Cloning of an intracellular protein that binds selectively to mitogenic acidic fibroblast growth factor

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In addition to its extracellular action, there is evidence that acidic fibroblast growth factor (aFGF) acts inside cells. To identify intracellular proteins interacting with aFGF, we screened a HeLa cell library in the yeast two-hybrid system using pLex-aFGF as a bait. A clone binding to aFGF, but not to the non-mitogenic mutant aFGF-K132E, was isolated and characterized. The insert contained an open reading frame corresponding to a novel protein of 42 kDa. The protein, termed aFGF intracellular binding protein (FIBP), is mainly hydrophilic and does not contain an N-terminal signal sequence. *In vitro*-translated FIBP bound specifically to a fusion protein of maltose-binding protein and aFGF. FIBP became post-translationally associated with microsomes added to the cell-free protein synthesizing system, and the membrane-associated protein bound aFGF with high efficiency. Immunoblots and fluorescence microscopy demonstrated that the protein is present in nuclei and, to a lesser extent, associated with mitochondria and other cytoplasmic membranes. The possibility is discussed that FIBP may be involved in the mitogenic action of aFGF.

INTRODUCTION

Acidic fibroblast growth factor (aFGF) acts on a variety of cells either by stimulating mitogenesis or by inducing morphological changes and differentiation [1–3]. There is evidence that the growth factor has both an extracellular and an intracellular mode of action [4–7].

The extracellular action consists of binding to receptors at the cell surface. Best characterized are transmembrane FGF receptors (FGFRs) that contain a split cytoplasmic tyrosine-kinase domain. There are four known FGFR genes (*FGFR1–4*) and, at least in the case of *FGFR1–3*, there are a number of splicing variants [8–11]. Upon binding of aFGF to these receptors, cross-linking of the receptors activates the intracellular tyrosine-kinase domain, thus stimulating cross-phosphorylation of the receptors. This activates intracellular signalling cascades, such as activation of phospholipase Cγ, and the phosphorylation cascade resulting in phosphorylation of mitogen-activated protein kinase, and, in some cases, transcription of early genes, such as c-*fos* [12,13]. As a result, the cells are induced to mitogenesis or differentiation, depending on the cell type. It is not known what differentiates between these two kinds of cellular response.

A specific binding protein without a cytoplasmic kinase domain (cysteine-rich receptor [14]) has also been described. Furthermore, the growth factor binds to cell-surface heparans. In contrast to the binding to the other receptors, the binding to surface heparans can be inhibited by the addition of heparin to the medium, or by treating the cells with a high-salt buffer [1]. The role of the binding to surface heparans may be to increase the local concentration of aFGF at the cell surface and, in addition, to assist in crosslinking of the transmembrane FGFR [16,17].

Recent evidence indicates that the growth factor also acts inside cells [4,5,7,18]. Thus, after binding to FGFR1 or FGFR4, the growth factor is able to enter the cytosol and the nucleus. When the growth factor was translocated as a fusion protein with diphtheria toxin into cells lacking FGFR, it entered the nucleus and stimulated DNA synthesis, but not cell proliferation [5]. Further experiments led to the conclusion that for proliferation to occur, translocation of the growth factor into the cells and to the nucleus, as well as activation of the tyrosine kinase of the FGFR, are required [7].

aFGF is synthesized as an intracellular protein lacking a signal sequence [1]. A certain amount of the growth factor is transported out of the cells by a mechanism that is so-far largely unknown [19,20], but which could be common for several exported proteins that do not contain a signal sequence, such as basic FGF (bFGF), interleukin 1α and 1β, ciliary neurotropic factor, HIV-TAT (transactivator of HIV-TAR), annexins, galectins and others [21–26].

A mutant aFGF has been characterized [27] that does not induce mitogenesis, except at high concentrations, but it may induce cell differentiation [28]. In spite of this, it binds to the receptors and activates the tyrosine kinase, resulting in induction of c-*fos*, c-*jun* and c-*myc* and tyrosine phosphorylation of phospholipase Cγ [27]. The mutation consists in changing a Lys to a Glu residue at an exposed loop in the aFGF molecule (aFGF-K132E). As a consequence, the growth factor binds less strongly to heparin and surface heparans, whereas the binding to FGFR is not reduced [27,29]. Upon binding to FGFR, aFGF-K132E is transported into the cells and to the nucleus, apparently in the same way as aFGF [29]. Cells transfected with the mutant did not acquire the elongated, polar phenotype seen in cells transfected with the wild-type growth factor [27]. We therefore reasoned that there might be an intracellular protein involved in regulation that binds aFGF, but not aFGF-K132E.

In an attempt to identify such a protein, we decided to use the yeast two-hybrid system [30,31] to search for proteins that bind to wild-type aFGF, but not to the aFGF-K132E mutant. We report here the identification of a protein with this property, which appears to be localized mainly in the nucleus.

Abbreviations used: aFGF, acidic fibroblast growth factor; FIBP, aFGF intracellular binding protein; FGFR, fibroblast growth factor receptor; bFGF, basic fibroblast growth factor; MBP, maltose-binding protein; ER, endoplasmic reticulum.

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The nucleotide sequences described in this paper have been submitted to GeneBank database under accession numbers AF010187 (human FIBP) and AF010188 (simian FIBP).

EXPERIMENTAL

Bacteria and yeast strains

Escherichia coli DH5α [F^-, *recA1*, *endA1*, *hsdR17*, *supE44*, *thi1*, *gyrA*, *relA1*] was the transformation recipient for all plasmid constructions. *E. coli* HB101 [F^-, *hsdS20*(*hsdR hsdM*), *recA13*, *proA2*, *leuB*, *leuB1*, *ara14*, *lacY1*, *galK2*, *xyl-5*, *mtl1*, *supE44*, *endA*, *rpsL20* (Str^r)] was used to recover expression plasmids from yeast.

The genotype of the *Saccharomyces cerevisiae* reporter strain L40 was *MATa*, *trp1*, *leu2*, *his3*, *LYS2::lexA-HIS3*, *URA3::lexA-lacZ*.

For the two-hybrid screening, pLexA-myc was constructed by cloning the linker CATGGCCCATATGGAACAAAAACTCATCTCAGAAGAGGATCTGAATTCCGGCCCTCGAGGGATCCTCTAGAGTCGACCTGCACGGGTATACCTTGTTTTTGAGTAGAGTCTTCTCCTAGACTTAAGGCCGGGAGCTCCCTAGGAGATCTCAGCTGG into the *Nco*I/*Pst*I sites of pLexA-Rab5 [32]. The plasmids pLexA-aFGF and pLexA-aFGF-K132E were obtained by cloning cDNAs encoding wild-type aFGF [7] and the aFGF-K132E mutant, respectively, between the *Nco*I and *Sal*I sites of pLexA-myc in-frame with the LexA coding sequence. The *myc*-epitope was lost in the process. For the screening, the reporter yeast strain L40 [31] was first transformed with pLexA-aFGF, using the lithium acetate-based protocol [33]. A 100 ml log-phase culture of L40 harbouring pLexA-aFGF was subsequently transformed with 50 μg of a HeLa cDNA library in pGAD GH (Clontech, Palo Alto, CA, U.S.A.), and then grown overnight in 100 ml of synthetic medium lacking tryptophan and leucine. Following this, the yeast was plated on 109 cm^2 plates containing synthetic medium without leucine, tryptophan or histidine. Colonies were picked 4.5 days after plating and tested for β-galactosidase activity, first using replica plate assay [31] and then in liquid culture [34]. Library plasmids from positive clones were rescued into *E. coli* HB101 cells plated on leucine-free medium and subsequently analysed by DNA sequencing and in co-transformation experiments.

