrid panel was screened using the 3′ noncoding segment of the GIF cDNA as a probe. As shown in Figure 4, a discrete band was observed in genomic DNA containing human chromosome 16. The result clearly confirms that the GIF gene is located on chromosome 16, as are the metallothionein genes (Karin *et al.*, 1984).

The protein encoded by the GIF cDNA has a growth inhibitory activity

To confirm that the protein encoded by the human GIF cDNA inhibits the growth of neonatal rat cortical neurons, the GIF cDNA was inserted into an *E.coli* expression vector, pKK223-2 (Amann and Brosius, 1985), which allows the synthesis of protein identical to native human GIF, starting at ATG at position 53 of the GIF cDNA. The production of GIF protein was induced by IPTG (isopropylthio-galactoside), and Western blotting analysis indicated that *E.coli* harboring the human GIF cDNA in pKK233-2 produced a 7 kDa protein which was detected by antibody raised against synthetic peptide corresponding to amino acid residues 53−64 of human GIF (Figure 5).

To perform a functional assay of the gene product, we purified the GIF protein from the *E.coli* lysate by the method described previously (Uchida *et al.*, 1991). As shown in Figure 6, the purified GIF protein inhibits growth in a dose-dependent manner, although the specific activity of growth inhibition on neonatal rat cortical neurons of the recombinant GIF was slightly lower than that of the native GIF.

GIF expression is confined to the nervous system

The tissue distribution of GIF expression was analyzed by Northern blot analysis using the 3′ noncoding region of the

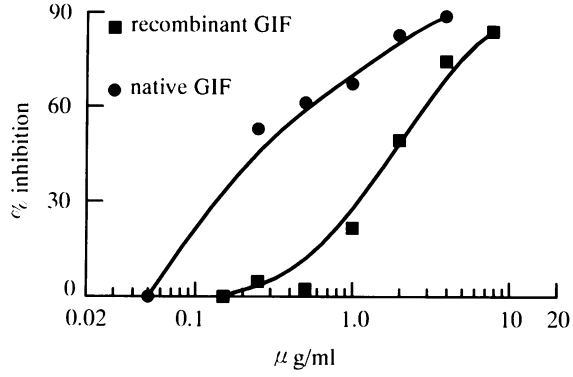

Fig. 6. Expression of growth inhibitory activity of recombinant GIF. The GIF protein was produced by *E.coli* harboring human GIF cDNA in an expression vector, pKK223-2, in the presence of 1 mM IPTG. The recombinant GIF protein was purified from the *E.coli* lysate and subjected to a bioassay using neonatal rat brain cultures (Uchida *et al.*, 1991).

GIF cDNA as a probe to avoid cross-hybridization to metallothionein mRNAs. The GIF expression was detected only in the cerebrum and cerebellum, not in liver, kidney, spleen, testis, muscle or fibroblasts (Figure 7). It is interesting to note that GIF is not expressed in liver or kidney, which are the major sites for expression of metallothionein mRNA.

Down-regulation of GIF mRNA in Alzheimer's disease

To see if the decreaed inhibition of growth in AD brain extract is due to decreased levels of GIF mRNA expression, RNAs were extracted from both normal and AD brains and analyzed by Northern blot hybridization. To avoid cross-hybridization of the GIF cDNA probe to metallothionein RNAs, a DNA fragment generated by PCR amplification of the 3′ noncoding sequence of the GIF cDNA was used

Fig. 7. Tissue distribution. Total RNAs were extracted from various human tissues or cells including HeLa cells, fibroblasts, cerebrum, cerebellum, liver, spleen, kidney, testis and muscle. 20 μg of total RNAs was electrophoresed through a denaturing 1.4% −2.2 M formaldehyde−agarose gel, transferred to a nitrocellulose membrane and hybridized with the ^{32}P-labeled DNA fragment generated by PCR amplification of the 3′ noncoding region of human GIF cDNA.

