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Purification and characterization of the 210-amino acid recombinant basic fibroblast growth factor form (FGF-2)

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Abstract

Four forms of basic fibroblast growth factor (bFGF or FGF-2), using one AUG (155 amino acids) and three upstream CUG (210, 201 and 196 amino acids) start codons, were synthesized through an alternative use of initiation codons. The 210-amino acid form of FGF-2 (210FGF-2) was expressed in a plasmid vector under the control of a bacteriophage T7 RNA polymerase promoter system in *Escherichia coli*. Characterization of the purified protein was performed by electrospray mass spectrometry and Edman degradation. The recombinant 210FGF-2 produced in *E. coli* had a mitogenic activity similar to the 146-amino acid form extracted from tissues.

Key words: Basic fibroblast growth factor; High molecular weight form; Recombinant protein; *E. coli*

1. Introduction

Basic fibroblast growth factor (bFGF or FGF-2) is a mitogen and differentiation factor for a variety of cells including vascular endothelial cells as well as being a potent angiogenic factor [1–3]. Its was originally identified in the bovine pituitary by D. Gospodarovicz in 1974 [4]. FGF-2 is prototypic of a family of nine heparin-binding structurally related peptides (for review, see [5]). At least four human genes encode high affinity cell surface protein tyrosine kinase receptors (FGFR) [6,7]. The complexity of the FGFR family is increased by the alternative splicing of mRNA [8]. The biological activity of FGF-2 also requires low affinity heparan-sulfate proteoglycans (HSPG) binding sites located both on the cell surface and in the extracellular matrix [9].

Because of the potential therapeutic value of FGF-2, laboratories have used the DNA technique to produce recombinant proteins for research purposes. Since cDNAs corresponding to bovine and human FGF-2 mRNA have been cloned [10–12], much work has been carried out to establish systems for the production of large quantities of recombinant FGF-2 (rFGF-2) [13–19].

High molecular weight forms of FGF-2 have been described [20,21]. Analysis of an extended hepatoma FGF-2 cDNA revealed unusual start codons [22]: proteins result from an alternative initiation of translation at an AUG (155FGF-2, 155 amino acids: 18 kDa) or at three inframe upstream CUGs (210FGF-2, 210 amino acids: 24 kDa, 201 amino acids: 22.5 kDa and 196 amino acids: 22 kDa) [22,23]. The high molecular weight forms of FGF-2 contain a glycine-arginine-rich domain where arginines can be dimethylated [24]. The multiple FGF-2 isoforms are localized in different subcellular compartments: the three high molecular weight forms contain a nuclear localization sequence (NLS) in their NH_2-terminal part and can address a reporter protein to the nucleus while the 18 kDa form remains mainly cytoplasmic [25]. The same distribution pattern was observed in transfected cells constitutively producing one or other form, and their expression correlated to modifications of the phenotypes, the different FGF-2 isoforms appear to play specific roles in the control of cell metabolism [26,27]. The high molecular weight form of FGF-2 (210 amino acids) is produced by a wide variety of cell lines including hepatoma cells in which it may be involved in hepatomacarcinogenesis [20,28].

The purpose of this study was to characterize the biological activities of the different FGF-2 to get an insight into the significance of such diversity. Therefore, in this communication, we report the construction of an expression plasmid that directs the synthesis of r210FGF-2 in *E. coli*. We also describe the purification and structural characterization of the purified recombinant 210FGF-2 and evaluate the biological activity of the purified form.

2. Materials and methods

2.1. Construction of FGF-2 expression vector

The cDNA encoding the 210FGF-2 [22] was cloned in the pT7 expression plasmid pET3a [29] between *Nde*I and *Bam*HI sites. In this construct the CTG initiation codon was substituted by an ATG which was part of the *Nde*I site. This mutagenesis was generated by double-stranded oligonucleotide (5'TATGGGGGACCGC3' and 5'GGTCCCCCA3') insertion harboring *Nde*I and *Sac*II cohesive ends.

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2.2. FGF-2 production in E. coli

The transformed bacteria, BL21(DE3)pLysS, were grown on plates containing 100 μg/ml ampicillin, 0.5 mg/ml methicillin and 25 μg/ml chloramphenicol at 37°C until colonies were visible. Colonies were grown overnight in Luria broth (Gibco) and inoculated into 'terrific broth' [30] with antibiotics, shaken at 37°C, to give 0.02 absorbance at 600 nm. When the absorbance reached 0.6 at 600 nm, IPTG was added to the medium to a final concentration of 1 mM and growth was prolonged for 2 h.

