Endogenous basic fibroblast growth factor isoforms involved in different intracellular protein complexes

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Four forms of basic fibroblast growth factor (bFGF or FGF-2) result from an alternative initiation of translation involving one AUG (155-amino acid form) and three CUGs (210-, 201- and 196-amino acid forms). These different forms of bFGF show different intracellular biological activities. To identify their intracellular targets, the 210- and 155-amino acid forms of bFGF were independently transfected into CHO cells and their correct subcellular localizations were verified, the 155-amino acid bFGF form being essentially cytoplasmic whereas the 210-amino acid protein was nuclear. The radiation fragmentation method was used to determine the target size of the different bFGF isoforms

in the transfected CHO cells and to show that the 210- and 155-amino acids bFGF isoforms were included in protein complexes of 320 and 130 kDa respectively. Similar results were obtained using the SK-Hep1 cell line, which naturally expressed all forms of bFGF. Co-immunoprecipitation assays using different chimaeric bFGF–chloramphenicol acetyltransferase proteins showed that different cellular proteins are associated with different parts of the bFGF molecule. We conclude that bFGF isoforms are involved in different molecular complexes in the cytosol and nucleus, which would reflect different functions for these proteins.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a member of the heparin-binding growth-factor family which is composed of nine proteins including the two prototypes acidic FGF (FGF-1) and bFGF (FGF-2) [1–3]. bFGF is a pleiotropic growth factor exhibiting various biological activities such as proliferation and differentiation of a wide range of cells of mesenchymal and neuroectodermal origin, wound healing and angiogenesis during embryological and developmental processes [4]. The biological activity of exogenous bFGF is mediated by a family of high-affinity cell-surface protein tyrosine kinase receptors that are encoded by four human genes [1,5]. Low-affinity heparan sulphate proteoglycan-binding sites, both on the cell surface and in the extracellular matrix, participate in the cellular response to exogenous bFGF [6].

We have previously shown that initiation at one AUG [155 amino acids (aas)] and three CUG (210, 201 and 196 aas) sites is responsible for multiple bFGF isoforms [7]. The AUG-initiated protein is cytoplasmic, whereas the CUG-initiated forms are nuclear [8,9]. A specific role in oncogenesis for the CUG-initiated bFGF forms has been proposed [10,11]. Moreover only the AUG-initiated bFGF is able to stimulate cell migration [12], to down-regulate its own receptor [13] and to stimulate integrin synthesis [14]. On the other hand, the nuclear CUG-initiated forms are involved in cell proliferation by a cell-surface receptor-independent pathway [13]. Concurrently with bFGF, the intracellular route has been recently analysed for acidic FGF [15,16], and the results strongly suggest two distinct but complementary endogenous and exogenous pathways [17].

The mechanism of action of the bFGF isoforms is sufficiently different to postulate that these proteins are able to interact with

different intracellular macromolecular components. The method of radiation fragmentation was used to examine further the status of the different bFGF isoforms. This technique has been used to determine the target size (TS) or functional mass of a large number of proteins [18,19] and, more recently, RNA [20]. It is particularly useful because it provides information about the protein without requiring its purification; thus TS can be determined even for complex unpurified samples such as whole frozen cells [21] and membranes [22]. Briefly, TS determination is based on the observation that absorption of ionizing radiation by the protein causes extensive breakage of covalent bonds leading to its complete inactivation, and a Poisson distribution function governs the probability of collision in relation to molecular mass. The estimated size is unaffected by lipid association or covalently bound carbohydrates [23].

The radiation-fragmentation method was used to determine the TS of the different bFGF isoforms in the whole cell. For this experiment, cell lines expressing the 210- or 155-aa bFGF form independently were established. Our results suggest that these bFGF isoforms exist as different molecular complexes. Co-immunoprecipitations were carried out that demonstrated that different proteins can strongly interact with different domains of bFGF proteins. These results indicate that the different complexes could involve protein species and may be related to distinct subcellular localizations and biological functions.