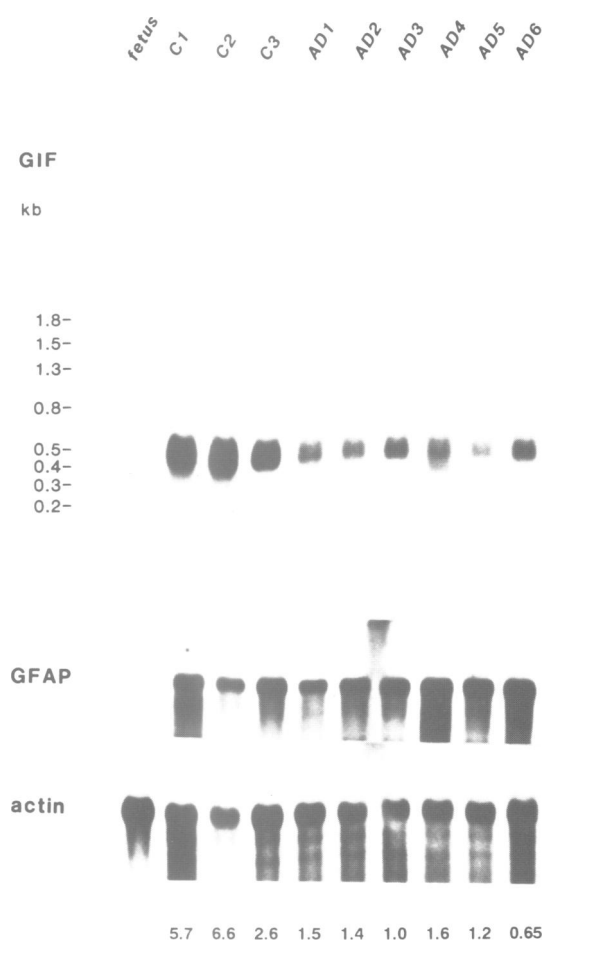

Fig. 8. Northern blot analysis of GIF mRNA in AD and normal brains. Total RNAs were extracted from normal human brains as well as AD brains. 20 μg of total RNAs was electrophoresed through a denaturing 1.4% agarose−2.2 M formaldehyde gel, transferred to a nitrocellulose membrane and hybridized to a ^{32}P-labeled DNA fragment which was prepared by PCR amplification of the 3′ noncoding region of human GIF cDNA. The ratios of human GIF mRNA to β-actin mRNA determined by a Laser Image Analyzer are shown below the autoradiograms.

as the probe. The amount of GIF mRNA was quantified using β-actin mRNA as a reference. As shown in Figure 8, AD brains showed dramatically less expression of the GIF mRNA than normal brains, with the ratios of GIF mRNA to β-actin mRNA being 5.0 ± 2.1 (mean ± SEM, n = 3) for normal control brains and 1.2 ± 0.4 (mean ± SEM, n = 6) for AD brains. Interestingly, the GIF mRNA level is relatively low in C3, a normal control aged 100. Although this case did not show clinical features of AD, histological examination showed numerous senile plaques but no neurofibrillary tangles, a common finding in normal aged human brains.

Discussion

We have shown here the complete nucleotide sequence of a full-length human GIF cDNA. The cDNA insert of pTKGIF6 is 396 bp long, excluding the poly(A) tail, and Northern blot analysis indicates that the GIF mRNA is ~500 nt long. The results suggest that we have isolated a full-length cDNA for human GIF. Indeed, the first ATG of the open reading frame lies at position 53 of the cDNA, and the deduced amino acid sequence starting at the first ATG is completely identical to that determined by chemical sequencing of the human GIF purified from normal human brains (Uchida *et al.*, 1991).

The expression of the growth inhibitory activity of the recombinant GIF produced by *E.coli* harboring GIF cDNA in a prokaryotic expression vector, as well as the complete colinearity of the deduced amino acid sequence with chemically determined amino acid sequences of human GIF, confirms the authenticity of the human GIF cDNA. As there are no additional ATG codons upstream of the ATG at position 53, the human GIF probably lacks a signal peptide that is required for the translocation of peptides into the luminal side of the endoplasmic reticulum and for the subsequent secretion (Rosenfeld *et al.*, 1982; Walter *et al.*, 1984; Watson, 1984). This point needs to be confirmed by detailed nucleotide sequence analysis of the human GIF gene and determination of the transcription initiation site; this is under way in our laboratory. Because of the absence of a signal peptide, it seems likely that the GIF is a cytoplasmic protein.

Immunohistochemical observations using antibody raised against a synthetic peptide (amino acid residues 61−68,

Uchida *et al.*, 1991) demonstrated that GIF is expressed exclusively in astrocytes in the gray matter (Uchida *et al.*, 1991). Therefore, it is not clear how the GIF inhibits the growth of neurons *in vivo*. Although the mechanism of secretion is also unclear, it has been shown that a metallothionein that is highly homologous to GIF and also lacks a signal peptide is present in rat serum (Garvey and Chang, 1981). This result raises the possibility that there is a mechanism for secretion of GIF from astrocytes. The transport mechanism of GIF, if present, should be thoroughly investigated. Another alternative possibility is that the first step of action of GIF takes place in astrocytes, and subsequent processes may follow for the expression of growth inhibitory activity. Therefore, it would be of interest to identify receptors for GIF, if present.