2.3. Purification of recombinant FGF-2

The cells were harvested 2 h after postinduction by centrifugation 5000 rpm at 4°C for 10 min. The cell pellets were resuspended in 50 mM Tris-HCl, pH 7.3, with protease inhibitors (PMSF 1 mM, aprotinin 2 μg/ml, leupeptin 2 μg/ml). The cells were lysed by three cycles of freezing at −80°C, and sonicated 5 × 20 s. The cell lysate was adjusted to 0.7 M NaCl and centrifuged 100,000 × *g* for 60 min at 4°C.

Heparin-Sepharose affinity chromatography. The supernatant fraction containing the recombinant FGF-2 was applied directly to a column packed with heparin-Sepharose CL-6B (Pharmacia, 1 ml of resin) previously equilibrated with 10 mM Tris-HCl, pH 7.3, NaCl 0.7 M. The column was washed with equilibration buffer and then with buffer containing 1 M NaCl until absorbance at 280 nm (A_{280}) of the column effluent reached the baseline value. The recombinant FGF-2 was eluted with 2.3 M NaCl in Tris buffer.

Cationic exchange chromatography. Fractions of the heparin-Sepharose affinity chromatography were desalted using gel filtration (NAP-10 column, Pharmacia) and loaded on to a column (6 × 90 mm) packed with 3 ml of S-Zephyr (Sepracor) equilibrated with 50 mM Tris-HCl, pH 8.0. The column was washed until A_{280} reached the baseline value. The proteins were eluted with a linear gradient of 0–2 M NaCl in Tris buffer for 40 min.

Protein determination and SDS-PAGE. The concentration of total protein at each step was determined with the Biorad Protein Assay (BioRad Labs) or the BCA technique (Pierce). 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described [31]. Proteins were revealed by Coomassie brillant blue R 250 or silver staining [32].

Immunoblot analysis. Immunoblot analysis was done as previously described [26] using rabbit anti-FGF-2 polyclonal antibody (Sigma) or rabbit anti-210 amino acid FGF-2-NH_2-terminus serum.

HPLC analysis. For mass spectrometric analysis, recombinant FGF-2 was desalted and concentrated by reverse phase HPLC on a nucleosil C4 column (250 × 4.6 mm, 300 Å, 5 μm). The elution system was 0.1% TFA (solvent A) and 0.07% TFA in acetonitrile (Solvent B). A portion of the heparin-Sepharose affinity chromatography fractions was loaded on to the column equilibrated with solvent A and washed with solvent A for 5 min. The protein was eluted with a gradient of acetonitrile (up to 60%) at a flow rate of 1 ml/min.

2.4. Structural characterization of FGF-2

Electrospray mass spectrometric analysis. A freeze-dried sample of HPLC-purified r210FGF-2 was dissolved in H_2O/acetonitrile (1:1) containing 1% formic acid to a final concentration of about 10 pmol/μl. Electrospray mass spectrometric analysis was carried out by injecting 10 μl of this solution into a Trio 2000 mass spectrometer (VG Analytical) consisting of an electrospray ion source followed by a quadrupole mass analyser, at a flow rate of 4 μl/min. The mass spectrometer was scanned over *m/z* 800–1400 in 20 s at a one mass unit resolution. Data were acquired by summing ten scans to obtain the final spectrum; molecular species produced a series of multiply charged ions from which the molecular mass was determined by simple data system routines. Mass scale calibration employed the multiply charged ions from a separate introduction of myoglobin (average molecular mass 16,950.6 kDa); the reported molecular masses are the average values which take into account all the natural isotopic contributions.

Protein sequencing. Automated Edman degradation of r210FGF-2 was carried out on a Applied Biosystems 470A protein sequencer. Phenylthioidantoin derivatives were identified by HPLC after automatic injection in to an Applied Biosystem analyser 120A, coupled to the sequencer.

2.5. Production of rabbit polyclonal antibodies directed against the N-terminal domain of FGF-2

Immune sera were raised in rabbit against a synthetic peptide, situated between residues 39–53 in the 210FGF-2 sequence, and conjugated to bovine serum albumin (BSA) with glutaraldehyde. Antibodies were purified on an affinity column bearing synthetic polypeptide antigens. After extensive washing with MOPS 0.1 M, pH 9, the bound material was eluted with glycine, 0.2 M, pH 2.8, immediately neutralized with Tris, pH 8.5 and dialysed against 10% glycerol in phosphate buffered saline, 0.2% BSA before use.

