Identification and characterization of an intracellular protein complex that binds fibroblast growth factor-2 in bovine brain

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The fibroblast growth factor (FGF) family is composed of polypeptides with sequence identity which signal through transmembrane tyrosine kinase receptors. We report here the purification from bovine brain microsomes of an FGF-2-binding complex composed of three proteins of apparent molecular masses 150 kDa, 79 kDa and 46 kDa. Only the 150 kDa and 79 kDa proteins bound FGF-2 in cross-linking and ligand-blotting experiments. Binding of FGF-2 to p79 is enhanced in the presence of calcium. Peptide sequences allowed the identification of p150 and the cloning of the cDNAs encoding p79 and p46. The deduced amino acid sequence of p79 reveals high similarity to those of gastrin-binding protein and mitochondrial enoyl-

CoA hydratase/hydroxyacyl-CoA dehydrogenase. p46 is similar to mitochondrial ketoacyl-CoA thiolase. Stable transfection of FR3T3 rat fibroblast cells with p79 cDNA analysed by electron microscopy following immunolabelling of ultra-thin cryosections revealed a localization of p79 in the secretory pathway, mainly in the endoplasmic reticulum and the Golgi region, where it is specifically associated with the molecular chaperone calnexin. *In vivo* a protein similar to the Golgi protein MG-160 forms a complex with FGF-2 and p79.

Key words: calnexin, endoplasmic reticulum, fatty acid β -oxidation, mitochondrial enzymes, targeting sequences.

INTRODUCTION

Fibroblast growth factor-2 (FGF-2) is a member of the FGF family, which currently includes 18 other proteins [1–3] and references therein). FGF-2 is a pleiotropic factor mediating various biological activities, such as proliferation and differentiation of a wide range of cells, wound healing and angiogenesis [3,4]. Multiple FGF-2 isoforms are translated from one AUG (155 amino acids) and three CUG (210, 201 and 196 amino acids) codons [5]. The AUG-initiated FGF-2 is cytoplasmic and found extracellularly [6], whereas the CUG-initiated forms are nuclear [7]. Different biological roles have been demonstrated for CUG- and AUG-initiated isoforms of FGF-2 [8].

The biological effects of exogenous FGF-2 are mediated by a family of high-affinity tyrosine kinase receptors and low-affinity heparan sulphate proteoglycan binding sites [9]. A protein binding FGF-1 and FGF-2 in the absence of heparin was also characterized in chick embryos by Burrus and co-workers [10,11]. This FGF-binding protein, named cysteine-rich FGF receptor (CFR), has an apparent molecular mass of 150 kDa from SDS/PAGE analysis, a large N-terminal extracellular domain, one transmembrane domain and 13 intracellular C-terminal amino acids devoid of tyrosine kinase activity. In these experiments CFR was co-purified with two other unidentified proteins of 70 kDa and 45 kDa [10]. These two proteins were associated with CFR when the latter was subjected to gel filtration or

purified by affinity chromatography on an anti-CFR–Sepharese gel [11]. Another FGF-2-binding protein was previously detected and partially purified from bovine brain membranes [12]. This protein, named heparin-binding FGF receptor (HB-FGFR), of apparent molecular mass 150 kDa, binds FGF-2 only in the presence of heparin [13]. We show herein that HB-FGFR is closely related to CFR. Two other proteins closely related to CFR were also identified: human and rat MG-160 [14,15] and mouse ESL-1 [16]. MG-160 is one of the most abundant proteins of the Golgi apparatus [15], and ESL-1 is a ligand for E-selectin, which participates in endothelial-leucocyte adhesion [17]. Both proteins are almost identical to CFR, and only 60–70 N-terminal amino acids differ among the three proteins. MG-160 binds FGF-2 [15]; however, the capacity of ESL-1 to bind FGFs has not yet been assessed. Unlike CFR, no other proteins have been reported to be associated with MG-160 or ESL-1 [14–16]. The exact role of this family of proteins in relation to FGF-2 remains unclear. Nevertheless, CFR was hypothesized to be involved in the targeting of intracellular FGF to the Golgi apparatus [18].

In the present work we identify a complex of three proteins of molecular mass 150 kDa (HB-FGFR), 79 kDa (p79) and 46 kDa (p46) which binds FGF-2 in bovine brain microsomes. The p79 cDNA sequence is almost identical with that of gastrin-binding protein (GBP) [19] and mitochondrial 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase (p79M; EC 1.1.1.35/4.2.1.17) [20]. The cDNA encoding p46 is identical to that

Abbreviations used: CFR, cysteine-rich fibroblast growth factor receptor; ER, endoplasmic reticulum; FGF, fibroblast growth factor; HB-FGFR, heparin-binding FGF receptor; GBP, gastrin-binding protein; MNN blot, human brain multiple nuclei Northern blot; MTN blot, human multiple tissue Northern blot; ORF, open reading frame; p79M, long-chain fatty acid mitochondrial enoyl-CoA hydratase/hydroxyacyl-CoA dehydrogenase; p79ER, endoplasmic reticulum homologue of p79M; p46M, long-chain fatty acid mitochondrial ketoacyl-CoA thiolase; rbFGF-2, recombinant bovine FGF-2; TS, targeting sequence.

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The nucleotide sequence data for bovine p79ER, p46M and p79M have been submitted to the EMBL/GenBank/DBJ Nucleotide Sequence Databases under accession nos. AJ222637 BTAJ637, AJ003066 BTAJ3066 and AJ003123 BTAJ3123 respectively.

encoding mitochondrial ketoacyl-CoA thiolase (p46M; EC 2.3.1.16) [20]. However, our studies reveal that p79, which binds FGF-2, is mainly localized in the endoplasmic reticulum (ER) and Golgi apparatus. Moreover, p79 is found *in vivo* in a complex with an MG-160/CFR-like protein and high-molecular-mass FGF-2 isoforms. The different subcellular localizations of MG-160/CFR-like proteins, p79 and FGF-2 may suggest their participation in a dynamic process involved in the regulation of intracellular FGFs.