DNA-sequence and protein-homology analyses

cDNA encoding aFGF intracellular binding protein (FIBP) from HeLa and Vero cells was sequenced using the dideoxynucleotide method and Sequenase 2.0 according to the manufacturer's protocol (U.S. Biochemical, Cleveland, OH, U.S.A.). The predicted protein sequences were used to search PIR, SWISS-PROT and PROSITE databases by FASTA and BLAST programs.

cDNA library screening

The identification of the FIBP Vero clone was carried out by *in situ* hybridization to bacterial colonies originating from a pCEPS-1 cDNA library from Vero cells [35]. Bacterial colonies were transferred to Hybond™-N nylon membrane (Amersham, Little Chalfont, Bucks, U.K.), lysed [2 × 0.15 M NaCl/0.015 M sodium citrate (2 × SSC)/5 % SDS] and baked in a microwave oven for 3 min to fix the bacterial DNA to the membrane. The hybridization was performed overnight at 65 °C with [^{32}P]dCTP (Amersham)-labelled, random-primed (Random Primers DNA Labelling System, Gibco BRL, Gaithersburg, MD, USA) cDNA for FIBP from HeLa cells as specific DNA probe.

Generation of 5′ FIBP sequences by PCR amplification

The 5′ end of the human FIBP was obtained by PCR amplification starting with an Rch (human lymphocyte) library from human leukaemia cells by priming with a T7 forward primer (5′-AATACGACTCACTATAC-3′) annealing to the pCDM8 vector containing the Rch library, and a reverse primer (5′-AGATGAGCTGGTGCAGTAGC-3′) complementary to residues 226–246 of the FIBP coding sequence.

Northern-blot hybridization

A human multiple-tissue Northern blot (Clontech MNT blot I) containing 2 μg per lane of poly(A^+) RNA was probed with a [^{32}P]dCTP-labelled, random-primed DNA probe using either the 300 bp 5′ terminal fragment of the HeLa clone of FIBP or the *Bam*HI–*Eco*RI fragment of human aFGF cDNA as templates.

The blot was hybridized overnight in 5 × [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA] (SSPE)/Denhardt's solution/100 mg/ml salmon sperm DNA/50 % formamide/2 % SDS containing 2×10^6 c.p.m./ml of the probe, washed twice with 2 × SSC/0.1 % SDS for 15 min at room temperature, followed by one wash with 2 × SSC/0.1 % SDS at 50 °C. Membranes were exposed to X-Omat AR film (Kodak, New York, NY, U.S.A.) at −70 °C using an intensifying screen.

In vitro transcription and translation

DNA was linearized downstream of the coding sequence and transcribed in 20 μl of reaction mixture with T3 or T7 RNA polymerase as described elsewhere [36]. The mRNA was precipitated with ethanol and dissolved in 10 μl of H_2O containing 10 mM dithiothreitol and 0.2 units/μl RNAsin (Promega, Madison, WI, U.S.A.). The translation was carried out for 1 h at 30 °C in micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) using 5 μl of the dissolved mRNA per 100 μl of lysate and 1 μM [^{35}S]methionine (Amersham).

Binding to microsomal membranes

For binding to canine pancreatic microsomal membranes (Promega) the transcription of mRNA was carried out in 25 μl of rabbit reticulocyte lysate in the presence of 1.25 μl microsomal membranes and 1 μM [^{35}S]methionine for 1 h at 30 °C. In some cases microsomal membranes were added post-translationally in the presence of 10 μg/ml of cycloheximide and the incubation was continued for 30 min more at 30 °C. The samples were centrifuged at 60000 revs./min (130000 ***g***) for 30 min at 4 °C. The membrane pellets were resuspended in JCT buffer (78 mM KCl/4 mM $MgCl_2$/83.7 mM $CaCl_2$/10 mM EGTA/1 mM dithiothreitol/50 mM Hepes/KOH, pH 7.2). Finally, the supernatants and the resuspended pellets were analysed by SDS/PAGE and fluorography [37].

In another series of experiments aFGF mRNA was used additionally in the translation mixture containing FIBP mRNA and microsomal membranes. As described above, in some cases the microsomes were added post-translationally.

Construction of maltose-binding protein (MBP)-fusion proteins and *in vitro* binding

To construct MBP-aFGF, HBGF-1α [4] cDNA was inserted between the *Nco*I and *Eco*RI sites in the polylinker region of pMal-cN [38]. MBP-aFGF-K132E was obtained in a similar way using PCR to generate a mutation changing Lys132 to Glu and simultaneously introducing a silent mutation, giving an *Stu*I site. MBP Il-1α was created by cloning into the polylinker region of pMal-cN a PCR fragment obtained using the synthetic human interleukin 1α cDNA (British Biotechnology, Abingdon, Oxon, UK) as template, 5′-ATTGCCCATGGGCGCGCCTTTCTCCTTCCTG-3′ as the forward primer and 5′-CGTCGCTCTAGA-

TTACATGATAACGCATGCTTGGTTCTCGAAGGATCT-G-3′ as the reverse primer, and then digested with *Nco*I and *Xba*I. To construct MBP-bFGF the coding region for the 18 kDa form of bFGF [39] was inserted between the *Nco*I and the *Eco*RI sites in the polylinker region of pMal-cN. p121-EGFP-encoding green fluorescent protein in front of FIBP was obtained by cloning the coding sequence of FIBP cDNA into the *Bgl*II site of pEGFP-C1 (Clontech).

Expression of MBP fusions was induced by 0.3 M isopropyl-β-D-galactoside for 2 h. Then cells were harvested and resuspended in 25 ml of column buffer (20 mM Tris/HCl, pH 7.5/200 mM NaCl/1 mM EDTA), frozen at -20 °C and thawed in cold water. The cells were broken by sonication and centrifuged for 30 min at 9000 ***g***. The supernatant was diluted 1:5 with column buffer, loaded on to an amylose resin column (New England Biolabs, Beverly, MA, U.S.A.) and washed with 8 volumes of column buffer. Fusion proteins were eluted with 10 mM maltose in the same buffer. Protein concentrations were estimated by Bradford assay (BioRad, Hercules, CA, USA). Fusion proteins and ovalbumin were coupled covalently to Affi-Gel-10 (BioRad) to a final concentration of 1 mg/ml.

