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Molecular Cloning of a Novel Cytokine cDNA Encoding the Ninth Member of the Fibroblast Growth Factor Family, Which Has a Unique Secretion Property

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Glia-activating factor (GAF) is a novel heparin-binding growth factor purified from the culture supernatant of a human glioma cell line. It shows a spectrum of activity slightly different from those of other known growth factors. We have isolated the cDNA which encodes human GAF. A homology search revealed that GAF would be the ninth member of the FGF family, and we therefore call it FGF-9. The human FGF-9 cDNA cloned by using oligonucleotide probes encoded a polypeptide consisting of 208 amino acids. Sequence similarity to other members of the FGF family was estimated to be around 30%. Two cysteine residues and other consensus sequences in family members were also well conserved in the FGF-9 sequence. FGF-9 was found to have no typical signal sequence in its N terminus like those in acidic FGF and basic FGF. Acidic FGF and basic FGF are known not to be secreted from cells in a conventional manner. However, FGF-9 was found to be secreted from cells after synthesis despite its lack of a typical signal sequence. It could be detected exclusively in the culture medium of cDNA-transfected COS cells. The amino acid sequence of proteins purified from culture supernatant of the CHO cell line, which was cDNA transfected and selected as a high producer of FGF-9, showed that no peptides were cleaved from the N terminus except the initiation methionine. The rat FGF-9 cDNA was also cloned, and the structural analysis indicated that the FGF-9 gene is highly conserved. Expression of the FGF-9 gene could be detected in the brain and kidney of the adult rat. Restricted gene expression in organs and the unique secretion nature of the protein suggest that FGF-9 plays a physiological role which differs from those of well-characterized acidic FGF and basic FGF.

Glial cells are known to play an important role in guiding the construction of the nervous system and controlling the chemical and ionic environment of nerve cells. In this regard, growth factors affecting glial cells are thought to play a crucial role in the development and maintenance of the nervous system. We have recently identified, in the culture supernatant of a human glioma cell line, a novel growth factor, glia-activating factor (GAF), that promotes glial cell proliferation (22). The factor was found to consist of a single polypeptide and to bind to heparin. Three molecular species varying in size (30, 29, and 25 kDa) were purified (the ratio of recovered proteins was 5:1:1), and the N-terminal sequence of each molecular species was determined. All molecular species were found to have an N-linked sugar chain of about 3 kDa. GAF has a unique spectrum of activity on cells, and it acts on cells of the central nervous systems. It activated O-2A progenitor cells, PC-12 cells, and BALB/c 3T3 cells as well as rat glial cells. It could not, however, activate human umbilical vein endothelial cells. In this paper, we describe molecular cloning and characterization of human and rat cDNA encoding GAF. From the analysis of the cDNA structure, GAF was revealed to be the ninth member of the FGF family (4) and hereafter we refer to the factor as FGF-9, in line with published recommendations (3). A unique secretion mechanism of the synthesized protein and the restricted expression pattern of the FGF-9 gene in adult rat organs are also described.

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MATERIALS AND METHODS

Polymerase chain reaction (PCR) and cDNA library. The N-terminal amino acid sequence of purified 30-kDa GAF was used to design two oligonucleotide mix primers that could be used for amplification of a portion of the FGF-9 gene. Sense primer 5′-AAGGATCCGTIGGIAA(T/C)TA(T/C)TT(T/C)GG-3′ corresponded to amino acids 4 to 9 of 30-kDa GAF (Val-Gly-Asn-Tyr-Phe-Gly) and an additional *Bam*HI site. Antisense primer 5′-AAGAATTCAC(A/G)TTICC(A/G)AAIGGIAC-3′ corresponded to amino acids 14 to 19 of 30-kDa GAF(Val-Pro-Phe-Gly-Asn-Val) and an additional *Eco*RI site. Human genomic DNA (1 μg) was subjected to PCR (Gene Amp; Cetus) by the addition of 560 ng of each primer and 2.5 U of *Taq* DNA polymerase. PCR was carried out under the following conditions: annealing at 50°C for 2 min, extension at 72°C for 3 min, and denaturation at 94°C for 1 min and for 25 cycles. An amplified DNA fragment of the predicted size was excised from the polyacrylamide gel and cloned into an M13 vector and sequenced (5′-GGATCCGTGGGGAACTATTTCGGGGTGCAGGATGCGGTCCCCTTCGGCAACGTGAATTC-3′). Two screening oligonucleotide probes derived from the DNA fragment were synthesized, and the sequences were 5′-TGGGGAACTATTTCGGGGTGCAGGATGCGG-3′ and 5′-ACGTTGCCGAAGGGGACCGCATCCTGCACC-3′. A human foreskin cDNA library (10) and rat brain cDNA library (23) were kind gifts from H. Okayama and Y. Ono, respectively. Human and rat GAF-enriched cDNA libraries were constructed by using specific primers. The sequences of the specific primers were 5′-AAGGGCTCAAGTGAAGAAATTGTC-3′ for the human

library and 5'-AAGGGCTCAAGTGAAGACACTGTC-3' for the rat library. cDNA synthesis and library construction were performed by the methods of Seed and Aruffo (29).