EXPERIMENTAL

Materials

A bovine brain cDNA library in λ gt10 (BL1027a), bovine brain poly(A)⁺ mRNA, human brain multiple nuclei Northern (MNN) blots and human multiple tissue Northern (MTN) blots were purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). All radiochemicals were from ICN (Costa Mesa, CA, U.S.A.). Recombinant FGF-2 was from Synergen (Boulder, CO, U.S.A.) or was purified as described [21].

Protein purification and peptide sequence determination

Proteins were purified from bovine brain as previously described [13], except that the DEAE-Sepharose flow-through was adsorbed on a wheat-germ-lectin-Sepharose gel. The adsorbed material was eluted by 0.3 M *N*-acetylglucosamine, diluted 3-fold and chromatographed on an FGF-2-Sepharose column. The bound material was eluted from the gel by a 0.15–2 M NaCl gradient and analysed by SDS/7.5%-PAGE. The resolved proteins were stained with silver nitrate or transferred on to Problott membranes and sequenced directly using an Applied Biosystems 470A sequencer.

Phage library screening and DNA sequencing

The Clontech bovine brain cDNA λ gt10 phage library (2×10^6 recombinants) was plated on *Escherichia coli* strain K-12 C600 Hfl and transferred on to positively charged Biohyllon Z⁺ nylon membranes (Bioprobe). The library was probed for the p79 cDNA using two 5' ³²P-radiolabelled antisense oligonucleotides, 5' GCCCTGCTCAATCCCGGT 3' (p79-o1) and 5' GAAGT-AGTGCATGCCAATCAC 3' (p79-o2), deduced from the internally sequenced peptides (see Figure 2A) after the bovine codon usage probability had been calculated as described previously [22]. For the p46 cDNA screening, an oligonucleotide identical to the rat p46M nucleotide sequence [20] corresponding to the region of a sequenced p46 peptide [5'GCTATGGATTCTGATTGTTTGCACAAACTACATGGGTAGG3' (p46-o1)] was used. Another bovine brain cDNA library (9×10^6 recombinants) was constructed by ligating retro-transcribed bovine brain poly(A)⁺ RNAs to the *Sfi*I-digested arms of the λ pCEV27 vector (M. D. Katinka, unpublished work). Recombinant phage (3×10^6) were screened with 5'-labelled antisense oligonucleotides (see Figure 2A) corresponding to the targeting sequence (TS) of either the p79 cDNA [5' GCGGCAAATAT-ACCGAAGTGGGAGAACCAGGGAAGCTTTCTGAAATATGATGGG 3' (p79-o3)] or the GBP-like cDNA [5' GCGGCAAATGTACCCTCGGCAGCCAGAGTCTGGGAG-GCAGTGAAGCGACGG 3' (pGBP-o1)]. A 1 kb *Eco*RI fragment (nucleotides 696–1675 in Figure 2B), isolated from the first p79 screened library, was also used as a probe. The positive isolated inserts were subcloned in pBluescript II KS⁺ and sequenced.

Cloning of the elements encoding TSs

The bovine brain λ gt10 cDNA library was screened with the 5'-³²P-labelled oligonucleotide 5' GGGTGAGTTAATTCGAAT-AACTGCC 3' (p79-o4; nucleotides 177–201 of the p79 cDNA sequence shown in Figure 2A) corresponding to part of the N-terminus of the putative mature p79. Single-strand cDNA retro-transcribed from p79-o4 on bovine brain poly(A)⁺ mRNA was then amplified by PCR, using the phosphorylated p79-o4 primer with either the phosphorylated sense primer p79-o5 (5' CACTC-ATTATCAAGTGAGG 3'; located 155 bp upstream of the p79 putative initiation codon [19]) or the phosphorylated sense primer pGBP-o2 (5' AGTCCATGGCCACCATGGTGCCTCCCG-GGCAATA 3'; starting 5 nucleotides upstream from the pig GBP translation initiation codon (see Figure 2A; [19]).

Northern blot analysis

MTN and MNN blots [2 μ g of poly(A)⁺ RNA per lane] were hybridized at 65 °C with the following probes: the 1 kb *Eco*RI fragment of p79 and the entire cDNA coding sequence of p46 (1.5 kb) radiolabelled with [α -³²P]dCTP by random priming. A probe corresponding to the predicted first 32 amino acids of the signal peptide was synthesized by PCR using the sense primer 5' CTCCTGAGAAAATCACGGAC 3' (p79-o6), located 11 bp upstream of the first ATG, and the antisense primer 5' CCT-GAGAAGTGGGAGAACCAG 3' (p79-o7), located 79 bp downstream from the initiation codon. PCR was carried out for 30 cycles at denaturing, annealing and elongation temperatures of 95 °C, 53 °C and 74 °C respectively. The PCR product (129 bp) was re-amplified on one strand using the antisense downstream oligonucleotide in the presence of [α -³²P]dCTP and [α -³²P]dATP (3000 Ci/mmol).

Nucleotide and amino acid sequence analysis

Nucleotide sequences were analysed using the NCBI Program BLASTN 1.4.9MP [23]. Hydrophobicity profiles were analysed according to Kyte and Doolittle [24].

¹²⁵I-FGF-2 cross-linking and ligand blotting

Human recombinant FGF-2 (10 μ g) was iodinated with 1 mCi of ¹²⁵I by the IodoGen method (Pierce, Rockford, IL, U.S.A.) to a specific radioactivity of $\sim 7.5 \times 10^4$ c.p.m./ng of protein. Binding and cross-linking of ¹²⁵I-FGF-2 to bovine brain membrane proteins or FR3T3 cells were performed as described elsewhere [9, 11]. Ligand-blotting experiments were performed as described in [10].