Affi-Gel-10 beads (30 μl) carrying MBP-fusion proteins were incubated for 3 h with 1 μl of *in vitro*-translated [^{35}S]methionine-labelled FIBP in 400 μl of PBS containing 0.05 % Triton X-100. Then the beads were washed 3 times with PBS containing 0.05 % Triton X-100 and once with H_2O. The protein bound to the beads was subjected to SDS/PAGE and visualized by fluorography.

Production and affinity purification of antibodies to FIBP

Antibodies against a fusion protein of MBP and a portion of FIBP (amino acids 57–372) were raised in rabbits. FIBP-specific antibodies were purified by affinity chromatography on an Affi-Gel-10 column with covalently bound MBP-FIBP. Material eluted from the column with 100 mM glycine (pH 2.8) was immediately neutralized with 3 M Tris/HCl (pH 8.8) and passed through an Affi-Gel-10 column containing bound MBP alone to remove MBP-specific antibodies. The flow-through fraction was collected and used in all immunocytochemistry and immunoblotting experiments.

Subcellular fractionation

Vero cells grown to $\approx$ 80 % confluence on 175 cm² Petri dishes were washed free of medium with PBS containing 5 mM EDTA, scraped from the dishes with a rubber policeman and recovered by centrifugation (130 ***g*** for 10 min). After resuspension in 2 ml of homogenization buffer [0.25 M sucrose/0.1 mM EDTA/20 mM Tris/HCl, pH 7.4/1 mM PMSF/200 units/ml aprotinin/1/50 tablet/ml of Protease Inhibitor Cocktail (Boehringer Mannheim, Mannheim, Germany)], the cells were again sedimented at 325 ***g*** for 7 min in a refrigerated microcentrifuge. Cells were then kept on ice for 5 min in 0.75 ml of hypotonic buffer (20 mM HCl, pH 7.4/0.1 mM EDTA/15 mM KCl). After addition of an equal volume of 2 × concentrated homogenization buffer, the cells were disrupted by 10 passages through a 22-gauge needle fitted to a 1 ml syringe and monitored by phase-contrast microscopy to ensure that the nuclei appeared intact and free of cytoplasmic material. Cell debris was removed by a 500 ***g*** spin, and the lysate was subjected to a 780 ***g*** centrifugation for 10 min to sediment the nuclei. The post-nuclear supernatant was collected. The nuclear pellet was washed twice by resuspension in lysis buffer (PBS containing 10 mM EDTA, 1 % Triton X-100), and once in the same buffer containing 0.3 M sucrose and then resuspended in 0.2 ml of lysis buffer containing 0.3 M sucrose, layered over 0.8 ml of 0.7 M sucrose in lysis buffer and centrifuged at 800 ***g*** for 15 min at 4 °C.

The post-nuclear supernatant was centrifuged at 9300 ***g*** for 10 min to sediment mitochondria. The post-mitochondrial supernatant was collected. The mitochondrial pellet was washed twice by resuspension in homogenization buffer adjusted to contain 0.4 M sucrose and centrifuged at 6000 ***g*** for 10 min. The post-mitochondrial supernatant was centrifuged at 15000 ***g*** for 10 min to obtain the heavy microsomal fraction.

Immunofluorescence microscopy

For indirect immunofluorescence experiments, COS-1 cells were seeded out on sterile coverslips, transiently transfected with cDNA for the indicated proteins in pcDNA3 using FuGENE™ transfection reagent (Boehringer Mannheim) and grown for 48 h. Then cells were rinsed briefly with PBS, submerged in fixative (3 % paraformaldehyde in PBS) for 20 min at room temperature, permeabilized with 0.5 % Triton X-100 in PBS and incubated in PBS containing 5 % non-fat dry milk powder and 40 μg/ml affinity-purified anti-FIBP, as well as 5 μg/ml monoclonal antibodies (9E10) against the *myc*-epitope [40]. Secondary antibodies were 5 μg/ml of FluoroLink™ Cy2™-labelled goat anti-mouse IgG (Amersham) and 3 μg/ml of Cy3™-conjugated AffiniPure goat anti-rabbit IgG(H + L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.). Immunofluorescence images were collected on a confocal scanning microscope (Leica Lasertechnik GmbH) and were processed using Adobe Photoshop software (Adobe Photosystems, Inc., Mountain View, CA, U.S.A.).

Immunoblotting

Nuclear and membrane fractions from Vero cells, obtained by differential centrifugation, were either left untreated or treated by pronase (3 mg/ml) for 10 min, boiled with an equal volume of SDS sample buffer (0.25 M Tris/HCl, pH 6.8/40 % glycerol/20 % mercaptoethanol/8 % SDS), separated on a 10 % SDS polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated overnight in blocking buffer (0.5 % Tween in PBS containing 5 % non-fat dry milk powder), and then incubated with anti-FIBP (1:200) and subsequently with anti-calreticulin (1:1000; StressGen Biotechnologies, Victoria, BC, Canada). Binding of the primary antibodies was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG and Super Signal CL-HRP-labelled goat anti-mouse IgG (Pierce, Rockford, IL, U.S.A.).

RESULTS

Two-hybrid screening for proteins interacting with wild-type aFGF, but not with a mutant aFGF unable to stimulate DNA synthesis

In order to identify proteins interacting with aFGF, we screened a HeLa cell library, using the yeast two-hybrid system [30]. Reporter yeast cells were first transformed with a plasmid encoding a C-terminal fusion of aFGF with the bacterial repressor protein LexA, which recognizes specific operator sequences upstream of the two reporter genes, *HIS3* and *lacZ* [31]. The resulting strain was subsequently transformed with a HeLa cell cDNA library fused with the transcriptional activation domain of the yeast transcription factor, Gal4 [30]. Library plasmids rescued from positive clones, which were able to grow on histidine-free medium as well as possessing β-galactosidase activity, were transformed into reporter yeast containing three different LexA fusions. Whereas aFGF was the positive control,

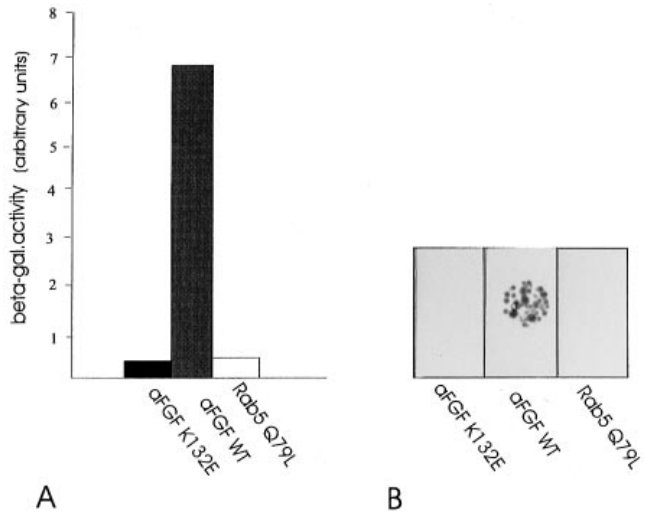

Figure 1 Specific interaction between aFGF and clone 121 in the yeast two-hybrid system