As expected from chemically determined amino acid sequences of human GIF, nucleotide sequence analysis of the human GIF cDNA has shown striking homology to those of metallothioneins (Karin and Richards, 1982; Schmidt *et al.*, 1985; Heguy *et al.*, 1986; Varshney *et al.*, 1986; Foster *et al.*, 1988). Metallothioneins are ubiquitous, low molecular weight proteins that are characterized by an unusually high cysteine content (23–33 mol%) and a selective capacity to bind heavy metal ions such as zinc, copper and cadmium (Hamer, 1986). There are two major subgroups of metallothioneins, MT1 and MT2, and each class is further resolved into several distinct isoforms. In man, MTs are encoded by a multigene family consisting of at least 12 genes. All the known functional human MT genes are clustered on chromosome 16, and the remaining MT-related sequences are dispersed to at least four other autosomal loci (Karin *et al.*, 1984; West *et al.*, 1990). Detoxification of heavy metals is thought to be a function of MTs, but other potential functions have also been suggested (Karin, 1985; Hamer, 1986).

As shown in Figure 2, the GIF molecule has striking similarities to previously described mammalian metallothioneins in the following aspects (Hamer, 1986). (i) The GIF cDNA shows a striking homology to metallothioneins (Karin and Richards, 1982; Schmidt *et al.*, 1985; Heguy *et al.*, 1986; Varshney *et al.*, 1986; Foster *et al.*, 1988) (54% identity in nucleotide sequence and 63% identity in amino acid sequence). (ii) All cysteine residues are conserved between GIF and the mammalian metallothioneins, and the cysteine content of the GIF molecule is as high (29 mol%) as that of mammalian metallothioneins. (iii) Basic amino acids (Lys) juxtaposed to Cys residues are also highly conserved. (iv) Aromatic amino acids are scarce. (v) The human GIF contains three zinc and four copper atoms per molecule (Uchida *et al.*, 1991). Taken together, the results indicate that the GIF is likely to be a new species of metallothionein. Furthermore, it was determined that the human GIF was on chromosome 16, as are the metallothionein genes. This suggests that GIF and metallothionein genes may be derived from a common ancestor gene. The fact that all cysteine residues of human GIF are conserved compared with metallothioneins suggests that metallothiolate bond formation is important for the function of GIF.

Compared with mammalian MT2A, the central domain (amino acid residues 11–39 of human GIF) shows the highest homology. There are, however, 3 bp and 18 bp insertions in the coding sequence for the amino-terminal and carboxy-terminal domains compared with MT2A, respectively. Despite the similarity of their amino acid sequence to that of GIF, metallothioneins do not exhibit growth inhibitory activity (Uchida *et al.*, 1991). As the central domain of the GIF shows the highest homology to metallothioneins, it is most likely that an amino acid insertion at the amino-terminus or a six amino acid insertion at the carboxy-terminus plays an important role in the expression of the growth inhibitory activity. This point should be clarified by mutagenesis of the GIF cDNA and expression of the mutant GIF.

Northern blotting analyses have shown that the expression of GIF mRNA is highly specific to the nervous system, which is in striking contrast to the tissue distribution of metallothioneins: metallothionein expression is highest in