Construction of N-terminally tagged p79 and p46, and *in vitro* binding

A 2242 bp *Bam*HI fragment (nt 456–2698 in Figure 2B) of the p79 coding sequence was used to generate a recombinant protein (without the 153 N-terminal amino acids). The *Bam*HI fragment was ligated to the *Bam*HI site of the pET3a vector [25], and the resulting fusion protein was named N-tagged p79. The cDNA encoding the entire mature p46 protein (1.5 kb) was inserted into *Nde*I-digested pET3a. Recombinant bovine p79 and p46 were amplified in the BL-21(DE3)-pLysS *E. coli* strain [25]. Recombinant bovine FGF-2 (rbFGF-2) [21] was incubated with N-

tagged p79 in the presence of 3 mM dithiothreitol, 5 mM CaCl_2 , 5 mM ATP or 5 mM EDTA; FGF-2 was then immunoprecipitated and the immunocomplexes were resolved by SDS/PAGE, and the presence of N-tagged p79 and FGF-2 was assessed by staining with Coomassie Blue R-250. In a parallel experiment, [^{35}S]Met-radiolabelled rbFGF-2 was incubated with p79 coupled to CH-4B-Sepharose under the optimal conditions established above, in the presence of boiled FGF-2, FGF-1, or increasing amounts of unlabelled FGF-2. After washing, the Sepharose beads were resuspended in Laemmli sample buffer and the proteins were resolved by SDS/15% PAGE, and the dried gel was submitted to autoradiography. Finally, 5 μg of [^{35}S]Met-radiolabelled rbFGF-2 was incubated with 1 mg of rat brain lysate protein in the presence or absence of a 200-fold excess of unlabelled rbFGF-2 for 3 h at 4 °C. MG-160 and p79 were then immunoprecipitated, and immunoprecipitates were resolved by SDS/15% PAGE. Radiolabelled proteins were visualized by autoradiography.

Western blotting, immunoprecipitation and production of antibodies to p79 and p46

Wistar rat tissues (heart, brain, testis, lung, liver, stomach, skeletal muscle and kidney) were homogenized in an Ultra-Turrax T25 mounted with an N-10G blade at maximum speed in 20 mM Hepes (pH 7.4), 150 mM NaCl, 2% SDS, 1% Triton X-100, 1 mM EDTA and anti-proteinases [13]. The homogenates were ultracentrifuged for 1 h at 200 000 g (2 °C). Supernatant proteins were quantified by a BCA assay (Pierce). Proteins (200 μg) were analysed by immunoblotting. Immunoprecipitations were carried out as described [26]. Recombinant bovine p79 and p46 were purified by SDS/PAGE and injected into rabbits at Biocytex (Marseille, France) to produce antibodies.

Electroporation of FR3T3 cells

The p79 cDNA was inserted between the *Xba*I and *Eco*RV sites of the pCI-neo eukaryotic expression vector (Promega, Madison, WI, U.S.A.). The plasmid (1 μg) was electroporated into FR3T3 rat fibroblasts (10^6 cells) using an Easyject^{Plus} electroporator (Eurogentec, Seraing, Belgium; 270 V, 1050 μF in 4 mm cuvettes). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn-calf serum at 37 °C in a 5% CO_2 atmosphere in presence of 600 $\mu\text{g}/\text{ml}$ G418. Resistant clones were tested for the presence of p79 by immunoblotting following isolation of the membrane cellular fraction and by cross-linking experiments.

Subcellular fractionation

FR3T3 cells (1.5×10^7 cells, at a density of 9×10^5 cells/ cm^2) were washed with 25 mM Tris/HCl (pH 7.5), 250 mM sucrose, 2.5 mM magnesium acetate, 10 mM β -mercaptoethanol, 10 mM benzimidazole and 10 mM NaF (buffer A). This buffer was replaced with 2 ml of buffer A containing 5 mM EDTA, 5 mM EGTA, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin and 1 mM PMSF, and the cells were scraped and homogenized with 15 strokes of a Dounce type A pestle and 20 strokes of a B type pestle. The homogenate was centrifuged for 1 h at 105 000 g in a Beckman vTi65 rotor at 2 °C. The pellet was resuspended for 30 min at 4 °C in 25 mM Tris/HCl (pH 7.5), 14 mM β -mercaptoethanol, 10 mM benzimidazole, 10 mM Na_3VO_4 , 5 mM EDTA, 1% Nonidet P40 and proteinase inhibitors as listed for buffer A (buffer B). The solubilized proteins were clarified by a 20 min centrifugation at 15 500 g in

a Jouan A14 Microfuge, and then either directly submitted to SDS/PAGE, or after dilution in buffer B without β -mercaptoethanol, gel analysis was preceded by immunoprecipitation as described above.

Metabolic labelling and sequential immunoprecipitation

FR3T3-316 cells (5×10^5) were cultivated in Dulbecco's modified Eagle's medium depleted in methionine and cysteine for 4 h with or without tunicamycin treatment as previously described [27]. Cells were then labelled with 100 mCi of TRAN ^{35}S -label (600 Ci/mmol) for 1 h. After removal of the medium, cells were washed in PBS and lysed in 40 mM Tris/HCl (pH 7.5), 137 mM NaCl, proteinase inhibitors as above and 1% CHAPS. All procedures were then carried out as described in [27].

Electron microscopy: fixation, cryoprotection and freezing

Preparation of cells for cryo-immune electron microscopy was carried out as previously described for rat liver [28], with some modifications. Briefly, FR3T3-316 and FR3T3-Neo4 fibroblasts were grown as above until approx. 75–80% confluence. Cells were gently washed three times with 0.1 M sodium phosphate buffer (pH 7.4) and then fixed directly in the Petri dish with 10 ml of freshly prepared 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 10–30 min. The fixative was discarded, and cells were scraped into ice-cold Microfuge tubes, pelleted for 5 min in a refrigerated microcentrifuge at 2500 g and kept on ice. Fixative was then gently added to the tube so as not to disturb the pellet. The cell pellet was left at 4 °C for 1–3 days in fixative. Small pieces (approx. 0.5–1.0 mm^3) were dissected from the cell pellet, washed four times in ice-cold 0.1 M sodium phosphate buffer (pH 7.4) to eliminate the fixative, cryoprotected in multiple changes of 2.3 M sucrose/0.1 M phosphate buffer [29] for 1 h and then mounted on stubs and frozen directly in liquid nitrogen.