Cell culture and DNA transfection. Human glioma cell line NMC-G1 was grown in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) containing 10% fetal calf serum (FCS; Whittaker Bioproducts). DNA transfection of COS cells was carried out by the method of Seed and Aruffo (29). Cells (5×10^5) were plated on a 6-cm dish and cultured in DMEM with 10% Nu-serum (Collaborative Research, Inc.). At 50% confluence, 50 μl of a DNA cocktail (2 or 10 μg of DNA in 10 mg of DEAE dextran per ml) and 5 μl of 5-mg/ml chloroquine were added to the 1.5 ml of medium. After 4 h of incubation at 37°C, cells were treated with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS). Cells were washed with PBS and then cultured in 4 ml of DMEM–10% Nu-serum for 3 days. Culture supernatant and cells were collected, and cells were sonicated for 90 s in 1 ml of PBS to prepare cell lysates. Cell lysate and culture supernatant were subjected to a glial cell proliferation assay and Western blotting (immunoblotting) analysis.

Glial cell proliferation assay. Rat primary cortical astrocytes, isolated from brains of 17-day Crj:CD rat (Japan Charles River, Kanagawa, Japan) fetuses (22), were cultured in DMEM containing 10% heat-inactivated FCS (DMEM–10% FCS). Cells were collected by trypsinization, and a cell suspension (3,000 cells per 100 μl) was added to each well of 96-well microplates (A/S Nunc, Roskilde, Denmark). After 2 or 3 days, the medium was changed to DMEM–0.125% FCS, and culturing was continued for a further 2 or 3 days. Samples were added to each well, and then 18 h later, 1 μCi of [^{3}H]thymidine (95 Ci/mmol; CEA) was added to each well. After the cells were incubated for 6 h, they were trypsinized and collected on glass filters with a Titertek cell harvester (Skatron, Lier, Norway), and the radioactivity was measured.

Western blotting analysis. Cell lysate or culture supernatant of DNA-transfected COS cells (mock transfected and with 10 μg of DNA) from one 6-cm dish was applied to a small heparin–Sepharose CL-6B column (bed volume, 0.6 ml) and washed with a 20 mM Tris-HCl (pH 7.6)–0.4 M NaCl solution. The adsorbed protein was then eluted with 20 mM Tris-HCl (pH 7.6)–2.0 M NaCl. The eluted protein was precipitated by adding ice-cold trichloroacetic acid and dissolved in application buffer for gel electrophoresis. One third of the sample was applied to a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel (17). The protein on the polyacrylamide gel was transferred to a nitrocellulose membrane by the Horizo-blot system (ATTO). Anti-FGF-9 antibodies were raised in rabbits against the synthesized N-terminal peptide (from the 5th to 17th amino acid and an additional cysteine) and C-terminal peptide (C-terminal 16 amino acid residues and an additional cysteine) of FGF-9. For immunization, the synthetic peptides were conjugated to keyhole limpet hemocyanin via the addition of a cysteine residue to the N terminus of the peptides. These antibodies were used for the staining of FGF-9 transferred to a nitrocellulose membrane with an alkaline phosphatase immunoblot kit (ProtoBlot Western Blot AP system; Promega) by using the supplier's protocol.

Establishment of FGF-9-producing CHO cells. Plasmid pDGAF1 harboring hamster DHFR cDNA downstream from the simian virus 40 early promoter and FGF-9 cDNA downstream from the murine leukemia virus long terminal repeat was constructed from pTB485 (28) and pGAF1. In this plasmid, the entire cDNA insert of pGAF1 was included. DHFR$^-$ CHO cells were cultured in Ham's F12 medium (Flow Laboratories) supplemented with 5% FCS and transfected with pDGAF1 (10 μg per 6-cm dish). After 2 days, the cells were trypsinized and seeded into 96-well microplates with a selective medium which consisted of DMEM, 5% dialyzed FCS, and 35 μg of proline per ml. The cells were maintained and fed every 3 to 4 days. After 3 weeks, the colonies were transferred to Linbro dishes. Cells were cultured in selective medium and exposed to stepwise increasing of methotrexate (Sigma) concentrations. Mitogenic activity of the supernatant of methotrexate-resistant cells was tested, and CDGAF4 cells which showed the highest activity were selected.

Purification of recombinant FGF-9 produced in CHO cells. Recombinant FGF-9 proteins were purified from the culture supernatant of CDGAF4 cells. The purification procedure was essentially the same as that adopted for the purification of native proteins (22). Briefly, CDGAF4 cells were grown to semiconfluence in a roller bottle, and medium was replaced with Ham's F12 medium-DMEM (1:1) containing 0.5% FCS. After 2 days, the medium was collected and NaCl and phenylmethylsulfonyl fluoride were added to concentrations of 0.1 M and 1 mM, respectively. This mixture was then passed through a heparin–Sepharose CL-6B column. Adsorbed protein was eluted from the column with 2 M NaCl solution in buffer (20 mM Tris-HCl [pH 7.6], 0.1% CHAPS) {CHAPS is 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate}, and the eluted protein was then subjected to Sephacryl S200 gel filtration. FGF-9 was monitored by Western blotting with anti-FGF-9 C-terminal peptide antibody. Fractions containing FGF-9 were pooled and applied to a heparin high-pressure liquid chromatography (HPLC) column (Shodex HR894; Showadenko). The proteins were eluted with a linear NaCl gradient from 0.4 to 2 M. Antibody-reacting proteins were eluted around 0.8 M NaCl, and they were pooled and applied to a C_4 reverse-phase HPLC column. Elution was accomplished with an acetonitrile gradient, and eluted protein was tested for reactivity with the antibody and in the glial cell proliferation assay. The N-terminal amino acid sequence of the purified protein was analyzed by a gas-phase protein sequencer (model 473A; Applied Biosystems). *N*-glycanase treatment of the purified protein was done under the supplier's protocol. Samples (about 20 ng of the protein) were treated with 0.5 U of *N*-glycanase (peptide:N^4-[*N*-acetyl-β-glucosaminyl]asparagine amidase; Genzyme) in a solution containing 20 mM Tris-HCl (pH 8.0), 0.2% SDS, 1% Nonidet P-40, 20 mM 2-mercaptoethanol, and 20 mM EDTA at 37°C for 24 h. The digested proteins were separated on SDS-polyacrylamide gels and detected by Western blotting with anti-FGF-9 N-terminal peptide antibody.