2.6. Biological assay

10^4 ABAE cells were seeded per 35 mm tissue culture dishes. The different forms of FGF-2 were then added every two days to the indicated final concentration. On day 4, the cells were harvested and viable cell numbers were quantitated by counting (Coulter counter Model ZM).

3. Results and discussion

3.1. Production of r210FGF-2

In order to express rFGF-2 at high levels in *E. coli*, the cDNA encoding the protein was cloned in the pT7 expression plasmid pET3a (Fig. 1). Bacterial strain *E. coli* BL21(DE3) pLysS was routinely used to produce rFGF-2. This system gave results for r146FGF-2 (unpublished results) comparable to those already described [13]. A maximum of 1 mg protein per 1 liter of culture medium was obtained for r210FGF-2. We postulate that this difference in production efficiency results from the 5′ end mRNA structure encoding the N-terminal extension of the r210FGF-2 which is 86% G/C rich (versus 58% for the 146 amino acids encoding part) and that is deleted in the mRNA encoding the 146 amino acid protein. In addition, the presence of repetitive sequences encoding Gly-Arg could induce some translational disorders.

3.2. Purification of the r210FGF-2

Like r146FGF-2, most of the r210FGF-2 remained soluble in bacterial cells and was recovered (60%) in the soluble fraction after cell breakage. This fraction was

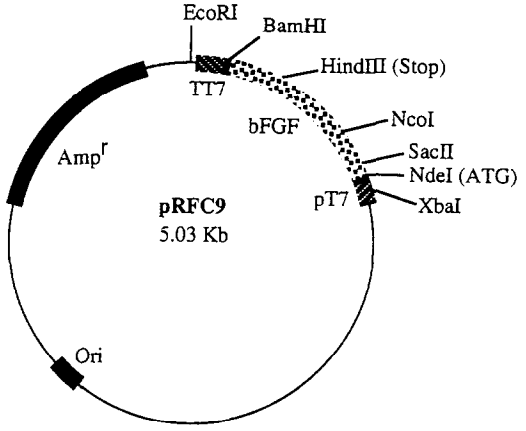

Fig. 1. Schematic diagram of the pRFC9 expression vector allowing the production of r210FGF-2.