Tissue sectioning, immunolabelling and contrasting

All steps followed procedures described by Dahan et al. ([28] and references therein). The antibodies and gold conjugates were diluted in PBS (pH 7.4) containing 2% BSA, 2% casein and 0.5% ovalbumin.

Analysis of gold labelling

Quantification of gold labelling was carried out as described elsewhere [30]. Three separate stubs of FR3T3-316 cells were used for the quantitative analysis of labelling for p79 and for an irrelevant control antibody against rat α -amylase. Cells were randomly selected, and nucleus-free intracellular regions were photographed at a primary magnification of $\times 11\,700$, this being increased to a final magnification of $\times 18\,500$ on prints. Gold particles were scored on the micrographs and were allocated to cellular compartments: mitochondria, lysosomes, ER, Golgi apparatus, multivesicular endosomes, plasma membrane, cytosol and unidentifiable structures.

RESULTS

Purification of FGF-2-binding proteins from bovine brain membranes

To isolate major FGF-binding proteins, a particulate fraction from 10 bovine brains was subjected to three chromatographic steps [13] after membrane solubilization. Analysis by SDS/7.5% in

Figure 1 Analysis of FGF-2-associated affinity-purified proteins from adult bovine brain membranes

(A) Elution from the FGF-2-Sepharose column with 0.8 M NaCl resolved by SDS/7.5%-PAGE and visualized by silver staining. (B) Cross-linking of the FGF-2-Sepharose column eluate with ^{125}I -FGF-2 (0.5 nM) in the presence (+) or absence (–) of 0.25 mM disuccinyl suberate (DSS), 10 mg/ml heparin and/or 200 nM unlabelled FGF-2. Arrows indicate complexes of molecular mass 170 kDa and 95 kDa. (C) Autoradiogram of a ligand blot experiment. The proteins (1 μg of total protein), prepared as in (A), were submitted to non-reducing SDS/PAGE, transferred on to nylon membranes and incubated with ^{125}I -FGF-2 (100 pM) in the presence or absence of 10 mg/ml heparin or unlabelled FGF-2 (100 nM), as indicated. The iodinated FGF-2 associated with 79 kDa (faint signal), 130 kDa and 150 kDa (faint signal) species is depicted by the arrows.

PAGE and silver nitrate staining of the material eluted from the FGF-2-Sepharose gel with 0.8 M NaCl revealed the presence of three major proteins with apparent molecular masses of 150 kDa, 79 kDa and 46 kDa (designated p150, p79 and p46; Figure 1A). Cross-linking of the FGF-2-Sepharose eluate with ^{125}I -FGF-2 revealed complexes of 170, 95 and 60 kDa in the presence of heparin, but only of 95 and 60 kDa in its absence. Only the 170 and 95 kDa complexes were competed with by an excess of unlabelled FGF-2 (Figure 1B, small arrowhead). As deduced from the molecular mass of FGF-2 (18 kDa), these data indicated heparin-dependent specific binding of FGF-2 to the 150 kDa polypeptide, heparin-independent binding to the 79 kDa polypeptide and a non-specific association with the 46 kDa polypeptide. Interestingly, a 200 kDa complex was observed in the presence or in the absence of heparin, and this bound FGF-2 in a specific manner. When proteins from the FGF-2-Sepharose eluate were fractionated by SDS/PAGE under non-reducing conditions, silver nitrate staining revealed the presence of a 130 kDa protein in addition to the 150 kDa, 79 kDa and 45 kDa

species (results not shown). An ^{125}I -FGF-2 blotting on non-reduced proteins was performed, either in the presence or in the absence of heparin and with or without a 100-fold excess of unlabelled FGF-2. In the presence of heparin, FGF-2 bound specifically to the 150 kDa and 130 kDa proteins, and to a lesser extent to the 79 kDa protein; in the absence of heparin FGF-2 bound to the 130 kDa and 79 kDa species only (Figure 1C).

Three internal endolysine peptides of p150 were sequenced (IIQEIALXY, RNLDRIEMWSYAAK and HTXSNNLAVL-ESLQD). The N-terminal amino acid sequences of p79 and p46 were determined, as well two p79 and one p46 internal peptide sequences (Figures 2B and 2C). The p150 sequences were highly similar to the CFR-like proteins [11], MG-160 [14,15] or ESL-1 [16]. The p79 sequences were very similar to those of GBP [19] or p79M [20], and the p46 sequences were identical to that of p46M [20]. Despite sharing greater than 90% amino acid identity, GBP and p79M were previously reported as being located in the plasma membrane and mitochondria respectively. To assess the degree of similarity between p79 and p46 and their homologues, we undertook the cloning of cDNAs encoding these proteins.

Cloning of the p79- and p46-encoding cDNAs

The screening of a $\lambda\text{gt}10$ bovine brain cDNA library with the p79 probes described in the Experimental section gave rise to 55 positive plaques from which DNA was extracted, analysed and sequenced. Three overlapping sequences were assembled into one contig containing an open reading frame (ORF) harbouring both a TGA stop codon and an oligo(dA)₂₀ tail, but not an initiator methionine codon. The BLAST search [23] comparing the p79 nucleotide sequence with those present in the GenBank and EMBL libraries demonstrated a high degree of identity between the p79 cDNA and the GBP and p79M coding sequences. Likewise, the deduced p79 ORF showed marked similarity with the GBP and p79M amino acid sequences (Table 1). To determine the putative upstream N-terminal coding sequences, two experimental approaches were employed. First, the bovine brain cDNA library was screened with an oligonucleotide corresponding to the ultimate 5'-nucleotide sequences established (Figure 2A). The DNA from 25 phages was analysed, but no further upstream sequences were recovered from 22 of the cDNAs. However, DNA from the last three independent recombinant phages harboured the whole mature GBP/p79M N-terminal region, preceded by a sequence encoding 46 or 43 amino acids, with two possible ATG initiation codons separated by 6 bp (Figure 2B). The sequence found in the three clones was different from the mitochondrial TS published for rat and human p79M or pig GBP (Figure 3A). We refer hereafter to these three cDNAs and the deduced protein as p79ER. Secondly, two types of sequences were detected by 5'-rapid amplification of cDNA ends/PCR, one closely related to the published pig GBP or rat or human p79M sequences, and the other identical to the p79ER cDNAs (Figure 3A). Both sets of data suggested the existence of at least two p79 cDNA species.