(**A**) β-galactosidase activity induced by *in vivo* interactions between clone 121 and aFGF. Yeast strains were co-transformed with the rescued pGAD clone 121 and plasmids containing LexA fusions of the proteins indicated. The cells were grown in synthetic media lacking tryptophan and leucine; liquid culture β-galactosidase activity assay was performed using *o*-nitrophenyl-β-D-galactopyranoside (Sigma) as substrate [54]. (**B**) *HIS3* reporter gene activation caused by specific interaction between clone 121 and aFGF in the two-hybrid system. L40 reporter yeast cells co-transformed as above were spotted on agar containing synthetic medium lacking tryptophan, leucine and histidine. Colonies were photographed after 3 days of incubation at 30 °C.

the aFGF-K132E mutant was used as a negative control. This mutant binds with high affinity to aFGF receptors and stimulates receptor tyrosine phosphorylation, but it is unable to stimulate DNA synthesis [27]. Rab5 Q79L, a GTPase-deficient mutant of the small GTPase Rab5, was selected as a second negative control because it has an isoelectric point close to that of aFGF and interacts strongly with its effector, rabaptin-5, in the two-hybrid system [32]. Neither of the plasmids were found to cause reporter gene stimulation by itself (results not shown).

Out of 1.4×10^5 clones screened, one clone, 121, was found to interact with aFGF, but not with Rab5 Q79L or aFGF-K132E, determined by its ability to induce expression of β-galactosidase (Figure 1A). Also, it did not interact with bFGF (results not shown). The same clone was able to grow on histidine-free medium (Figure 1B) in the presence of pLexA-aFGF, but not in the presence of pLexA-aFGF-K132E or pLexA-Rab5 Q79L.

Analysis of the positive clone

The plasmid recovered from the positive clone was found to contain a 1.2 kb insert. DNA sequencing demonstrated that the major part represented an open reading frame not found in sequence databases. Since the possibility existed that the sequence was incomplete at the 5′ end, we amplified the cDNA extending further in the 5′ direction, using a human cDNA library as template and an antisense primer based upon the 5′ end of the cloned sequence, to establish the complete coding sequence. The amplified sequence extended the open reading frame by 12 amino acids and contained a Kozak translation-initiation consensus site [41]. There was an in-frame stop codon 94 bp upstream of the

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CTAGAATTCAGCGACCGCTTTTTTTTTTTTTTGCGGCGCGCCAGTCCCGAGCAGTGCTCGCTCCTGCTCGGGGCGGTGCGGCCCCGGGCGTCGCCATGACC 100
                                                                                                  M  T

AGTGAGCTGGACATCTTCGTGGGGAACACGACCCTTATCGACGAGGACGTGTATCGCCTCTGGCTCGATGGTTACTCGGTGACCGACGCGGTGGCCCTGC 200
 S  E  L  D  I  F  V  G  N  T  T  L  I  D  E  D  V  Y  R  L  W  L  D  G  Y  S  V  T  D  A  V  A  L

GGGTGCGCTCGGGAATCCTGGAGCAGACTGGCGCCACGGCAGCGGTGCTGCAGAGCGACACCATGGACCATTACCGCACCTTCCACATGCTCGAGCGGCT 300
R  V  R  S  G  I  L  E  Q  T  G  A  T  A  A  V  L  Q  S  D  T  M  D  H  Y  R  T  F  H  M  L  E  R  L

GCTGCATGCGCCGCCCAAGCTACTGCACCAGCTCATCTTCCAGATTCCGCCCTCCCGGCAGGCACTACTCATCGAGAGGTACTATGCCTTTGATGAGGCC 400
  L  H  A  P  P  K  L  L  H  Q  L  I  F  Q  I  P  P  S  R  Q  A  L  L  I  E  R  Y  Y  A  F  D  E  A

TTTGTTCGGGAGGTGCTGGGCAAGAAGCTGTCCAAAGGCACCAAGAAAGACCTGGATGACATCAGCACCAAAACAGGCATCACCCTCAAGAGCTGCCGGA 500
 F  V  R  E  V  L  G  K  K  L  S  K  G  T  K  K  D  L  D  D  I  S  T  K  T  G  I  T  L  K  S  C  R

GACAGTTTGACAACTTTAAACGGGTCTTCAAGGTGGTAGAGGAAATGCGGGGCTCCCTGGTGGACAATATTCAGCAACACTTCCTCCTCTCTGACCGGTT 600
R  Q  F  D  N  F  K  R  V  F  K  V  V  E  E  M  R  G  S  L  V  D  N  I  Q  Q  H  F  L  L  S  D  R  L

GGCCAGGGACTATGCAGCCATCGTCTTCTTTGCTAACAACCGCTTTGAGACAGGGAAGAAAAAAACTGCAGTATCTGAGCTTCGGTGACTTTGCCTTCTGC 700
  A  R  D  Y  A  A  I  V  F  F  A  N  N  R  F  E  T  G  K  K  K  L  Q  Y  L  S  F  G  D  F  A  F  C

GCTGAGCTCATGATCCAAAACTGGACCCTTGGAGCCGTCGACTCACAGATGGATGACATGGACATGGACTTAGACAAGGAATTTCTCCAGGACTTGAAGG 800
 A  E  L  M  I  Q  N  W  T  L  G  A  V  D  S  Q  M  D  D  M  D  M  D  L  D  K  E  F  L  Q  D  L  K

AGCTCAAGGTGCTAGTGGCTGACAAGGACCTTCTGGACCTGCACAAGAGCCTGGTGTGCACTGCTCTCCGGGGAAAGCTGGGCGTCTTCTCTGAGATGGA 900
E  L  K  V  L  V  A  D  K  D  L  L  D  L  H  K  S  L  V  C  T  A  L  R  G  K  L  G  V  F  S  E  M  E

AGCCAACTTCAAGAACCTGTCCCGGGGGCTGGTGAACGTGGCCGCCAAGCTGACCCACAATAAAGATGTCAGAGACCTGTTTGTGGACCTCGTGGAGAAG 1000
  A  N  F  K  N  L  S  R  G  L  V  N  V  A  A  K  L  T  H  N  K  D  V  R  D  L  F  V  D  L  V  E  K

TTTGTGGAACCCTGCCGCTCCGACCACTGGCCACTCAGCGACGTGCGGTTCTTCCTGAATCAGTATTCAGCGTCTGTCCACTCCCTCGATGGCTTCCGAC 1100
 F  V  E  P  C  R  S  D  H  W  P  L  S  D  V  R  F  F  L  N  Q  Y  S  A  S  V  H  S  L  D  G  F  R

ACCAGGCCTCTGGGACCGCTACATGGGCACCCTCCGCGGCTGCCTCCTGCGCCTGTATCATGACTGAGGTGCCTCCCAACCGTCCGCCCACGCTGACAAT 1200
H  Q  A  S  G  T  A  T  W  A  P  S  A  A  A  S  C  A  C  I  M  T  E  V  P  P  N  R  P  P  T  L  T  I

AAAGTTGCTCTGA 1213
  K  L  L  .
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Figure 2 DNA and amino acid sequences of human FIBP

The DNA sequence was determined by dideoxy sequencing of two clones, the first one isolated by the yeast screen from the activation-tagged HeLa expression library (residues 126–1213), and the second obtained from Rch cDNA library by PCR screening (residues 1–247).