MATERIALS AND METHODS

Plasmid construction

The construction of vectors allowing permanent expression of the 210-aa bFGF, (pRFENeo) or the 155-aa bFGF (pRF11AEN)

Abbreviations used: TS, target size; bFGF, basic fibroblast growth factor; CAT, chloramphenicol acetyltransferase; PBS-CMF, Ca²⁺- and Mg²⁺-free PBS; ECL, enhanced chemiluminescence assay; aa, amino acid.

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has been detailed in a previous report [24]. Vector control was similar to pRFENeo or pRF11AEN but without the bFGF gene.

FGF2–chloramphenicol acetyltransferase (CAT) plasmid constructions

The basic pSCTCAT plasmid which contains the CAT gene under the control of cytomegalovirus promoter was used to subclone each FGF2–CAT construct [25]. Human bFGF fragment *XhoI*–*ApaI* corresponding to the N-terminal translated sequence upstream of the AUG initiation codon was inserted in frame with the CAT gene, lacking its own initiation codon, at the *ApaI* site by PCR amplification as already described [8]. The resulting pSCT4 plasmid encoded three fusion proteins initiated at the three CUG codons comprising the N-terminal sequence up to aa 52 in frame with CAT (see Figure 5B; *ApaI* site). To construct pSCT5 encoding only the 36 first FGF aas in frame with CAT, *NcoI*-site-creating oligonucleotides generated an AUG initiation codon in place of the CUG1 and *ApaI* site at position 424 from nucleotide +1 of the transcription-start site, allowing CAT fusion. The pSCT6 comprised the *NcoI* site at position 434 to create an artificial AUG initiation codon and encoded the FGF sequence 36–52 aa in frame with CAT at the native *ApaI* site. The protein derived from pSCT70 included the entire 18 kDa coding sequence in frame with CAT as previously described [8].

Cell culture and transfection

CHO cells were grown as previously described [24]. Some 2×10^5 cells per 50 mm plate were permanently transfected with a calcium phosphate precipitate containing 2 µg of plasmid DNA. After 48 h culture, the cells were cultured in the presence of 1 mg/ml G418 (Geneticin; Gibco). Two weeks later, G418-resistant clones were isolated, grown on and tested for the expression of bFGF by immunoblot analysis. The SK-Hep1 human hepatoma cell line was grown in Dulbecco modified Eagle's medium (Gibco) containing 10% foetal calf serum and antibiotics.

COS-7 cells were plated at 3.5×10^5 cells per 50 mm dishes and transiently transfected using the DEAE-dextran method as previously described [26] before labelling and immunoprecipitation.

Immunocytochemical analysis

Transfected CHO cells grown for 24 h on glass coverslips were fixed in 3% paraformaldehyde in PBS and permeabilized in 0.05% Triton X-100 and 0.05% saponin. After preincubation in goat non-immune serum, the coverslips were incubated for 1 h with a 1:144 dilution of serum against bFGF (a gift from N. Quarto, Naples, Italy). After being washed, immune complexes were detected with FITC-conjugated goat anti-rabbit IgG (Nordic Immunology) and visualized by fluorescence microscopy under a Leitz Ortholux II microscope after being mounted in 50% glycerol.

Co-immunoprecipitation

At 48 h after transfection, cells were incubated in methionine- and cysteine-free medium for 45 min and then pulse-labelled for 4 h with [³⁵S]methionine/cysteine (150 µCi/ml; Trans-label; ICN). At the end of each pulse, cell pellets were washed with Ca²⁺- and Mg²⁺-free PBS (PBS-CMF), resuspended in 200 µl of lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) containing 1 mM PMSF, 1 µM aprotinin and

5 µg/ml DNase-1, and then allowed to lyse for 1 h at 4 °C. After centrifugation for 15 min at 13 000 g (10 000 rev./min), the supernatants were treated with non-immune serum and 25 µl of Protein A–Sepharose (Pharmacia) for 30 min. The pellets were then discarded and the supernatants incubated with antibodies directed against CAT (Tebu) and 25 µl of Protein A–Sepharose. After 1 h and two PBS washes, the pellets were resuspended in 20 µl of sample buffer containing fresh dithiothreitol and 2-mercaptoethanol, then heated at 60 °C for 10 min. Immuno-complexes were analysed by SDS/PAGE (12% gel) and autoradiographed for 48–72 h after drying.