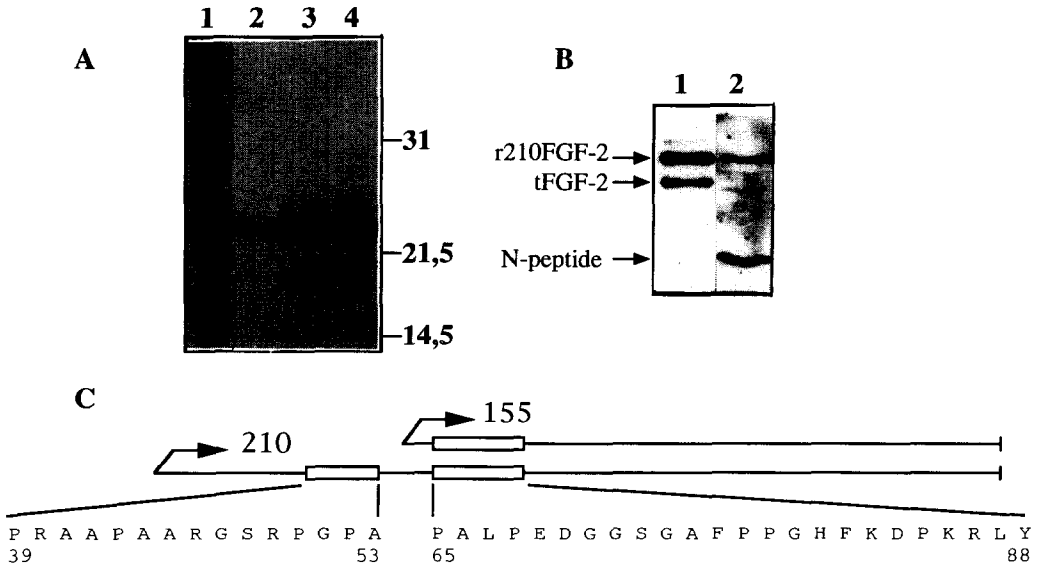

Fig. 2. SDS-PAGE analysis of bacterial proteins from *E. coli* IPTG induced cells transformed with the pRFC9 plasmid. (A) Coomassie blue staining. Lane 1 = total proteins solubilized (S100); lane 2–4 = fractions from the heparin-Sepharose affinity column eluted by 2.3 M NaCl. (B) Immunoblot analysis of the heparin-Sepharose affinity purified r210FGF-2 with a serum raised against residues 65 to 88 in the 210 amino acid sequence (lane 1) and with an anti-peptide serum raised against residues 39 to 53 in the sequence of the r210FGF-2 (lane 2). Arrows show the truncated form of r210FGF-2 (trFGF-2) and the N-terminal peptide (N-peptide) resulting from the cleveage of the r210FGF-2. (C) Shematic representation of the peptide residues recognized by the two different sera.

directly applied to heparin-Sepharose affinity chromatography. Fig. 2 shows SDS-PAGE analysis of individual fractions from the heparin-Sepharose affinity chromatography, visualized by Coomassie blue staining. The apparent molecular weight of r210FGF-2 was 24 kDa but an additional major contaminant of about 17 kDa (Fig. 2A) was routinely observed. This additional 17 kDa heparin binding protein was related to FGF-2 since it was recognized by serum directed against the peptide from residues 65 to 88 in the r210FGF-2 sequence (Fig. 2B, lane 1). In order to further characterize the r210FGF-2, we developed a specific polyclonal antibody directed against a peptide only present in the 210FGF-2 form (residue 39 to 53 in the r210FGF-2 sequence). In immunoblot analysis (Fig. 2B), this serum recognizes the 24 kDa form and an additional 6.5 kDa peptide, but not the 17 kDa form (Fig. 2B, lane 2). This result suggests that, even if all steps were done in the presence of a cocktail of protease inhibitors, a proteolysis event occurred during preparation. The 6.5 kDa peptide must correspond to the N-terminal part of 210FGF-2 and the 17 kDa form to the C-terminal part (Fig. 2B). Further characterization of the 17 kDa form was done using Edmann degradation (Table 1). The sequence established that the N-terminus corresponds to residue 63 in the sequence of r210FGF-2 leading to a protein of 148 amino acids. The 148 amino acid form could not result from an internal initiation of the translation since there is no methionine or other unusual initiation codon near the cleavage site (SIT$^{\downarrow}$TLP). The same truncated form was also observed in extracts of eukaryotic cells producing the 210FGF-2 form (data not shown) and was described as artefactual degradation occurring during storage of tissues at −20°C [28,33]. Futhermore, we and others never identified such a maturation product after the 155FGF-2 form production suggesting that the processing recognition site is not fully present in the 155 amino acid form [15,18,19]. So the 148 amino acid form seems to be a specific degradation product of the high molecular weight form of FGF-2. The three dimentional structure of the high molecular weight form of FGF-2 could allow the exposure of this sequence that would become a highly sensitive endoproteolytic site. Nevertheless we cannot exclude that the 210FGF-2 presents a self-cleaving activity in its NH_2-terminus domain as has been demonstrated for nucleolin, a protein that also exhibits a glycine-arginine-rich domain [34]. In all events

Table 1
Amino-terminal sequences of rFGF-2 isolated from *E. coli*, expressed in amino acid single letter code

210 amino acid FGF-2	GDRGR
210 amino acid rFGF-2 predicted from gene[+]	MGDRGR
146 amino acid FGF-2#	PALPE
trFGF-2	(T)LPALPE
heFGF-2*	TLPALPE

[+]The predicted sequence of human basic FGF based on the nucleotide sequence of the basic FGF gene [22].
#The sequence of the bovine brain and pituitary basic FGF as determined [1].
*The sequence of a trypic peptide of heFGF that has been isolated after extraction at neutral pH [38].