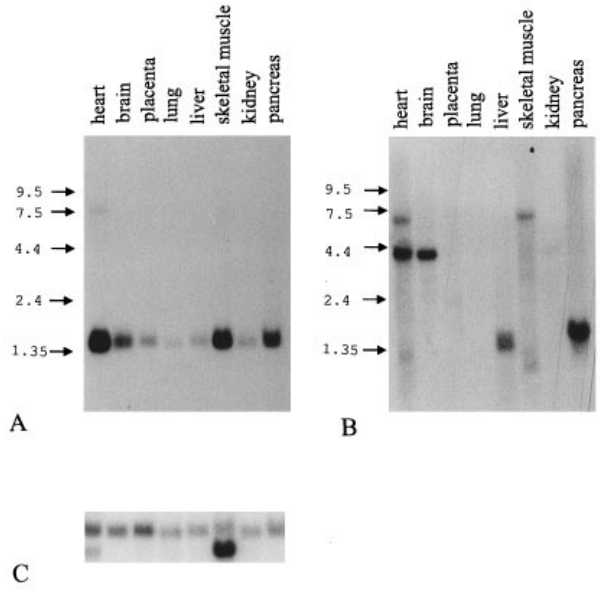

Figure 3 Expression of mRNA for FIBP (A), aFGF (B) and actin (C) in different human tissues

A multiple-tissue Northern blot containing 2 μg of poly(A$^+$) mRNA from the indicated tissues was hybridized with a [^{32}P]dCTP-labelled, random-primed FIBP probe, then stripped and hybridized with an aFGF probe, stripped again and hybridized with a probe for human β-actin.

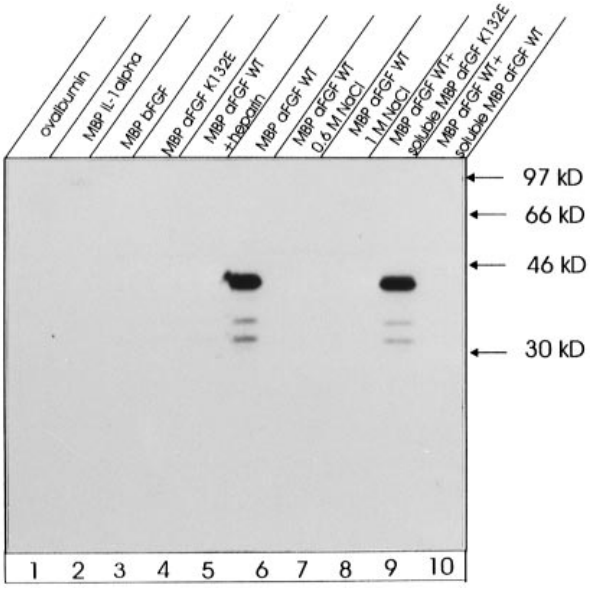

Figure 4 Interaction of FIBP and aFGF *in vitro*

Affi-Gel-10 with covalently bound ovalbumin (lane 1), or the fusion proteins MBP-IL-1α (lane 2), MBP-bFGF (lane 3), MBP-aFGF-K132E (lane 4) and MBP-aFGF (lanes 5–10) were incubated with *in vitro*-translated [^{35}S]methionine-labelled FIBP. In some cases the binding was carried out in the presence of the following additions: 10 units/ml heparin (lane 5), 0.6 M NaCl (lane 7), 1 M NaCl (lane 8), 60 μg/ml unlabelled soluble MBP-aFGF-K132E (lane 9) or 30 μg/ml unlabelled soluble MBP-aFGF (lane 10). The beads were then washed and subjected to SDS/PAGE and autoradiography.

initiation codon, indicating that we have obtained the full coding sequence. Protein database searches using BLAST and FASTA programs still did not reveal similarity with any known protein.

We also cloned the simian variant of the cDNA by colony screening of a Vero cell library. The obtained DNA sequence was of almost the same length as the extended human sequence. The two nucleotide sequences differed at a few positions without change in the amino acid sequence.

The complete open reading frame encodes a mainly hydrophilic protein of 372 amino acids, with a predicted molecular mass of $\approx$ 42 kDa (Figure 2) and a calculated pI of 6.6. Protein sequence analysis did not give any clue as to the biological function of the protein, which we termed FIBP. Also, the full-length FIBP bound selectively to aFGF in the two-hybrid system (results not shown).

Expression of FIBP and aFGF mRNA in different tissues

To determine the size and tissue distribution of FIBP mRNA, a Northern blot containing 2 μg of poly(A$^+$) RNA from different human tissues was hybridized with a FIBP probe. Only one RNA transcript with an apparent size of $\approx$ 1.4 kb was detected in all tissues. FIBP was highly expressed in heart, skeletal muscle and pancreas, and was somewhat less abundant in brain. It was also clearly detectable in placenta, liver and kidney, whereas very little was found in lung (Figure 3A).

When the membrane was stripped and hybridized with a probe corresponding to cDNA for human aFGF, at least six different transcripts were detected (Figure 3B). The largest transcript ($\approx$ 6 kb) was found in heart and skeletal muscle, whereas a somewhat smaller transcript ($\approx$ 4.2 kb) was present in heart, brain and, in low amounts, in kidney. This transcript may correspond to one described previously [42,43]. In addition, we detected 1.4 and 1.8 kb transcripts in liver and pancreas, respectively. In skeletal muscle several of the transcripts were found. Like the expression of FIBP, the strongest hybridization signal was found in heart, brain, skeletal muscle and pancreas, and little in kidney, placenta and lung. But, differently from the distribution pattern for FIBP mRNA, aFGF mRNA was abundant in liver.

The membrane was stripped again and hybridized with a probe for β-actin (Figure 3C, upper band). This mRNA was present to almost the same extent in all tissues tested, indicating that similar amounts of total RNA were present in the different lanes.

The observation that mRNAs for aFGF and FIBP are similarly expressed in most human tissues examined is consistent with the possibility that they interact functionally.

Specific binding of FIBP to aFGF *in vitro*

To test the association of FIBP with aFGF biochemically, *in vitro* binding assays were performed. In these experiments we tested the ability of different immobilized proteins to bind *in vitro*-translated, [^{35}S]methionine-labelled FIBP. An MBP-aFGF-fusion protein covalently coupled to Affi-Gel-10 readily bound FIBP (Figure 4, lane 6), whereas a fusion protein of MBP and the non-mitogenic mutant, aFGF-K132E, did not (lane 4). Also, there was no binding to a number of control proteins such as ovalbumin (lane 1), MBP-interleukin 1α (lane 2) and MBP-bFGF (lane 3). Whereas the presence of excess (60 μg) unlabelled aFGF-K132E was not able to prevent the binding of aFGF (lane 9), the presence of unlabelled aFGF at this concentration inhibited the binding completely (lane 10). No binding of FIBP was detected in the presence of heparin (lane 5), which is able to stabilize the conformation of aFGF and which interacts with the region of aFGF close to K132 [44–47].