The cloning of p46 cDNA from the $\lambda\text{gt}10$ bovine brain cDNA library was carried out using rat specific p46M oligonucleotides, as described in the Experimental section. Analysis of recombinant cDNA from 14 phages by restriction endonucleases and nucleotide sequencing revealed a 1.4 kb ORF (Figure 2C). Comparison with nucleotide and amino acid sequence libraries using BLAST revealed that the p46 cDNA and the deduced protein sequences were very closely related, if not identical, to those of p46M (Table 1). No further ORFs were revealed using rapid amplification of cDNA ends/PCR experiments.

Figure 2 Sequences of p79 and p46 cDNAs

(A) Localization of the oligonucleotides used for cloning of p79ER. Also shown are nucleotide sequences and deduced amino acid sequences established for p79ER (B) and p46 (C). Nucleotide sequences used in PCR experiments discussed in the text are underlined. Sequenced peptides are doubly underlined.

Table 1 Amino acid and nucleotide sequence identity comparisons between bovine p79, pig GBP and rat and human p79M, and between bovine p46 and rat and human p46M

Nucleotide sequence identity is given in parentheses.

	Identity (%)				
	Pig GBP	Rat p79M	Human p79M	Rat p46M	Human p46M
Bovine p79ER	88.3 (90)	80.1 (81)	86.4 (87.3)	—	—
Pig GBP	—	82.1 (81)	89.1 (87)	—	—
Rat p46M	—	—	83.9 (83)	—	—
Bovine p46	—	—	—	91.6 (87.0)	92 (90)
Rat p46M	—	—	—	—	89.9 (88)

A

Bovine p79 MRKMQKGLFYLIWVTDNRNPIIFQKASLVLP LLRYICRSFTGSSALL
 Bovine GBP MVASRAIGSLRRFT--QT-GCRG---N--R---
 Pig GBP -----R-P-----
 Human GBP ---C---S--S-FRI--SR-----G---
 Human p79M -----
 Rat p79M ---S-----C--TALQLLP---

B

Bovine p46 MISLLTYTLKNLPNTSKWALRFCMRPLSCSSQLQA
 Human p46M -T*I---PF---TA-----SI-----R-
 Rat p46M -----ST---S-T-----V---S---VQS

C **Figure 3** Comparison between related TSs

(A) Comparison of TSs between bovine p79ER, bovine, pig and human GBP, and human and rat p79M. Sequences were compared two-by-two, and identical amino acids are depicted by dashes. (B) Comparison of bovine p46 and human and rat p46M TSs. The asterisk depicts a missing amino acid. (C) Hydrophobicity pattern analysis of bovine p79ER and human p79M (upper panel) and bovine and pig GBP (lower panel).

Analysis of the p79 and p46 amino acid and nucleotide sequences

The nucleotide sequence and deduced amino acid sequence of mature p79ER are very closely related (Table 1) to those of pig GBP, rat p79M and human p79M [31]. The bovine p79ER ORF contains two putative methionine initiation codons (Met-Arg-Lys-Met). Comparison between the GBP and p79M putative TSs reveals an identical length of 36 residues (Figure 3A). Interestingly, the p79ER putative TS peptide is 43 or 46 residues

A **B** **Figure 4** Immunoblotting study of the FGF-2 binding complex

(A) Proteins extracted from bovine brain membranes and submitted to two chromatographic steps were adsorbed on an FGF-2-Sepharose column. The elution was performed with a 0.15–1 M NaCl gradient and then with a step to 2 M NaCl (○). Proteins were monitored by measuring UV absorbance at 280 nm (□). (B) Proteins from peak 2 were submitted to SDS/10%-PAGE, electroblotted and probed with anti-p79, anti-p46 or anti-MG-160 antibodies (lb).

long, and is different overall from the above TS in its N-terminal moiety. Hydrophilic profile analysis [24] of the various TSs showed that the two N-terminal sequences of the p79 TS are hydrophobic, whereas the TS termini of GBP or p79M are hydrophilic (Figure 3B). Overall, the data suggested that p79ER on the one hand and GBP or p79M on the other may be destined for different cellular compartments. The nucleotide and protein sequences of bovine p46 were also compared with those of rat and human p46M. The three mature protein amino acid sequences are highly conserved (Table 1). The TS sequences were, likewise, fairly well conserved among the three species (Figure 3B).

Analysis of protein–protein interactions

Antibodies raised against p79, p46 and MG-160 were used to assess if the FGF-2-binding proteins were associated. The membrane fraction proteins retained on the FGF-2-Sepharose

Figure 5 Characterization of the interaction between FGF-2 and p79

(A) rbFGF-2 (25 or 50 μ g) was incubated with 25 μ g of N-tagged p79 in the presence or absence of 3 mM dithiothreitol (DTT), 5 mM CaCl_2 , 5 mM ATP or 5 mM EDTA. FGF-2 was then immunoprecipitated and immunocomplexes were separated by SDS/12.5%-PAGE. Proteins were visualized by Coomassie Blue staining. (B) [^{35}S]Met-radiolabelled rbFGF-2 (2 μ g) was incubated with p79-Sepharose beads in the presence of increasing amounts of unlabelled rbFGF-2 (2, 20, 50, 200 and 1000 μ g) in the presence of 200 μ g of boiled FGF-2 or 200 μ g of FGF-1. After extensive washing, beads were resuspended in Laemmli sample buffer and proteins were resolved by SDS/PAGE. Radiolabelled FGF-2 associated with p79 was visualized by autoradiography. (C) Incubation of [^{35}S]Met-labelled rbFGF-2 (5 μ g; precleared by incubation with Protein A-Sepharose beads) with a rat brain total lysate in the presence of 5 mM CaCl_2 , followed by immunoprecipitation by anti-MG-160 or anti-p79 antibodies. The immunoprecipitates were then resolved by SDS/15%-PAGE and the dried gel was autoradiographed. Lane 1, anti-p79 (Ip α p79); lane 2, anti-MG-160 (Ip α MG-160); lane 3, Ip α p79 plus 200-fold excess of unlabelled FGF-2; lane 4, Ip α MG-160 plus 200-fold excess of unlabelled FGF-2.