Northern (RNA) blot analysis. Total RNAs were isolated by the guanidine thiocyanate method by using an RNA extraction kit (Pharmacia). RNAs were applied to an oligo(dT) cellulose column, and poly(A)$^+$ RNAs were eluted. mRNAs (10 to 20 μg) were fractionated in a 1.0% agarose gel (1.1 M formaldehyde, 10 mM Na-phosphate buffer, pH 7.2) and blotted onto a nylon membrane (BioDine; Pall BioSupport). Hybridization was performed in a solution containing 6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM Na phosphate buffer, 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.1% SDS, 170 μg of salmon sperm DNA, and probes (2×10^9 cpm/μg, 5×10^6 cpm/ml) at 65°C. Membranes were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C for 30 min. Autoradiograms were produced by using X-AR film (Kodak) for Fig. 6A and an

image analyzer (Bio-image analyzer BAS2000; Fuji) for Fig. 6B.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases with the following accession numbers: D14838 for human FGF-9 mRNA and D14839 for rat FGF-9 mRNA.

RESULTS

Isolation of human FGF-9 cDNA clones. Degenerate oligonucleotides corresponding to two short segments of the N-terminal 30-kDa GAF amino acid sequence were synthesized and used for PCR amplification of a segment of the FGF-9 gene of human genomic DNA. The PCR product of the predicted size was subcloned into an M13 vector and sequenced. Amino acids encoded by the PCR-amplified fragment and those of the purified protein were found to be identical. The human foreskin cDNA library was screened by using two oligonucleotide probes derived from the PCR fragment. Two positive clones (pGAF1 and pGAF2) were isolated from 10^6 clones, but both were found to have a 1.5-kb insert, suggesting the two clones were the same. Clone pGAF1 was subjected to further analysis. By partial nucleotide sequence of pGAF1 cDNA insert, it encoded amino acid sequences of GAF protein purified from the culture supernatant of the glioma cell line. Therefore, we concluded that pGAF1 encodes GAF protein.

Structure of human FGF-9 cDNA and protein. From the entire nucleotide sequence analysis of the cDNA of pGAF1, a long open reading frame was found (Fig. 1). A TAA stop codon was found 147 bases upstream from the first ATG codon in the frame, so the first ATG was thought to be an initiation codon. To confirm the FGF-9 cDNA structure, we attempted to isolate other clones and constructed an enriched cDNA library from NMC-G1 mRNA by using a specific primer. Six positive clones were obtained from the enriched library, and among them, one clone containing the longest insert (H6-2) was analyzed. No difference in nucleotide sequence of the overlapping cDNA region of pGAF1 and H6-2 was found. From the analysis of the cDNA structure, the primary translation product of FGF-9 was shown to consist of 208 amino acids. The N-terminal amino acid sequences identified in the purified protein of the three molecular species, 30, 29, and 25 kDa (22), were all included in the sequence deduced from the cDNA. This indicated that the three purified factors varying in size were encoded by a single gene and that the smaller ones might be products of proteolytic digestion. One canonical N glycosylation site was found, agreeing well with the earlier finding that native GAF has an N-glycosyl-type sugar chain of 3 kDa. It might be worth noting that the sequence ATTTATTTA, found in many cytokines and proto-oncogene mRNAs, was found to be present in the 3′ noncoding region of the FGF-9 cDNA (30). This sequence is thought to be involved in the stability of mRNA for posttranscriptional regulation. Sequence homology with other known proteins was examined. It was revealed that FGF-9 has about 30% sequence similarity with each member of the FGF family (1, 4, 5, 7, 9, 11, 12, 15, 19, 21, 26, 31–33, 35, 38). Recently, Tanaka et al. reported isolation of an androgen-induced growth factor (AIGF) gene which might correspond to the eighth member of the FGF family (33). We therefore refer to GAF as FGF-9 (3). In Fig. 2, the amino acid sequence of FGF-9 is aligned with that of the first seven members of the FGF family so as to give the highest similarity. Two cysteine residues and other sequence