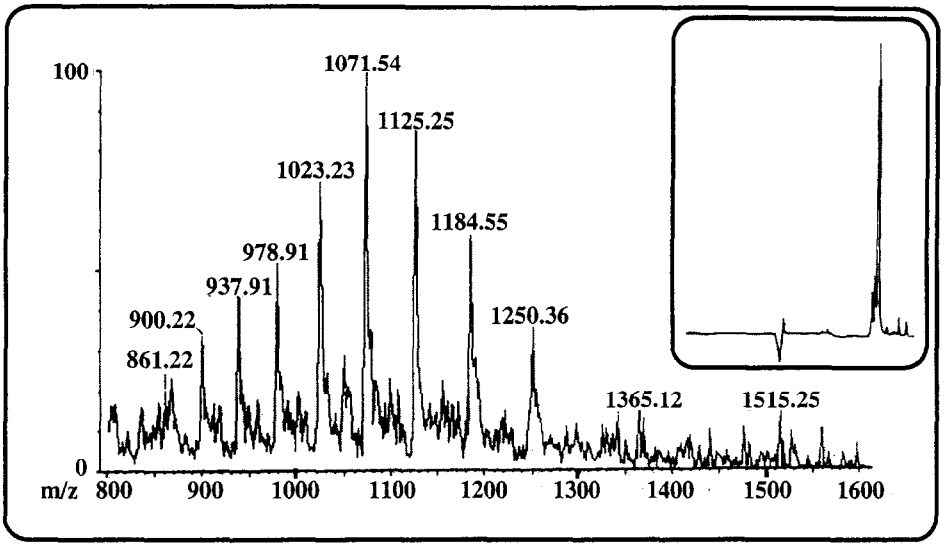

Fig. 3. Electrospray mass spectrometric analysis of r210FGF-2 purified by HPLC. All peaks correspond to the same protein associated with a variable number of protons. The calculated molecular mass was 22,485.75 Da. Inset: HPLC elution pattern of the 210 amino acid form containing in the HSAC fraction. The protein was injected onto a C4 column and eluted in 20 min (1.0 ml/min) with a linear gradient of 20% to 60% acetonitrile containing 0.1% trifluoroacetic acid.

the presence of the 17 kDa form in FGF-2 producing cells must be correlated to the synthesis of the large forms.

3.3. Structural characterization

Structural characterization of the r210FGF-2 purified from the bacterial cells was done using a mass spectrometric technique which allowed accurate determination of the molecular masses of large biomolecules.

Fractions from the heparin-Sepharose affinity chromatography were subjected to reverse phase HPLC analysis. The Fig. 3 inset shows a typical chromatogram obtained using a C4 column. Analysis of the major peak by SDS-PAGE and immunoblotting revealed homogenous r210FGF-2 (data not shown). The minor peaks were unrelated contaminants or correspond to the 17 kDa FGF-2 form present in the heparin-Sepharose affinity chromatography fractions. A sample of r210FGF-2 was subjected to electrospray mass spectrometry. Multiply charged ions were obtained allowing calculation of the molecular mass (Fig. 3). The average molecular mass of 22,485.75 ± 4.3 Da agreed with that predicted on the basis of the amino acid sequence (22,487.8; with the mass of the initial formylmethionine that is not present in the mature protein substracted [35]).

3.4. Purification of biological active r210FGF-2 to homogeneity

A further purification step was carried out by running the r210FGF-2 fractions, from the heparin-Sepharose

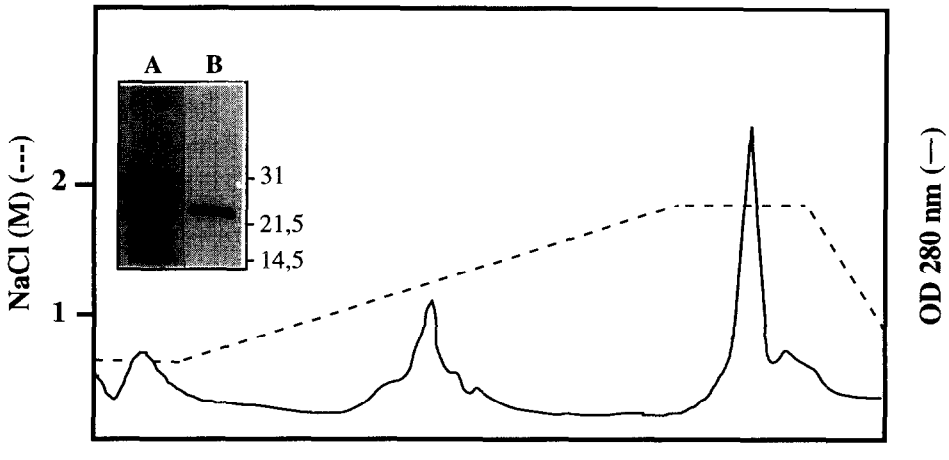

Fig. 4. Cationic exchange S-Zephyr chromatography and SDS-PAGE analysis of the eluted fractions. The r210FGF-2 (fraction from the heparin-Sepharose affinity chromatography) was purified on anion exchange chromatography, S-Zephyr (Sepracor) using a linear gradient from 0 to 2 M NaCl. Inset: (A) Coomassie blue staining of the major pic. (B) Immunoblotting with anti-FGF-2 antibody and peroxidase linked second antibody.