column were also eluted by a 0.15–2 M NaCl gradient (Figure 4A). Three major peaks detected by UV absorbance at 280 nm were revealed in the elution profile. The proteins from the different peaks were immunoblotted with antibodies raised against MG-160, p79 and p46. The material in peak 2 (0.75–0.9 M NaCl) revealed the presence of the three proteins (Figure 4B). No immunoreactive material could be detected in peak 3 (2 M NaCl) (results not shown). No immunoreactive MG-160 or p46 was detectable in peak 1, although a small amount of a 70–75 kDa species was detected by anti-p79 antibodies (results not shown).

Figure 6 Tissue distribution of p79 and p46 mRNAs and proteins

(A) Northern blots (Clontech) containing 2 μ g of poly(A)⁺ RNA from various human tissues (MTN; left panel) and from multiple brain nuclei (MNN; right panel) were probed with p79 and p46 cDNAs. MTN blots contained RNA from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7) and pancreas (lane 8). MNN blots contained RNA from the following nuclei: amygdala (lane 1), caudate nucleus (lane 2), corpus callosum (lane 3), hippocampus (lane 4), total brain (lane 5), substantia nigra (lane 6), subthalamic nucleus (lane 7) and thalamus (lane 8). (B) The MTN blot described in (A), probed with the p79 TS. (C) Proteins from various rat tissues were extracted, electrophoresed and probed after electroblotting on to nylon membranes with either anti-p79 (α p79) or anti-p46 (α p46) antibodies (Ib). Proteins were from heart (lane 1), brain (lane 2), testis (lane 3), lung (lane 4), liver (lane 5), stomach (lane 6), kidney (lane 7) and skeletal muscle (lane 8).

FGF-2 was shown previously to bind CFR and MG-160 [10,15]. To elucidate the interactions between FGF-2 and p79, rbFGF-2 and N-tagged p79 were incubated in the presence of 3 mM dithiothreitol, 5 mM CaCl_2 , 5 mM ATP or 5 mM EDTA. The co-precipitation of p79 with FGF-2 was somewhat increased in the presence of 5 mM CaCl_2 , but was abolished in the presence of 3 mM dithiothreitol (Figure 5A). EDTA or ATP had no effect on this association (Figure 5A). When [^{35}S]Met-labelled rbFGF-2 was incubated with N-tagged-p79-Sepharose beads in the presence of 5 mM CaCl_2 , increasing amounts of unlabelled FGF-2 (from 1- to 500-fold excess) competed with FGF-2 binding, but boiled FGF-2 had no effect and FGF-1 was only slightly competitive (Figure 5B). Moreover, on incubating [^{35}S]Met-labelled rbFGF-2 with a rat brain total lysate and immunoprecipitation with anti-MG-160 or anti-p79 antibodies, radiolabelled FGF-2 co-immunoprecipitated with these two proteins (Figure 5C); a 500-fold excess of unlabelled FGF-2 competed with this co-precipitation (Figure 5C).

Tissue distribution of p79 and p46

To determine the tissue distribution of p79 mRNA, an MTN blot was hybridized with the p79 1 kb probe (Figure 2B). RNA species of 3.1 kb were abundant in heart and skeletal muscle

Figure 7 Overexpression of p79 in FR3T3 fibroblasts and cross-linking with ^{125}I -FGF-2

FR3T3 cells were electroporated either by an empty expression vector (FR3T3-Neo4) or by an expression vector harbouring the p79ER cDNA (FR3T3-316). (**A**) Phenotypic modifications observed in FR3T3-316 as compared with FR3T3-Neo4 cells ($\times 120$ magnification). (**B**) For each cell type, the soluble cytosolic (S) and insoluble membrane (P) proteins were prepared, electrophoresed, electroblotted and probed with anti-p79 antibodies (lb: α p79). Arrowheads indicate the p70 and p79 species detected. (**C**) ^{125}I -FGF-2 (0.5 nM) cross-linked with disuccinyl suberate to proteins from FR3T3-316 and FR3T3-Neo4 clarified cell lysates, resolved by SDS/7.5%-PAGE and autoradiographed for 5 days.

(Figure 6A, lanes 1 and 6). Multiple transcripts centred around 3 kb were observed in the lane containing lung RNA. An MNN blot was also probed, and only very low quantities of 3.1 kb transcripts were detected in all of the nuclei, with a very slightly increased abundance in the corpus callosum material (Figure 6A, lane 3). The same MTN and MNN blots were also probed with the entire p46 ORF. Transcripts of 2.3–2.5 kb were weakly detected in all of the tissues present in the MTN blot. In the heart and skeletal muscle, however, the 2.5 kb transcripts were more abundant, and additional 2.0 kb transcripts of lesser abundance were also present (Figure 6A). The MNN blot (Figure 6A) revealed p46 transcripts of 2.5 kb in size, but these were only weakly expressed and their expression was correlated with that of p79 transcripts. The MTN blot was also probed with the p79ER TS probe. Only lung and, in lower amounts, brain transcripts hybridized with this probe with the same smeared distribution observed when using the p79 probe (Figure 6B). Immunoblotting

Figure 8 Interaction of p79 with calnexin in p79-transfected FR3T3 fibroblasts

Cell lysates from FR3T3-316 metabolically labelled for 1 h with [^{35}S]methionine were first immunoprecipitated (Ip 1) with anti-calnexin (CNX) or anti-p79 antibodies following a 4 h treatment with or without tunicamycin. Immune complexes were collected on Protein A-Sepharose beads, and released by heating in 1% (w/v) SDS. After a 10-fold dilution in lysis buffer, the released proteins were then immunoprecipitated with anti-p79 or anti-calnexin antibodies respectively (Ip 2), collected on Protein A-Sepharose beads, electrophoresed and autoradiographed for 24 h.

experiments were carried out on total clarified protein extracts from various Wistar rat tissues. Anti-p79 antibodies revealed a 79 kDa protein in the testis, liver and kidney (Figure 6C, upper panel, lanes 3, 5 and 7), and to a lesser extent in the brain (Figure 6C, upper panel, lane 2), which was co-distributed with an anti-p46 immunoreactive doublet of approx. 45 kDa (Figure 6C, lower panel, lanes 2, 3, 5 and 7).