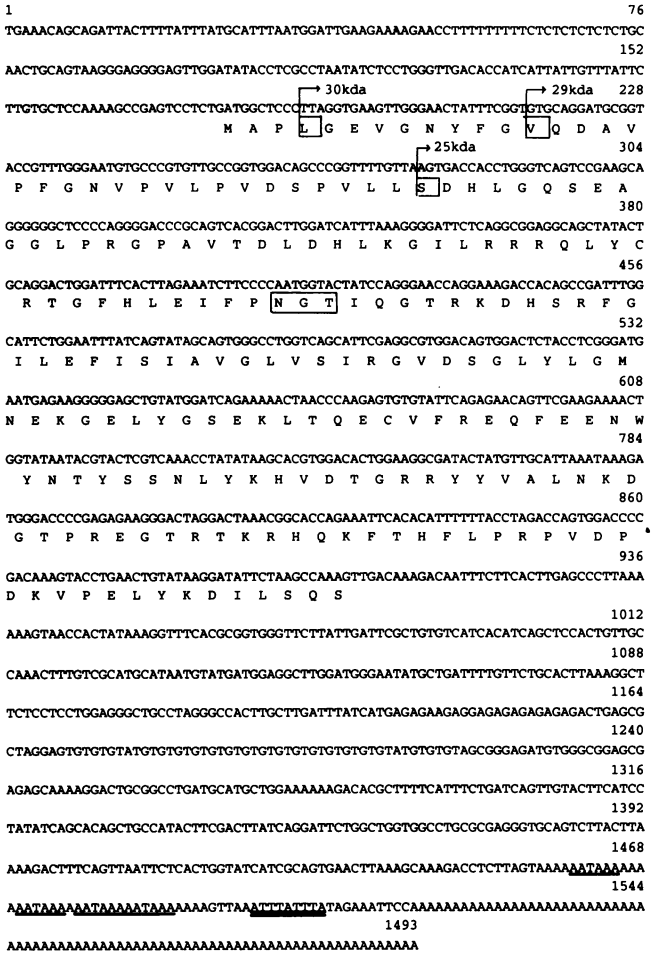

FIG. 1. cDNA structure and deduced amino acid sequence of human FGF-9. The nucleotide sequence of pGAF1 cDNA and deduced amino acid sequence are shown. The amino acid sequence is described with a one-letter code. N-terminal sequences of purified GAF protein are indicated by arrows, and the boxed sequence NGT is the canonical N-type glycosylation site. AATAAA is a polyadenylation signal sequence, and the ATTTATTTA sequence is thought to be involved in mRNA destabilization.

consensuses in the family members were also well conserved in the FGF-9 sequence.

Hydropathy plot analysis was performed, and the results indicated that hydrophobicity of the FGF-9 N-terminal portion was low compared with that of other typical secreted proteins (34) (Fig. 3). The profile resembled that of bFGF, which is known not to be secreted from cells in an ordinary way. FGF-9 has been purified from the culture supernatant of glioma cells. The N-terminal sequence of the 30-kDa protein, the major constituent of the purified factor, was shown to start at the fourth amino acid leucine (22). The peptide of only three amino acids might be too short to work as a secretion signal.

Genomic Southern blot analysis suggested that the FGF-9 gene is a single copy (data not shown).

COS cell expression of FGF-9 cDNA. Biologically active FGF-9 was detected by expressing cloned cDNA. In initially cloned pGAF1, cDNA was designed to be expressed under the control of the simian virus 40 early promoter. COS-7 cells were transfected with pGAF1, and culture supernatant

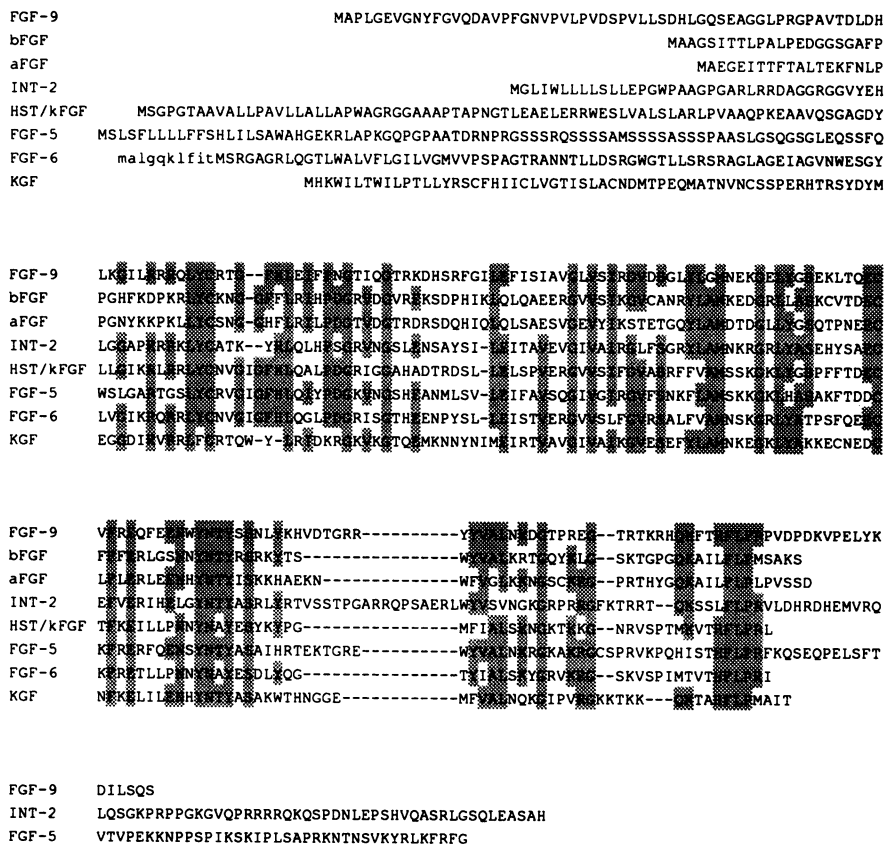

FIG. 2. Alignment of amino acid sequences of FGF-9 and other members of the FGF family. Amino acid sequences of the human FGF family are aligned, and well-conserved amino acids are shaded.

and cell lysate were subjected to the rat glial cell proliferation assay. Culture supernatant of cDNA transfected COS cells promoted the growth of glial cells (Fig. 4A). The lysate of cDNA-transfected COS cells activated glial cell growth, but the activity was of same level as that of untransfected cells (Fig. 4B). By the Western blotting analysis using the antibody raised against the C-terminal oligopeptide, FGF-9 protein of about 3 kDa was detected in the culture supernatant of cDNA-transfected cells (Fig. 4C). The same results could be obtained by using the antibody raised against the N-terminal oligopeptide (data not shown).