Table I. Hybrid cell lines

Cell line	Chromosome no.																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
GM/NA09925	74	24	0	74	76	60	82	78	0	0	4	68	6	86	78	14	98	96	46	84	0	76	0	0
GM/NA09926	69	75	75	65	2	88	85	69	0	68	0	2	77	73	93	2	81	75	84	96	2	4	2	0
GM/NA09927	69	83	75	77	0	93	79	73	0	82	0	0	77	79	90	0	81	73	87	89	0	0	0	0
GM/NA09928	0	84	58	0	48	32	0	66	0	2	0	0	4	76	92	0	98	0	28	0	70	82	0	78
GM/NA09929	0	0	61	59	0	43	2	49	0	0	33	49	0	59	2	0	96	0	2	31	0	0	2	0
GM/NA09930A	0	34	62	4	12	0	26	4	0	0	6	22	56	82	12	0	86	78	0	22	82	76	6	8
GM/NA09931	0	0	0	0	26	0	78	0	0	46	0	64	0	100	0	0	100	0	0	78	90	0	0	14
GM/NA09932	0	0	0	68	86	46	0	80	0	2	28	26	0	0	0	0	96	0	2	0	92	0	0	0
GM/NA09933	50	0	84	16	54	76	92	54	0	6	0	50	84	78	92	0	88	70	80	32	94	88	0	32
GM/NA09934	0	50	0	0	83	79	4	87	0	0	77	87	0	2	89	0	90	89	0	91	89	2	0	0
GM/NA09935A	0	0	52	10	28	12	0	0	0	8	0	22	74	72	0	0	93	59	0	9	91	71	0	0
GM/NA09936	0	0	0	18	0	46	70	10	0	16	34	0	2	88	2	0	100	0	44	24	0	18	0	0
GM/NA09937	0	0	54	38	0	62	54	70	0	4	0	42	0	70	60	0	96	66	0	0	0	0	0	0
GM/NA09938	0	0	2	88	60	88	86	4	0	0	36	92	0	80	4	0	92	0	4	80	76	60	0	2
GM/NA09940	0	0	46	0	0	0	84	62	0	0	0	0	0	0	62	0	100	0	0	0	0	0	0	0
GM/NA10324	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	90	0
GM/NA10567	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	98	0	0	0	0	0	0	0	0
GM/NA10611	0	0	0	0	0	0	0	0	69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The numbers presented represent the percentages of cells with human chromosomes.

liver and kidney, and lowest in the brain. Furthermore, the GIF mRNA is not expressed in fetal brain. Transcription of the GIF gene is thus regulated in a tissue-specific manner as well as developmentally.

Northern blotting analysis has shown that expression of the GIF mRNA is dramatically decreased in AD brains, especially in AD brains with numerous neurofibrillary tangles. The result is concordant with previous immunohistochemical observations and Western blotting analysis using the antibody against GIF (Uchida *et al.*, 1988, 1991; Uchida and Tomonaga, 1989). Furthermore, the finding indicates that the decreased growth inhibitory activity in AD brain is due to the decreased expression of GIF mRNA. Immunohistochemical observations of normal brains showed that the GIF is expressed primarily in astrocytes in gray matter, but not in astrocytes in the white matter. Immunohistochemical observation has indicated that GIF staining is decreased even though the astrocytes do not seem to be degenerating (Uchida *et al.*, 1991). These observations strongly indicate that the decrease of GIF mRNA is a result of decreased transcription of the GIF gene, but not an epiphenomenon resulting from degeneration of astrocytes in the cortex of AD brain. Therefore the elucidation of the mechanisms of transcription regulation of the human GIF gene is the most important issue. The question of how the transcription of the GIF gene is down-regulated in AD brain is an intriguing one.

From observations of a limited number of AD brains, the down-regulation of GIF mRNA seems to be a consistent finding in AD brains. The result raises the possibility that down-regulation of GIF mRNA in turn results in decreased inhibition of growth and leads to massive somatodendritic sprouting. An intriguing hypothesis is that decreased expression of GIF mRNA is the primary cause of the accumulation of curly fibers and neurofibrillary tangles. To test this hypothesis, we will have to look carefully at the timing of the appearance of curly fibers and neurofibrillary tangles, and disappearance of GIF in AD brain; it will be possible to test this now that a GIF cDNA is availabe.

The availability of a full-length GIF cDNA and expression system will provide important information on the physiological functions of GIF. Analysis of the GIF gene, particularly its promoter region, will provide us with a better understanding of the mechanisms of down-regulation of GIF gene transcription in AD.

Materials and methods

Design of oligonucleotides for amplification of human GIF cDNA

For amplification of human GIF cDNA, a pair of unique oligonucleotide primers was designed: primer 488, 5′-ATGGATCCCGAGACCTGCCC; and primer 487, 5′-CTGGCAGCAGCTGCACTTCTC. The sense primer (488) was designed from the amino-terminus of human GIF (amino acid residues 1−7), and the antisense primer (487) was designed from the carboxy-terminus of GIF (amino acid residues 61−68). Taking account of the codon usage preference of human metallothionein genes (Karin and Richards, 1982; Schmidt *et al.*, 1985; Heguy *et al.*, 1986; Varshney *et al.*, 1986; Foster *et al.*, 1988), we selected a unique codon for each amino acid. Oligonucleotides were synthesized using an automated DNA synthesizer (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions.