Biological changes induced by overexpression of p79 in FR3T3 cells

A total of 22 FR3T3 clones harbouring pCIneo-p79ER were selected on the basis of G418 resistance and subsequent p79ER expression. FR3T3 cells expressing low amounts of transfected p79ER (Figure 7A; and results not shown) underwent morphological changes, with the loss of their original fibroblast morphology. These cells were more elongated than the mock-transfected (FR3T3-Neo4) cells, and presented a fish-net-like array (Figure 7A; FR3T3-316). Cells doubled every 32–34 h, as compared with 22–23 h for the mock cells. After separation of the cytosol and membrane fractions, protein composition was studied by immunoblotting following immunoprecipitation with anti-p79 antibodies (Figure 7B). An immunoreactive 70 kDa band was detected in cytosolic fractions of mock- and p79ER-transfected FR3T3 cells, whereas two immunoreactive species (apparent molecular masses 70 kDa and 79 kDa) were revealed in the membrane fraction of p79ER-expressing cells (FR3T3-316, FR3T3-317, FR3T3-305). No 79 kDa immunoreactive material was observed in the cytosolic fractions. The binding of FGF-2 to FR3T3-Neo4 and FR3T3-316 clarified cell lysates was studied by ^{125}I -FGF-2 cross-linking experiments. As shown in Figure 7(C), ^{125}I -FGF-2 was able to form two complexes of approx. 130 and 170 kDa in FR3T3-Neo4 cell lysates, and three complexes (approx. 130, 170 and 260 kDa) in FR3T3-316 cell lysates. The exact nature of these complexes remains to be determined.

Overexpressed p79 associates with calnexin

The amino acid sequence of p79ER comprises in its C-terminal domain a KKXXX motif which has been previously defined as an ER retrieval motif ([32] and references therein). Because p79ER presents a hydrophobic TS and a putative ER retrieval

Figure 9 Intracellular distribution of p79 in FR3T3-316 and FR3T3-Neo4 cells

Ultrathin cryosections of FR3T3-316 (**A**, **B**) and FR3T3-Neo4 (**C**) cells were immunolabelled with rabbit anti-(rat p79) antibodies followed by goat anti-(rabbit IgG) antibodies conjugated to 10 nm gold particles. The ER compartment (*) of FR3T3-316 fibroblasts was identified by looking at the distribution of the ER marker protein calnexin (CNX) using a rabbit antibody against the cytoplasmic tail of calnexin (CN4) followed by anti-(rabbit IgG) conjugated to 10 nm gold particles (**D** and **E**). Calnexin-labelled ER cisternae (*) were characterized by irregularly shaped membrane-bound compartments with a heterogeneous mottled content; the cytosolic compartment, in contrast, revealed an electron-dense homogeneous staining pattern (cy; **D** and **E**). The Golgi apparatus (G) exhibited negligible labelling for calnexin (**D**, **E**). In FR3T3-316 cells, p79 immunoreactivity was mostly distributed throughout the ER cisternae (*) (**A** and **B**) and within the Golgi apparatus (G). The latter, which typically consisted of 2–4 saccules (**A**), revealed p79 immunoreactivity predominantly at the immediate periphery of Golgi stacks (**A**; small arrows). In FR3T3-Neo4 cells, gold particle labelling for p79 was similarly distributed throughout the ER (*) and Golgi apparatus (G); however, the labelling density was noticeably lower (**C**). Lysosomes (L), mitochondria (M) and nuclei (Nu) revealed negligible labelling. Bars = 400 nm.

motif, its localization in an ER compartment was studied. Furthermore, the amino acid sequence of p79 presents two putative N-glycosylation sites (Asn¹⁵² and Asn⁴⁸⁵; bovine se-

Table 2 Intracellular distribution of p79ER in FR3T3-316 cells

Labelling is given as the percentage of the total number of gold particles counted over the indicated compartments in an approx. 1100 μm^2 profile surface area. The number of gold particles scored over cell compartments in sections labelled for p79ER were subtracted from background labelling over respective compartments in sections labelled with a control antibody against α -amylase over a 1100 μm^2 sectional profile area. This value corresponded to 3553 gold particles that were scored over 39 micrographs of FR3T3-316 fibroblasts; 72.5% of gold particles were over the compartments indicated, and 27.5% of particles were over irrelevant structures (lysosomes, multivesicular endosomes, cytosol and unidentifiable structures).

Cell compartment	Immunogold labelling (%)
Cell surface	3.5
ER	78.1
Golgi apparatus	14.2
Mitochondria	4.1

quence). To confirm an ER localization of this protein, we assessed the interactions between p79ER and calnexin by metabolic labelling followed by sequential immunoprecipitation. Immunoprecipitates of calnexin or p79ER from ³⁵S-labelled FR3T3-316 clarified cell lysate proteins were then sequentially immunoprecipitated with anti-p79 or anti-calnexin-C3 immunosera respectively. The results presented in Figure 8 show an association between p79ER and calnexin. Moreover, treatment of the FR3T3-316 cells with tunicamycin reduced the apparent p79ER–calnexin association by 90% (Figure 8). No difference in the calnexin expression level was observed between FR3T3-Neo4 and FR3T3-316 cells, however. Finally, the hydroxyacyl-CoA dehydrogenase activity was quantified, using the protocol described by Kamijo et al. [20], but no significant difference in enzymic activity was observed between the FR3T3-Neo4 and FR3T3-316 cells (results not shown).