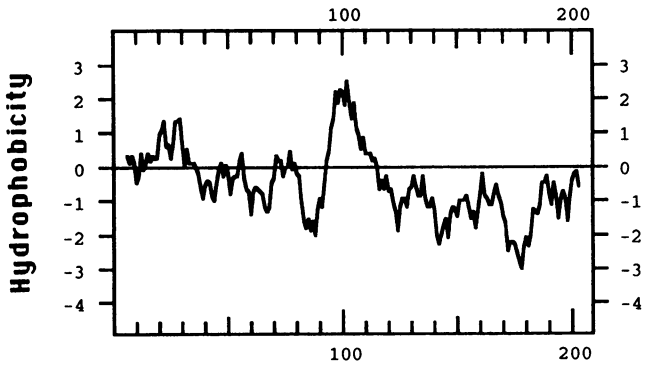

FIG. 3. Hydropathy plot analysis of FGF-9 protein. The hydrophobicity index was calculated by the method of Kyte and Doolittle (16).

Purification of recombinant proteins produced in CHO cells. Plasmid pDGAF1, which was designed to express both FGF-9 and hamster DHFR cDNA, was constructed. $DHFR^-$ CHO cells were transfected with pDGAF1, and $DHFR^+$ CHO cells were selected. The introduced gene was amplified by increasing methotrexate resistance of the cells. Mitogenic activity of the culture medium of 10 μM methotrexate-resistant cells was examined, and CDGAF4 cells whose culture medium showed the highest mitogenic activity were selected. Localization of the FGF-9 protein produced in CDGAF4 cells was examined by Western blotting, and the reactive proteins were detected mainly in the culture supernatant, as in the case of cDNA-transfected COS cells (data not shown). FGF-9 protein was purified from the culture supernatant of CDGAF4 cells. Purification procedures followed the method used in the purification of native FGF-9 protein, including repeated heparin column chromatography, gel filtration, and reverse-phase HPLC. The elution pattern of the reverse-phase HPLC, the final step of purification, and the profile of polyacrylamide gel electrophoresis of eluted fractions are shown in Fig. 5. FGF-9 proteins were separated into three peaks, the constituents of which were 21-, 27-, and 30-kDa proteins, in this column step. The recovery ratio of these proteins was calculated to be 1:20:5, and a total of 600 μg of FGF-9 proteins was recovered from 5 liters of culture supernatant. The constituents of all three peaks showed mitogenic activity and reacted with the anti-C-terminal peptide antibody. The N-terminal amino acid sequence of both 30- and 27-kDa proteins was revealed to be Ala-Pro-Leu-Gly-Glu-Val-Gly-, and they were therefore