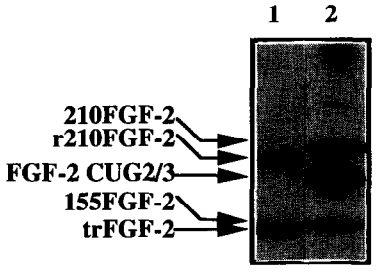

Fig. 5. Immunoblot analysis of FGF-2 proteins produced in *E. coli* or SK-Hep1 cells. Immunoblot analysis of FGF-2 proteins was carried out with an anti FGF-2 antibody (Oncoscience). Lane 1 = purified protein expressed in *E. coli*; lane 2 = FGF-2 from SK-Hep1 producing cells.

affinity chromatography, on anion exchange chromatography, S-Zephyr (Sepracor). The samples were desalted using gel filtration and applied to the column. r210FGF-2 protein was separated from the 17 kDa form by a linear salt gradient (0 to 2 M NaCl) as shown in Fig. 4. The Fig. 4 inset indicates that the major pic was r210FGF-2 as revealed by silver staining (lane A) and immunoblotting (lane B). This purified form was used as follows to test biological activities.

3.5. Comparison of r210FGF-2 with the protein produced in eukaryotic cells

Fig. 5 shows that the r210FGF-2 produced in *E. coli* (lane 1) has a smaller apparent molecular weight than the 210FGF-2 naturally expressed in SK-Hep1 (lane 2). The apparent size of the proteins expressed in mammalian cells was approximately 1 kDa higher than r210FGF-2. We suspected a post-translational modification of the high molecular weight form of FGF-2 as was already suggested [36]. According to Florkiewicz this modification is specific of the high molecular weight forms since 155FGF-2 is not modified; phosphorylation was ruled out [36]. So we suggest that the post-translational modification of the 210FGF-2 is located in the N-terminal domain. The only post-translational modification as yet identified in the N-terminal part of the high molecular weight FGF-2 is the dimethylation of arginines which takes place in glycine-arginine blocks [24]. The additional mass brought by the dimethylation of arginines (11 putative sites) cannot totally explain the 1 kDa difference between 210FGF-2 and r210FGF-2. Conformation induced by methylation could explain the observed abnormal electrophoretic migration. However additional uncharacterized modifications cannot be excluded.

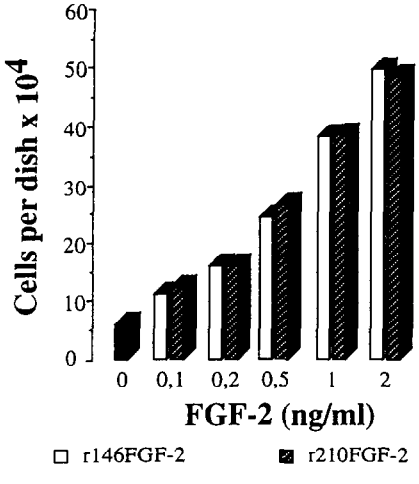

Fig. 6. Biological activity of the r210FGF-2 in ABAE cells. 10^4 cells were seeded per 35 mm tissue culture dishes, r146FGF-2 or r210FGF-2 were added every two days to the indicated final concentration. On day 4, the cells were harvested and counted.

3.6. Biological assay

S-Zephyr purified r210FGF-2 was tested for its mitogenic activity on ABAE cells and compared with the r146FGF-2 (Fig. 6). All proteins presented the same ability to support growth and were comparable to the 146 amino acid FGF-2 form isolated from tissue [37].

4. Conclusion

This paper reports the production and purification to homogeneity of biologically active r210FGF-2. The protein is synthetized in the native form from a T7 RNA polymerase expression vector. Structural characterization by mass spectrometric analysis reveals a correct molecular mass with regard to the primary sequence. The high molecular weight molecule of FGF-2 is however susceptible to maturation into a 148 amino acid form. The complexicity of FGF-2 with four proteins encoded by a single mRNA is poorly understood and our system provides an abundant source of new recombinant FGF-2 to analyse structure/function or to test particular biological activities of the 210 amino acid FGF-2 form.

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