Irradiation procedure

Confluent cells were washed with cold PBS-CMF, scraped with a rubber 'policeman', distributed equally (10^6 cells/tube) in Eppendorf microcentrifuge tubes, centrifuged at 400 g for 10 min and the supernatant discarded. Pellets were gently homogenized with 50 µl of PBS-CMF containing 20% glycerol. The tubes were then frozen and stored at –80 °C. The frozen samples were irradiated in a Gammacell model 220 irradiator as previously described [27]. An empirical equation resulting from calibration with standard proteins was used to compute TS:

$$TS = (1.77 \times 10^6)/D_{37}$$

D_{37} is the dose in Mrad necessary to decrease the measured protein to 37% of its initial amount. The D_{37} of each protein was obtained from the curve of the Napierian logarithm of the remaining intact protein versus radiation dose [27].

Protein extraction and immunoblot analysis

Irradiated cells were homogenized with 150 µl of PBS-CMF containing 0.2% SDS for the CHO cells, 50 µl for the SK-Hep1 cells and lysed by sonication (3×10 s) in ice. An aliquot was removed for protein determination by the bicinchoninic acid protein assay (Pierce). Aliquots (5 µg for the CHO cell extracts and 15 µg for the SK-Hep1 cell extracts) were analysed by SDS/PAGE (12% gel), and then transferred to a nitrocellulose filter. bFGF was detected by using an anti-bFGF antibody (1:200 dilution; Oncogene Science Inc.) and visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham).

RESULTS

Construction and characterization of cell lines constitutively expressing bFGF

The vectors pRFENeo and pRF11AEN encoding the 210- and 155-aa bFGF forms respectively, and the control vector were transfected into CHO cells. G418-resistant clones were picked after two weeks. About ten clones for each construct were isolated and grown on. As can be seen in Figures 1(A) and 1(B), no bFGF can be detected in wild-type CHO cells or in control-vector-transfected cells. bFGF was expressed at various levels in all pRFENeo- and pRF11AEN-transfected clones. Two clones expressing a high level of bFGF were chosen for further experiments (Figure 1A, lane 7 and Figure 1B, lane 7). Transfected cells were morphologically different from the parental cells (Figure 1C). CHO cells expressing 210-aa bFGF (Figure 1C, CHO/210) adhered poorly and grew in a disorganized pattern. CHO cells expressing 155-aa bFGF (Figure 1C, CHO/155) looked larger and more organized than the parental cells. The growth properties (generation time) of the transfected cells and parental cells were identical.

Figure 1 Immunoblot analysis and morphology of transfected CHO cells

Cell extracts were subjected to SDS/PAGE and then transferred to a nitrocellulose filter. Blots were probed with rabbit anti-bFGF antibody and an ECL kit. **(A)** CHO cells transfected with the 210-aa bFGF (pRFENeo). Lanes 1–4 and 6–9, different clones of transfected cells; lane 5, wild-type CHO; lane 10, CHO cells transfected with control vector (without bFGF gene). **(B)** CHO cells transfected with the 155-aa bFGF (pRF11AEN). Lane 1, wild-type CHO; lanes 2–9, different clones expressing the 155-aa bFGF; lane 10, recombinant 146-aa bFGF. **(C)** Morphology of transfected cells: phase-contrast micrographs of untransfected CHO cells (CHO) and CHO cells transfected with vector encoding the 210-aa bFGF form (CHO/210) or the 155-aa bFGF form (CHO/155).

Figure 2 Subcellular localization of bFGF proteins

Phase-contrast (top) and corresponding fluorescence-staining (bottom) micrographs of wild-type CHO cells (**A, B**) or CHO cells transfected with 210-aa bFGF (**C, D**) or 155 aa bFGF (**E, F**).