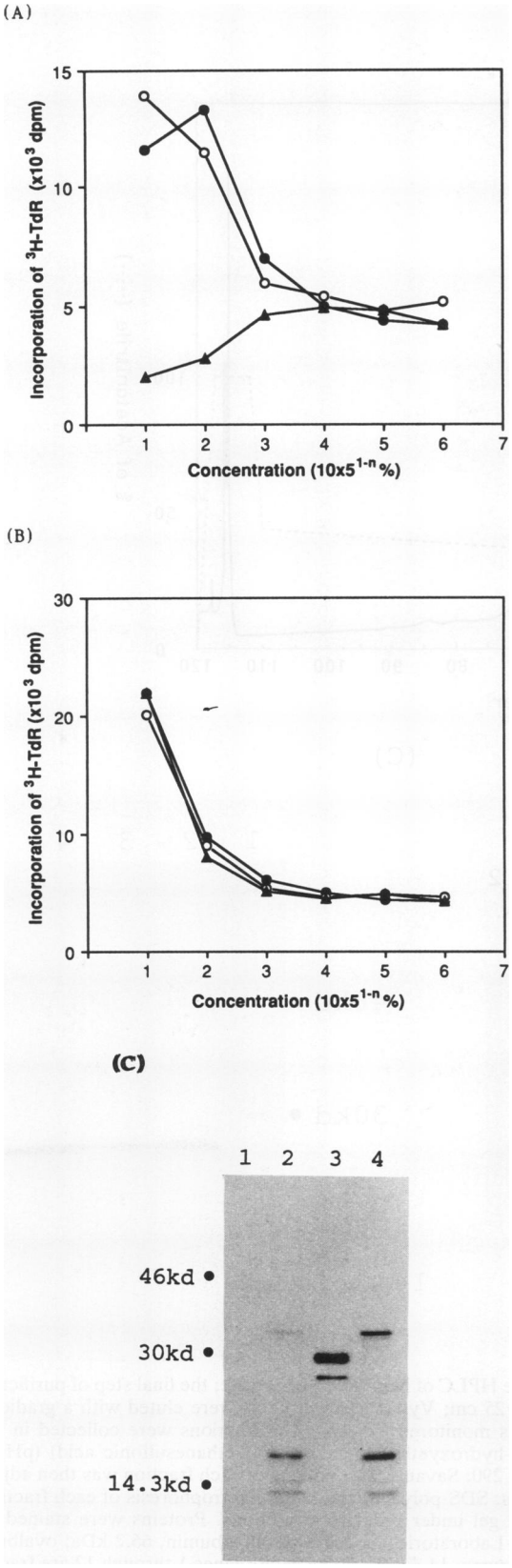

FIG. 4. Detection of FGF-9 in COS cells after cDNA transfection. COS cells were transfected with pGAF1, and mitogenic activity against glial cell in culture supernatant (A) and cell lysate (B) was measured. Samples were serially fivefold diluted and were added to 1/10 volume of the assay mixture. ▲, no DNA; ○, 2 μg of pGAF1; ●, 10 μg of pGAF1. The culture supernatant and cell lysate of DNA-transfected COS cells were applied to a heparin-Sepharose CL-6B column, and fractions eluted with 2 M NaCl were analyzed by SDS-polyacrylamide gel electrophoresis. (C) FGF-9 protein was detected by Western blotting using anti-FGF-9 C-terminal peptide antibody. Lanes: 1 and 3, culture supernatant of mock transfected and pGAF1-transfected cells, respectively; 2 and 4, cell lysate of mock transfected and pGAF1-transfected cells, respectively.

thought to start from the second amino acid alanine. This indicated that these proteins were secreted from the cells without cleavage of the N-terminal peptide except the initiating methionine. The difference in the molecular size of these proteins might be due to glycosylation. FGF-9 molecules purified from human glioma have been shown to have a single sugar chain of 3 kDa (22). The 30-kDa protein might have a sugar chain of this size as observed in the native protein, since it showed the same molecular size on the SDS-polyacrylamide gel as the glycosylated native FGF-9 molecule whose N terminus was the fourth amino acid glycine. *N*-glycanase treatment of the 30-kDa protein was shown to reduce its molecular size, and the product migrated at the same position as the 27-kDa protein on SDS-polyacrylamide gel electrophoresis (Fig. 5C). The minor component 21-kDa protein was shown to start from the 33rd amino acid leucine, and it might be produced by proteolytic cleavage of longer products.

Rat FGF-9. To isolate rat FGF-9 cDNA, a rat brain cDNA library was screened by using a human FGF-9 cDNA fragment as a probe. One positive clone was obtained. An additional six clones were isolated from the enriched library constructed by using an FGF-9-specific primer and rat brain mRNA. These were subjected to structural analysis, and no difference in sequence could be detected. Rat cDNA was highly homologous to human cDNA. Sequence homology of these two was 94%. The amino acid sequences of these two species were almost the same; the difference was only one amino acid, Asn-9 in human cDNA versus Ser-9 in rat cDNA. These results indicate that FGF-9 genes are highly conserved.

Expression of the FGF-9 gene. Three mRNA species of FGF-9, one major and two minor bands, could be detected in human glioma cell NMC-G1 by Northern blot analysis (Fig. 6A). The approximate molecular sizes of these transcripts were calculated to be 4.3 kb for the major band and 3.4 and 2.7 kb for the minor bands. Expression levels seemed to be low and corresponded well to the low yield of FGF-9 from the culture supernatant of NMC-G1 cells (22). We examined expression of the FGF-9 gene in rat tissues. When the rat cDNA was used as a probe, 2.4 kb of the major band and 3.8 kb of the minor band were detected in brain and kidney mRNA, although expression levels in these tissues were very low (Fig. 6B). In other organs so far examined, FGF-9 mRNA could not be detected. The level of expression in the kidney was higher than that in the brain.

DISCUSSION

The attempt of the cDNA cloning of a novel growth factor, GAF, resulted in acquisition of the cDNA of the ninth member of the FGF family (FGF-9). The FGF family is known to contain eight structurally related proteins: basic FGF (1, 15), acidic FGF (12), int 2 (21), hst 1/k-FGF (5, 27, 32, 35), FGF-5 (38), FGF-6 (11, 19), keratinocyte growth factor (KGF) (7, 26), and recently identified AIGF (33).