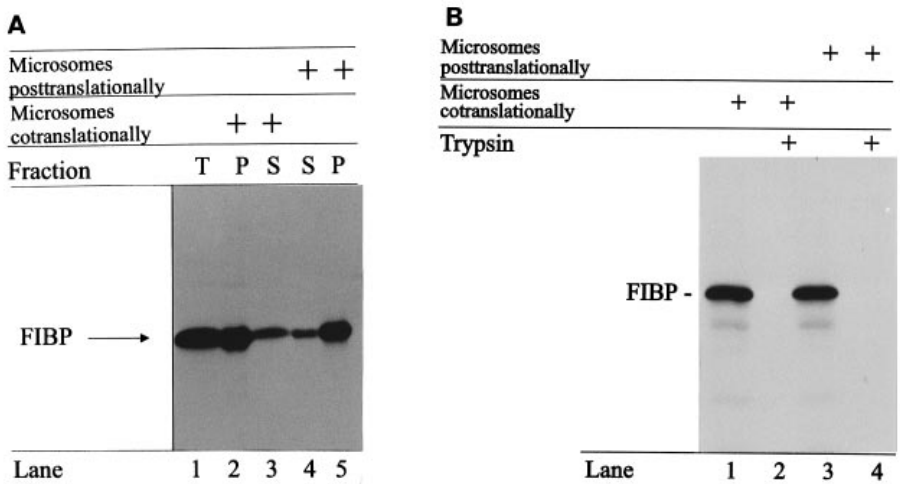

Figure 5 Ability of FIBP to associate with microsomal membranes *in vitro*

(**A**) FIBP was translated in a rabbit reticulocyte lysate. Canine pancreatic microsomal membranes were added either co-translationally (lanes 2 and 3) or post-translationally (lanes 4 and 5). In the latter case the samples were incubated for 30 min more at 30 °C. Subsequently, the translation mixtures were fractionated into a microsomal pellet (P) and a supernatant fraction (S). In one case (lane 1) the translation was in the absence of microsomes, and an aliquot of the total (T) translation mixture was analysed. (**B**) FIBP was translated as above, the microsomes were sedimented and then incubated in the absence and presence of trypsin. Subsequently, PMSF was added and the samples were analysed by SDS/PAGE and fluorography.

FIBP associates with, but does not translocate into, microsomal membranes *in vitro*

FIBP does not contain an N-terminal signal sequence, but, to test if it contains an internal signal sequence, we studied if it could be translocated into microsomes. *In vitro*-transcription of the FIBP cDNA was followed by translation in a rabbit reticulocyte lysate in the presence of canine pancreas microsomal membranes. This system is well established as efficient in directing integral, luminal and secreted proteins into the microsomes where they are completely or partially protected from digestion by exogenous protease [48,49].

Microsomal membranes were either present in the rabbit reticulocyte lysate co-translationally (Figure 5A, lanes 2 and 3) or added post-translationally (Figure 5A, lanes 4 and 5). To our surprise, after centrifugation the major part of the translated FIBP was in both cases recovered in the microsomal pellet (Figure 5A, lanes 2 and 5), and only a minor fraction was found in the supernatant (Figure 5A, lanes 3 and 4).

To test if FIBP was translocated into the microsomes or only attached to them, the microsomal pellet was treated with trypsin for 30 min. In this case no labelled material was obtained (Figure 5B, lanes 2 and 4), demonstrating that the membranes did not provide any protection for the labelled FIBP. Control experiments with mRNA for carboxypeptidase Y demonstrated transport into the microsomes as measured by glycosylation and pronase protection of the translation product (results not shown). Clearly therefore, the microsomes used were fully active. The data indicate that FIBP is able to become peripherally associated with microsomes and that this association is not linked to the translation process.

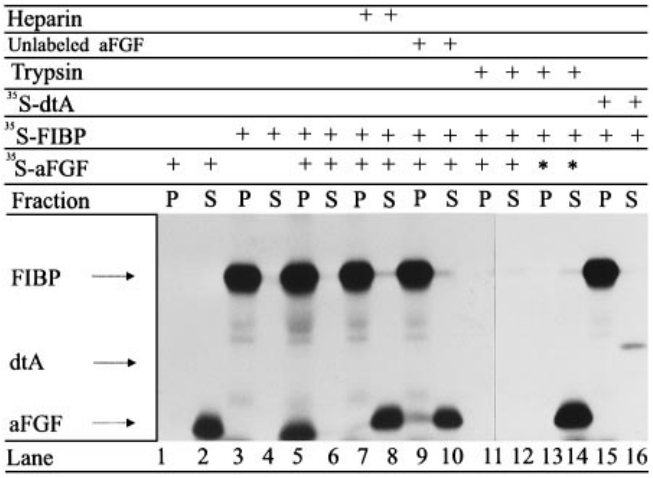

Figure 6 Ability of aFGF to bind FIBP associated with microsomal membranes

Translation of mRNAs encoding aFGF alone (lanes 1 and 2), FIBP alone (lanes 3, 4, 13 and 14), FIBP and aFGF (lanes 5–12) or FIBP and dtA (fragment A of diptheria toxin; lanes 15 and 16) was carried out in the presence of microsomal membranes. Certain samples were subsequently treated with trypsin for 30 min at 4 °C (lanes 11–14). Digestion was arrested by addition of 5 mM PMSF. To some samples, 10 units/ml heparin (lanes 7 and 8) or 10 μg of unlabelled aFGF (lanes 9 and 10) were added. In one case, [^{35}S]methionine-labelled aFGF was added *after* the trypsin digestion and incubated for 30 min at 30 °C (*, lanes 13 and 14). The incubation mixtures were fractionated into a microsomal pellet (P) and a supernatant fraction (S) and analysed by SDS/PAGE and fluorography.

Binding of aFGF to membrane-associated FIBP

To test whether membrane-associated FIBP binds aFGF, the two proteins were translated *in vitro*, either separately or together, in the presence of microsomal membranes. When translated alone, no aFGF was associated with microsomes (Figure 6, lanes 1 and 2), whereas all FIBP was associated with the microsomes (Figure 6, lanes 3 and 4). When the two proteins were translated together, essentially all aFGF was found in the microsomal pellet together with FIBP (Figure 6, lanes 5 and 6).

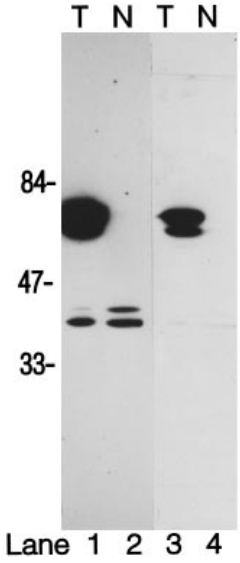

Figure 7 Characterization of antibodies raised against FIBP

COS-1 cells were transfected with p121-EGFP-encoding green fluorescent protein in front of FIBP. After 2 days the cells were collected and submitted to SDS/PAGE, the proteins were transferred to a PVDF membrane and probed with affinity-purified anti-FIBP. Lanes 1 and 3, transfected cells (T); lanes 2 and 4, non-transfected controls (N). Lanes 3 and 4 represent a shorter exposure of the same membrane as shown in lanes 1 and 2. The positions of molecular-mass markers (in kDa) are given to the left.