The intracellular localization of p79ER in ultra-thin cryosections of FR3T3-316 and FR3T3-Neo4 fibroblasts was studied by immunocytochemical electron microscopy, in parallel with that of a known membrane protein marker of the ER, calnexin. Calnexin-immunoreactive ER cisternae made up a large fraction of the fibroblast cytoplasm, and had a characteristic dilated morphology (Figures 9D and 9E); the lumen revealed a heterogeneous flocculent or mottled content that could easily be distinguished from the electron-dense homogeneously staining cytosol (cf. Figures 9D and 9E). FR3T3-316 fibroblasts immunolabelled with rabbit anti-p79 antibody followed by anti-(rabbit IgG) conjugated with 10 nm gold particles revealed that p79ER is predominantly distributed throughout the distended, electron-lucent ER cisternae and within the Golgi apparatus. Quantification of gold particle labelling revealed that, of the cellular compartments indicated in Table 2, p79 was most abundant within the ER and Golgi apparatus, which harboured respectively 78.1% and 14.2% of the total number of gold particles scored. This may imply that p79ER was retained in the secretion pathway, and this was further confirmed by the weak gold labelling at the cell surface (3.5%). Furthermore, only negligible labelling could be detected within mitochondria (4.1%), suggesting a non-mitochondrial localization of p79ER. In FR3T3-Neo4 cells, quantification of gold particle labelling revealed a similar distribution along compartments of the secretory pathway, with approx. 10% less labelling within the ER as compared with that within FR3T3-316 fibroblasts (results not shown). This slight difference notwithstanding, the frequency distributions of gold particle labelling within endomembrane compartments (ER, Golgi apparatus, multivesicular endosomes, cell surface, lysosomes and mitochondria) of FR3T3-

316 and FR3T3-Neo4 cells were found to be significantly different ($\chi^2 = 199.42$, $P < 0.001$).

DISCUSSION

Three proteins of 150 kDa, 79 kDa and 46 kDa were purified from bovine brain membranes on the basis of their capacity to bind both wheat-germ lectin and FGF-2 in chromatography experiments. We found that the p150 nucleotide and amino acid sequences were closely related to those of chick CFR [11], MG-160 [14] and ESL-1 [16]. The mature p79 coding sequence was 80–88% identical with that of rat and human p79M [20,31] or pig GBP [19]. However, we detected in the cDNA libraries and by PCR experiments a different TS (p79ER). The cloned p46 coding sequence was, moreover, shown to be identical with that of p46M [20].

Expression of mRNAs encoding p79 and p46 was studied in different tissues and presented identical expression patterns for both genes, except in the lung, in which p79-like transcripts were present whereas p46 transcripts were undetectable. Using a probe specific for the putative N-terminal TS of p79ER, expression was detected in the lung but not in the brain. The presence of the p79M-encoding mRNA was assessed by probing a cDNA library and by PCR and Northern blot experiments, and this was found to be an extremely rare species in the brain; p79ER, although rare, was the major isoform in this tissue [one p79 mRNA per $(2-5) \times 10^3$ transcripts]. We have also observed that rat p79-like proteins (in liver, kidney, testis and less so in brain) were not located in the tissues where human mRNAs were the most abundant (heart, lung and skeletal muscle). This may be due to precise tissue-specific regulation of transcription and translation, and/or mRNA and protein stability. Such fine regulation has been described in a number of p79M-linked pathologies, such as cardiomyopathy or hepatomegaly [20,33,34].

At the subcellular level, we were able to show, by stable transfection of FR3T3 cells, that the p79ER ORF was indeed mostly routed to the secretory pathway. Quantitative electron microscopy and immunocytochemical analysis of p79ER-expressing rat fibroblasts (FR3T3-316) confirmed the localization of p79ER in structures morphologically indistinguishable from those labelled for the ER membrane protein marker calnexin. This revealed that more than 75% of anti-p79 gold labelling was located in the ER compartment and almost 15% was located in the Golgi apparatus, where MG-160-like proteins are mainly localized [15,18,35]. These data could suggest a rapid recycling of p79ER between the ER and the Golgi, with an accumulation of p79 in the ER, as previously described for other proteins [36,37]. With regard to p46, only a mitochondrial-type TS is present in the mRNA isolated from bovine brain. This TS is very similar in its sequence to those detected in rat and human mitochondrial p46 [20,31]. How then is p46 localized in the ER or in the cytosol? This remains to be established, but the situation is reminiscent of that with mitochondrial aspartate aminotransferase which, although containing a mitochondrial TS, was nevertheless localized to the plasma membrane when overexpressed in mouse 3T3 fibroblasts [38].

Finally, we demonstrated the presence of FGF-2 in a complex with CFR/MG-160-like proteins and p79, and we observed an association between p79 and FGF-2 *in vitro*. Previously, only CFR and MG-160 have been shown to bind FGF-2 [11,15]. Zhou et al. [39] have shown that CFR possibly harbours two distinct FGF-binding sites; one of these is heparin-dependent, whereas the other is not. It is noteworthy that CFR may be implicated in FGF-2 intracellular transport [39] and in the regulation of intracellular FGF-1 and FGF-2 levels [18]. These

interactions may be independent of the release of FGF-1 from cytosol, shown to involve synaptotagmin-1 ([40] and references therein), or that of FGF-2 via a pathway implicating the catalytic subunit of Na^+/K^+ -ATPase [41]. The complex of p150 and p79ER (and p46) may play a role in the regulation of membrane traffic, and/or as secretory pathway resident proteins recognizing FGF-2 during intracellular transport [42]. Recently Kolpakova et al. [43] described a new FGF-1-binding protein found intracellularly and localized mainly in the nucleus, i.e. a protein also associated with intracellular membranes. This protein was hypothesized to be involved in the mitogenic action of FGF-1. Elucidation of the roles of intracellular proteins that regulate the trafficking of FGFs will be the next step towards gaining a better understanding of the functions of FGFs, particularly FGF-2, which has been described to have several different roles in the cell [8].

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