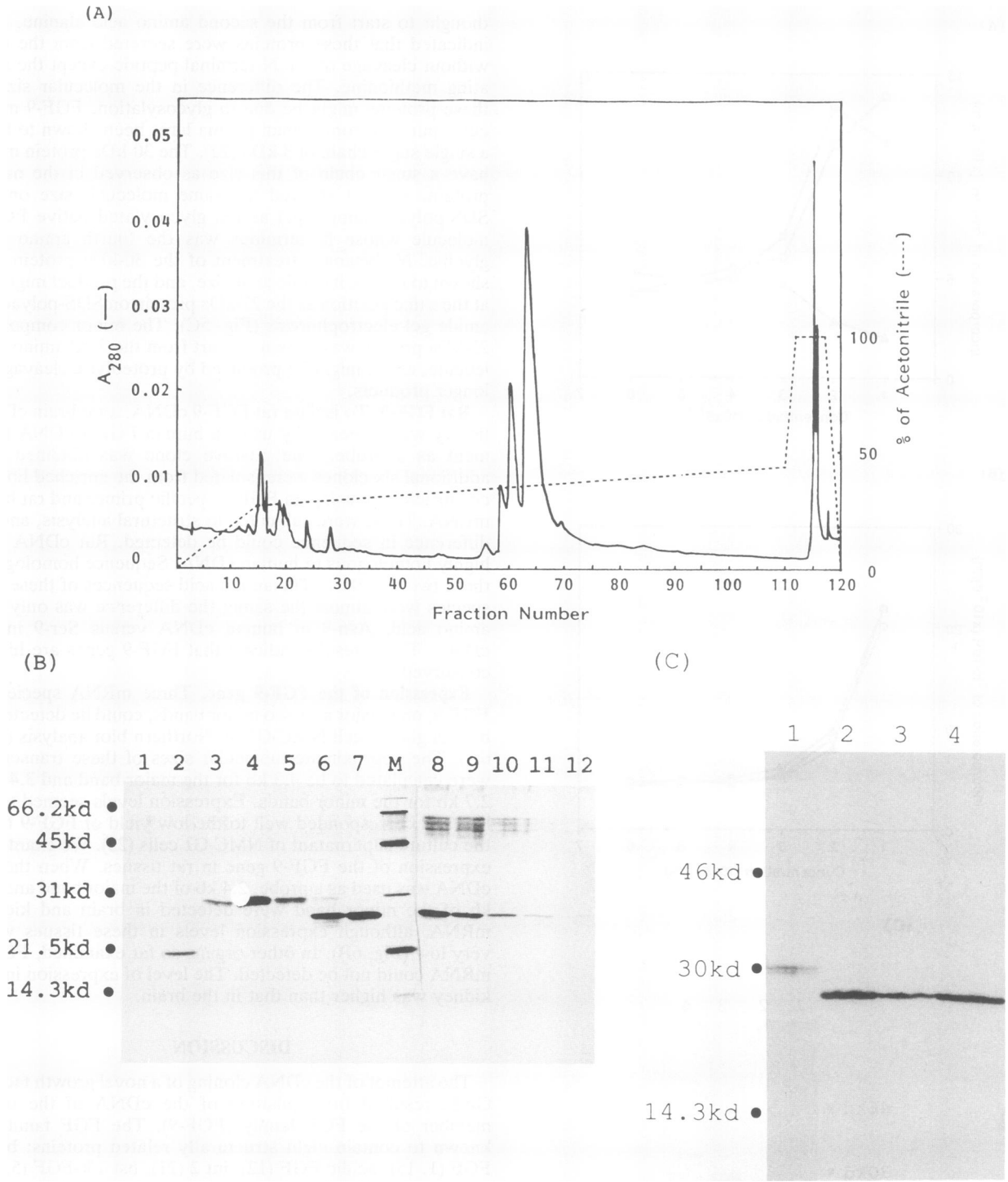

FIG. 5. Purification of FGF-9 produced in CDGAF4 cells. (A) Reverse-phase HPLC of heparin HPLC eluate; the final step of purification. Heparin HPLC-eluted fractions were applied to a Vydac C4 column (0.46 by 25 cm; Vydac), and proteins were eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid (0.8 ml/min/fraction). The effluent was monitored for A_{280}. The fractions were collected in tubes containing 20 μl of 5% (wt/vol) CHAPS and 100 μl of 0.5 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2). Acetonitrile in the fractions was removed by a Speed Vac Concentrator (model 290; Savant). The volume of each fraction was then adjusted to 0.5 ml with distilled water. (B) Gel electrophoresis pattern of eluted fractions. SDS-polyacrylamide gel electrophoresis of each fraction (3 μl) upon reverse-phase HPLC was carried out with a 12.5% polyacrylamide gel under reducing conditions. Proteins were stained by a silver-staining method. Lane M, low-molecular-size-marker proteins (Bio-Rad Laboratories) (bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; 50 ng each); lanes 1 through 12 are fractions 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, and 69, respectively. (C) *N*-glycanase treatment of the purified proteins. Samples were treated with *N*-glycanase as described in the text. The digested proteins were analyzed by SDS-polyacrylamide gel electrophoresis and detected by Western blotting using anti-FGF-9 N-terminal peptide antibody. Lanes 1 and 3, 30- and 27-kDa proteins without treatment; lanes 2 and 4, 30 and the 27-kDa proteins with *N*-glycanase treatment.