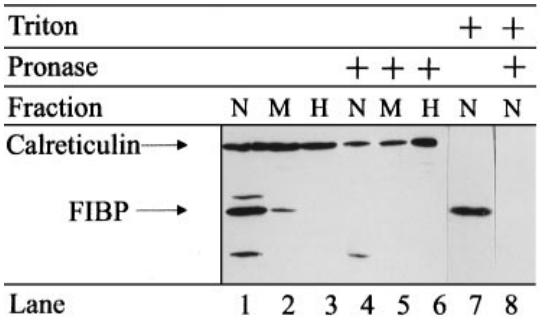

Figure 8 Distribution of FIBP in subcellular fractions of Vero cells

Cells were disrupted mechanically and subjected to differential centrifugation to obtain nuclear (N), mitochondrial (M) and heavy microsomal (H) fractions. In some cases the nuclear fraction was treated with lysis buffer containing 1% Triton X-100 to remove membrane material (lanes 7 and 8). Half of the fractions were incubated with pronase (3 mg/ml) for 10 min at 37 °C. The digestion was terminated by addition of 5 mM PMSF. Proteins in the different fractions were separated by SDS/PAGE and blotted on to a PVDF membrane. The blot was probed with anti-calreticulin and anti-FIBP antibodies.

Heparin (Figure 6, lanes 7 and 8) and excess of unlabelled aFGF (Figure 6, lanes 9 and 10) added to the translation mixture prevented the binding of aFGF to the microsomes and directed it to the supernatant instead. No translocation of FIBP or aFGF into the interior of microsomal vesicles was observed, since both proteins were easily degraded by treating the translation mixture with trypsin post-translationally (Figure 6, lanes 11 and 12). When labelled aFGF was added after the trypsin treatment, the growth factor was unable to bind to the microsomes (Figure 6, lanes 13 and 14). Fragment A of diphtheria toxin, used as a negative control, exhibited no microsomal binding in the presence of FIBP (Figure 6, lanes 15–16). It therefore appears that FIBP is able to become attached to microsomal membranes in such a way that it is capable of binding aFGF with high efficiency.

Subcellular localization of FIBP

To study the subcellular localization of FIBP, we produced antibodies using a fusion protein of MBP and the major part of FIBP (amino acids 53–372) to immunize rabbits. Anti-FIBP was obtained by immunopurification of the serum on immobilized MBP-FIBP followed by passing the eluted antibodies through a column with immobilized MBP alone. On immunoblots with total cell lysate from COS-1 cells, the antibodies recognized two bands migrating corresponding to 38 and 41 kDa (Figure 7, lanes 2 and 4). Presumably, the more slowly migrating band corresponds to the full-length FIBP, since it migrates like *in vitro*-synthesized FIBP (Figure 4). The 38 kDa band probably represents a proteolytically cleaved or otherwise modified form of the same protein.

To test the specificity of the antibodies, COS-1 cells were transfected with a plasmid encoding a fusion protein of green fluorescent protein linked to the N-terminus of full-length FIBP, and total cell lysate was analysed by Western blotting and probed with the anti-FIBP antibodies. In this case, two additional bands of $\approx$ 70 kDa were obtained (Figure 5, lanes 1 and 3), as expected for fusion proteins of green fluorescent protein (27 kDa) and the full-length and the processed form of FIBP.

The affinity-purified anti-FIBP antibodies were used to study the presence of FIBP in different subcellular fractions obtained after differential centrifugation of mechanically disrupted cells. The major part of the FIBP was found in the nuclear pellet (Figure 8, lane 1), whereas a smaller amount was present in the mitochondrial pellet (lane 2) and in some cases also in the microsomal fraction (results not shown).

We also tested the distribution of calreticulin, a marker protein for the endoplasmic reticulum (ER). As shown in Figure 8, lanes 1–3, calreticulin was present not only in the heavy microsomal fraction, but also in the mitochondrial and nuclear fractions. This demonstrates that both the nuclear and mitochondrial fractions contain ER membranes.

To study further the topology of FIBP, we treated the subcellular fractions with pronase. Calreticulin, which is a known luminal resident of ER [50], and therefore not exposed to pronase, was protected in all fractions (Figure 8, lanes 4–6). FIBP on the other hand was not protected from degradation by exogenous protease (Figure 8, lanes 4–6 and 8). It therefore appears that FIBP associated with membranes is bound externally. The finding that it also disappeared from the nuclear fraction can be explained by the small size of the proteases allowing them to enter through the nuclear pores.

To investigate whether FIBP is firmly bound in the nucleus, the nuclear pellet was washed with 1% Triton X-100 to dissolve the membranes. The amount of FIBP in the nuclear fraction was only moderately reduced by this treatment, whereas calreticulin was now completely absent (Figure 8, lane 7). Subsequent treatment with pronase completely degraded the FIBP (Figure 8, lane 8). It may be concluded that FIBP found in the nuclei is not due to contamination with cytoplasmic membrane structures, and that in fact the major part of FIBP is present in or associated with the nuclei as such.