Amplification of GIF cDNA using the unique oligonucleotide primers

1 μg of total plasmid DNA or phage DNA, prepared from human brain cDNA libraries (Kobayashi *et al.*, 1990, 1991), was subjected to 30 cycles of PCR, each consisting of denaturation at 94°C for 1 min, annealing at various temperatures (45−60°C) for 1 min and primer extension at 72°C for 3 min (Saiki *et al.*, 1988). PCR products were electrophoresed through a 1.4% agarose gel and stained with ethidium bromide. The PCR products were subsequently subcloned into the *Eco*RV site of pBluescriptKS(+) (pBSHGIF).

Molecular cloning of the human GIF cDNA

A human brain cDNA library (Kobayashi *et al.*, 1990) was screened by colony hybridization using the insert of pBSHGIF as the probe (Wahl *et al.*, 1979; Maniatis *et al.*, 1982). The filters were washed to a final stringency of 0.1×SSC (1×SSC = 150 mM NaCl and 15 mM sodium citrate)−0.1% SDS at 65°C. Of 24 cDNA clones, pTKGIF6, which contains the longest cDNA insert, was selected for detailed analysis.

Nucleotide sequence analysis

Nucleotide sequences were analyzed by the dideoxynucleotide chain terminator method using double-stranded plasmid DNA as a template (Sanger *et al.*, 1977; Chen and Seeburg, 1985).

Expression of human GIF in E.coli

To insert human GIF cDNA into an *E.coli* expression vector, pKK223-2 (Amann and Brosius, 1985), a pair of primers was designed: primer 516, 5′-AGCCATGGACCCTGAGACCT; and primer 517, 5′-GCAAGCTT-CACCACAGGGCATAGGT. For ligation to pKK223-2, an *Nco*I linker sequence and a *Hin*dIII linker sequence were added to primers 516 and 517, respectively. The pTKGIF6 was subjected to PCR amplification using the primer pair of 516 and 517. The PCR products were digested with *Nco*I and *Hin*dIII, and ligated to *Nco*I- and *Hin*dIII-cleaved pKK223-2. The ligation products were used to transform *E.coli*, JM105. The production of the GIF protein was induced by 1 mM IPTG for 5.5 h.

Bioassay of growth inhibitory activities

Purification of the recombinant GIF produced by *E.coli* and bioassay of growth inhibitory activities using newborn rat cortical cells were performed as described (Uchida *et al.*, 1991).

Northern blot analysis

Total RNA was extracted from human brains [three control brains aged 79 (C1), 87 (C2) and 100 (C3), and six pathologically proven AD brains aged 80 (AD1), 63 (AD2), 62 (AD3), 56 (AD4), 71 (AD5) and 81 (AD6)] by a modification of the method of Chirgwin *et al.* (1979) (Kobayashi *et al.*, 1990). The average post-mortem delay was 4.5 h, which is well within a desirable interval for RNA extraction (Kobayashi *et al.*, 1990). 20 μg of total RNA extracted from control and AD brains was electrophoresed through a denaturing 1.2% agarose−2.2 M formaldehyde gel (Lehrach *et al.*, 1977) and transferred to nitrocellulose membranes. Hybridization was performed as described previously (Kobayashi *et al.*, 1990). The radioactivities of the bands were measured using a Laser Image Analyzer (Fuji BAS2000 system, Fuji Film, Tokyo, Japan).

DNA probes

To avoid cross-hybridization to metallothionein mRNAs or genes, the unique 3′ non-coding sequence of human GIF cDNA was amplified using a primer pair of 511 and 518: primer 511, 5′-TTTATTGTCATTCCTCCAAG; and primer 518, 5′-GAAGGCACCCCTCCGTGTGG. Human glial fibrillary acidic protein (GFAP) cDNA was cloned in previous studies in our laboratory (unpublished). Human metallothionein 2A cDNA was obtained from the American Type Culture Collection, Rockville, MD, USA. Human β-actin genomic DNA was kindly provided by Dr T.Hamada of University of Tokyo.

Determination of chromosome localization

A somatic cell hybrid panel, NIGMS Human Rodent Somatic Cell Hybrid Mapping Panel #1, was obtained from Coriell Institute for Medical Science Research, Camden, NJ, USA. 7 μg of genomic DNA of each cell line was digested with *Pvu*II, run through 0.8% agarose gels, transferred to nitrocellulose membranes and hybridized with the 3′ noncoding segment of pTKGIF6, which was generated by PCR with a primer pair of 511 and 518. The filter was washed to a final stringency of 0.1×SSC−0.1% SDS at 65°C. The details of the hybrid cell lines are given in Table I.

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