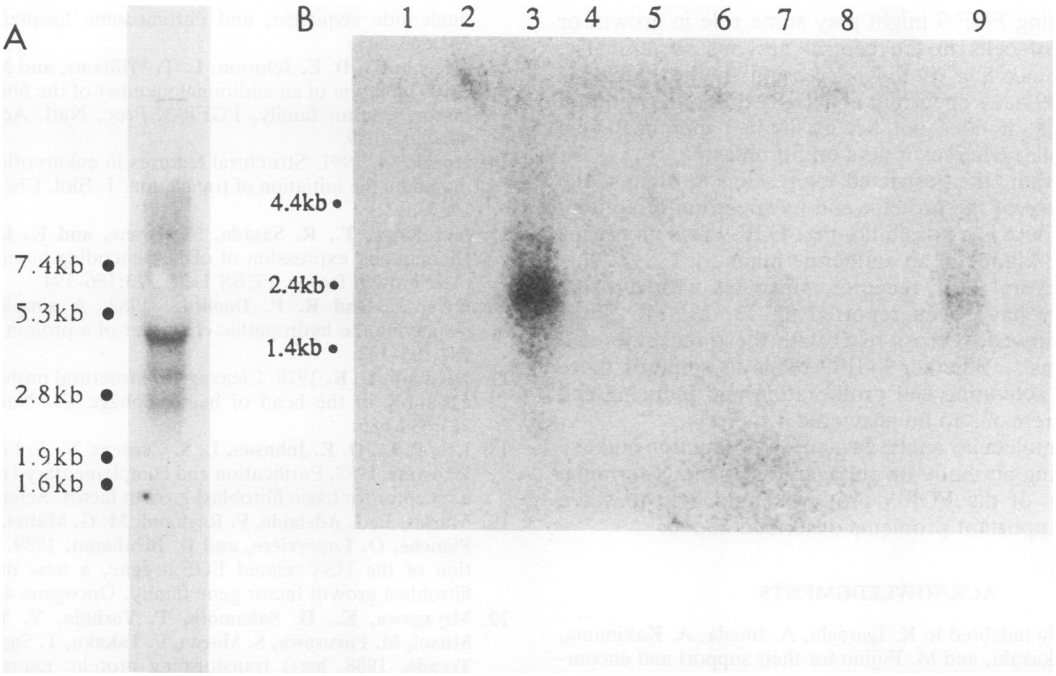

FIG. 6. Analysis of FGF-9 mRNA in a human glioma cell line and rat tissues. FGF-9 transcripts were detected by Northern blot analysis. Poly(A)$^+$ RNAs were prepared from human glioma cell line NMC-G1(A) and rat tissues (B). mRNAs (20 μg of NMC-G1 mRNA and 10 μg of rat tissue mRNAs) were size fractionated and blotted onto a nylon membrane. Tissues used (lanes): 1, liver; 2, lung; 3, kidney; 4, spleen; 5, thymus; 6, testis; 7, heart; 8, adrenal gland; 9, brain.

Basic FGF and acidic FGF have been well characterized. They are potent modulators of cell proliferation, cell motility, differentiation, and survival and act on cell types from ectoderm, mesoderm, and endoderm. These two FGFs, along with KGF and AIGF, were initially identified by protein purification. However, the other four members were isolated as oncogenes (5, 9, 19, 21, 27, 32, 35, 38), expression of which was restricted to embryogenesis and certain types of cancers (20, 30, 33, 36, 37). Reports indicate that they are generally not found in adult tissues. FGF-9 was purified from the supernatant of an established cell line (NMC-G1) derived from a human glioma patient and was demonstrated to be a mitogen against glial cells. Members of the FGF family are reported to have oncogenic potency. FGF-9 cDNA showed transforming potency when transfected into BALB/c 3T3 cells (unpublished results). FGF-9 might therefore be involved in oncogenesis of NMC-G1 cells.

From the structural analysis of the cDNA, the primary translation product of FGF-9 was showed to consist of 208 amino acids. The sequence similarity with each family member was about 30%. FGF-9 lacks a typical signal sequence; hydrophobicity of the N-terminal 20 amino acids from the initiation methionine is low. It was reported that in some cases translation of basic FGF and int 2 might start at the upstream CUG codon producing a longer product which might be translocated differently from the original protein (2, 8, 25). Upstream from the FGF-9 cDNA coding region, an in-frame CTG codon was found only just ahead of the initiation codon. Introduction of point mutation into the initiating ATG codon to change it to a GTG codon resulted in the expression of no mitogenic activity when the mutated plasmid was transfected into COS cells. Thus, alternative initiation sites might not exist in the 5′ untranslated region, and translation was thought to start exclusively at the methionine codon indicated in Fig. 1, although the neighboring nucleotide sequences did not fit Kozak's criteria (14). The N-terminal structure of FGF-9 resembles that of two of the family members, basic FGF and acidic FGF. Other members have typical signal sequences and are thought to be secreted from cells. Basic FGF and acidic FGF are primarily cell-associated proteins and are regarded as extracellular matrix components. The pathway of release and sequestration of these factors is not well understood. However, the cDNA transfection experiment with COS cells clearly showed that FGF-9 was secreted from the cells. We could find few proteins in the cell lysate reactive to the anti-FGF-9 antibody. The protein product purified from a high-producer CHO cell line reacted with the antibodies raised against both the N-terminal and C-terminal peptides. The N-terminal sequence analysis of the proteins showed that no peptide was cleaved but the initiating methionine. From the analysis of purified native protein, FGF-9 was thought to have an N-linked carbohydrate chain, and it was ascertained from the presence of a N-glycosylation site in the sequence. Part of the proteins produced in CHO cells were showed to be glycosylated, and this indicated that FGF-9 might go through the constitutive ER/Golgi secretion pathway. In the FGF family, int 2, hst/kFGF, and FGF-5 are thought to have a carbohydrate chain (11, 20, 38), but these molecules are reported to have typical signal sequences. Whether other unknown internal signal sequences or a novel secretion pathway exist is left unclear. The presence of a functional signal sequence in the N-terminal domain of the FGF-9, which is uncleavable in the secretion step, has not been eliminated.

FGF-9 was originally isolated from a glioma cell line as the growth-promoting factor for glial cells (22). By Northern blot analysis, the FGF-9 gene was revealed to be expressed in the

brain, suggesting FGF-9 might play some role in growth or maintenance of cells in the central nervous system. The physiological meaning of the expression in the kidney is unclear. FGF-9 has a spectrum of activity different from that of other FGFs; it does not act on human umbilical vein endothelial cells, whereas it acts on fibroblasts.

Taken together, the restricted expression in organs, the secretion nature of the protein, and its spectrum of activity indicate that there is a possibility that FGF-9 acts on organs other than the kidney in an endocrine manner.

Recently several FGF receptor molecules with tyrosine kinase activity have been reported (6, 13, 18, 24). Some FGFs were showed to cross-react with these receptors and transduce signals. Whether FGF-9 binds to some of these receptors for activating cell proliferation and inducing cell development remains to be answered.

Additional molecular analyses such as production and use of a neutralizing antibody or mutagenesis in the N-terminal or other parts of the FGF-9 protein should help to solve many of the important problems described above.

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