We also carried out immunofluorescence experiments on

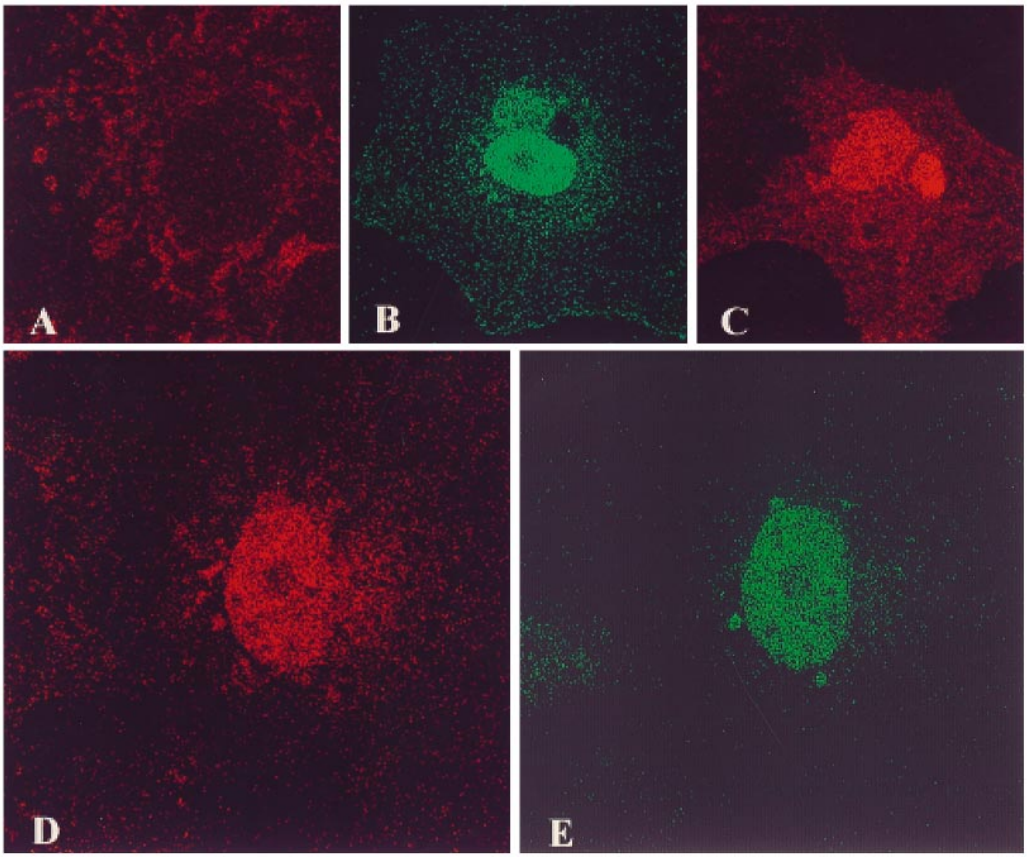

Figure 9 Localization of FIBP and aFGF in COS-1 cells

Cells were grown on coverslips, transfected with pcDNA3 alone (**A**), *myc*-tagged FIBP in pcDNA3 (**B**, **C**) or co-transfected with both FIBP and *myc*-tagged wild-type aFGF (**D**, **E**), fixed in 3% paraformaldehyde, permeabilized and labelled with polyclonal affinity-purified FIBP-specific antibody (**A**, **C**) or monoclonal antibodies against *myc*-epitope (**B**) or double-labelled with both (**D**, **E**). Subsequently, the cells were stained with fluorescent secondary antibodies [goat anti-rabbit IgG (H + L)–Cy3™ (**A**, **C**, **D**) or goat anti-mouse IgG (H + L)–Cy2™ (**B**, **E**)] and examined using confocal microscopy.

untransfected and transfected COS cells. Confocal microscopic images of untransfected cells labelled with fluorescent anti-FIBP (Figure 9A) showed labelling of membranous structures in the cytoplasm, particularly of mitochondria identified by antibodies against a marker for mitochondria, cytochrome *c* oxidase subunit 1 (results not shown). In addition, we could observe a somewhat weaker labelling throughout the nucleus. When FIBP containing a *myc* tag was over-expressed in COS cells and the cells were stained with an antibody against the *myc* epitope, most of the protein was found in the nucleus (Figure 9B). This was also the case when the transfected cells were stained with anti-FIBP (Figure 9C).

With the available antibodies it was difficult to visualize aFGF even when over-expressed in COS cells. We therefore over-expressed aFGF labelled with an *myc* tag together with FIBP, which in this case did not have this tag. The cells were then double-stained with antibodies against the *myc* epitope as well as against FIBP. The results showed that FIBP (Figure 9D) as well as aFGF (Figure 9E) were found mainly in the nucleus and showed largely the same distribution. aFGF-K132E, which did not bind to FIBP, was also found in the nucleus (results not shown) in accordance with our earlier findings [29]. Clearly therefore, binding to FIBP does not target aFGF to the nucleus, but the two proteins may interact in the nucleus.

DISCUSSION

In the present paper we have identified a protein, FIBP, that binds mitogenic aFGF, but not a mutant growth factor that is unable to stimulate DNA synthesis. FIBP is mainly found in the nucleus and to a lesser extent associated with mitochondria and probably other membranes, such as the ER. In fact, when the protein was translated in a cell-free system, it readily associated with microsomes. Even when the microsomes were added *after* synthesis of FIBP, the protein was able to become associated with the membranes, indicating that the folded protein had affinity for microsomes.

When bound to microsomes in the cell-free system, FIBP readily binds wild-type aFGF, but not the mutant aFGF-K132E. Based on estimations from the amount of [^{35}S]methionine incorporated into the two proteins (Figure 6) and their content of methionine (on the assumption that the N-terminal methionine is removed co-translationally from both proteins [51,52], FIBP has nine methionines and aFGF has two), it appears that somewhat less aFGF than FIBP was synthesized in the cell-free system. Since essentially all aFGF was bound, the affinity between the two proteins must be high.

Trypsin and pronase completely removed FIBP from microsomes, mitochondria and nuclei. Since no protected fragment was seen, these data indicate that FIBP is only attached to the

membranes, and not inserted. In accordance with this, the protein does not contain an N-terminal signal sequence for transport into ER or mitochondria.

The binding of FIBP to aFGF was completely prevented by 0.6 M NaCl, suggesting that the binding is of an electrostatic nature. It was also inhibited by heparin. Heparin binds tightly to aFGF, inducing a more compact conformation of the protein [44] that could be the reason why it does not bind to FIBP. Another heparin-binding protein, bFGF, did not bind to FIBP, indicating that bridging of aFGF and FIBP by heparin molecules that could be present in the reticulocyte lysate is not causing the interaction.

Western-blot analysis of whole cells demonstrated that FIBP is present in two forms, with migration rates that differ by ≈ 3 kDa. It is not clear if this is due to a modification or to proteolytic cleavage. In the latter case, the cleavage must occur at the C-terminal end, since a fusion protein of green fluorescent protein added to the N-terminal end of FIBP was also expressed in the cells as two bands differing by ≈ 3 kDa.

FIBP appears to be widely distributed in the human body, but the amount of transcript varies considerably between organs. The gene is expressed in particularly high amounts in muscle, brain and pancreas. In all organs only a single transcript was detected. aFGF on the other hand is expressed as several different transcripts, but the relative amount of total message correlates largely with that of FIBP.

The role of FIBP in the cells is not clear. The fact that it is found on membrane structures both in the cytoplasm and in the nucleus is consistent with the possibility that it cycles between the two compartments. If so, it is unlikely that it is responsible for transport of aFGF into the nucleus, since the mutant aFGF-K132E is transported to the nucleus equally efficiently as the wild-type growth factor [29]. On the other hand it is possible that aFGF entering the nucleus could bind to FIBP and somehow act on initiation of DNA synthesis. Unfortunately, so far the sequence of FIBP has not given any clue as to its function.

It is not clear why anti-FIBP is less efficient at labelling FIBP in nuclei of non-transfected cells than in labelling membrane-associated material. Possibly the protein interacts with components in the nuclei, making them less accessible for the antibodies.

In the case of bFGF it has been found that the β-subunit of casein kinase 2 binds to the growth factor [53]. Possibly, the growth factor transports the kinase into the nucleus to modify proteins required for DNA synthesis. FIBP does not have a kinase domain and must therefore act differently. Since, in the untransfected cells that we have used, the growth factor is not visible on Western blots, although mRNA is detectable by reverse transcriptase PCR, there may be an excess of FIBP over aFGF. Possibly, the function of external aFGF entering the cells is to bind to FIBP, which may then activate a nuclear component involved in initiation of DNA synthesis.

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