Localization of bFGF

Immunocytochemical analyses were performed on the two clones characterized above to determine the intracellular distribution of the different forms of bFGF. As shown in Figure 2, the 210-aa bFGF was clearly nuclear (Figures 2C and 2D). Immunocytochemical analysis of cells expressing only the 155-aa bFGF form (Figures 2E and 2F) revealed a more diffuse pattern. Compared with the parental cells, which did not express bFGF (Figures 2A and 2B), and the staining of cells producing the 210-aa bFGF form, we concluded that the 155-aa bFGF form was present in whole cells and predominantly in the cytosol.

TS analysis

The classical experimental procedure to determine TS is to

irradiate a sample preparation with various radiation doses and to follow the decrease in protein-specific intensity or radioactivity related to the protein studied.

In this study, the TS analysis was applied to frozen cells under conditions that ensure their viability. The feasibility of such an investigation has already been described [21]. However, it was essential to test if the use of an antibody to detect the remaining protein was suitable. Purified proteins added to a disrupted cell preparation (wild-type CHO cells) were used to study antibody response by analysis of two parameters: bFGF concentration and exposure time after ECL visualization of the immunoblot. The antibody response was evaluated by densitometric scanning (Biocom) and appeared to be linear between 0.05 and 0.4 ng of bFGF for 2.5 min exposure (results not shown). This control scale was reproduced for each experiment.

Figure 3 Determination of the TS of bFGF proteins in transfected CHO cells

Quantification of bFGF proteins in irradiated samples of CHO cells transfected with 210-aa bFGF (A) or 155-aa bFGF (B). Intensities of bFGF immunoreactive bands (top) were scanned with a densitometer and reported as integrated values expressed in arbitrary units (bottom) according to the radiation dose. (C) Determination of the TS of bFGF proteins in transfected CHO. The percentage of the remaining protein measured by densitometric scanning as shown in (A) and (B) was plotted as a logarithmic function (Ln) of the radiation dose. Analyses were performed independently for the 210-aa bFGF ($\square$) and the 155-aa bFGF ($\blacklozenge$). The results are a typical determination from three separate analyses.

TS analysis of bFGF expressed in transfected CHO cells

The two CHO cell lines expressing the 210- or the 155-aa bFGF independently were used to determine TS. After exposure of the cells to increasing doses of radiation, the remaining bFGF was quantified by immunoblot analysis (Figure 3). We found a progressive decrease in the amount of bFGF in the two cell lines studied. Higher doses were necessary to visualize the decrease in the 155-aa bFGF (above 30 Mrad; Figure 3B) compared with the 210-aa bFGF (Figure 3A). The plot of the Napierian logarithm of the integrated peak area of the immunoblot bands (Figure 3C) as a function of the radiation dose gave a TS of 317 ± 32 kDa and 132 ± 11 kDa for the 210- and 155-aa bFGF proteins respectively (mean $\pm$ S.D.; $n = 4$). It should be noted that no fragmentation products were observed. The protocol could therefore be applied to cells that express all forms of bFGF without contamination of the lower bands with fragmentation products of the upper ones.

TS analysis of bFGF expressed in SK-Hep1 cells

Because previous results were obtained with non-human transfected cells, we investigated the TS of bFGF in the SK-Hep1 cell line, a well-characterized human strain that naturally expresses all bFGF isoforms. Figure 4 shows the immunoblot analysis of the bFGF isoforms present in SK-Hep1 cells and the decrease in the amount of protein for each form versus radiation dose. Analysis yields a TS of 270 ± 60 kDa for the CUG1-initiated bFGF (210 aas), 180 ± 20 kDa for CUG2/3-initiated bFGF (these two proteins of 201 and 196 aas were unresolved in our SDS/PAGE conditions) and 80 ± 11 kDa for AUG-initiated bFGF (155 aas) protein (mean $\pm$ S.D.; $n = 4$).

Figure 4 Determination of the TS of bFGF proteins in SK-Hep1 cells

(A) bFGF analysis was performed by immunoblotting. (B) TS was calculated for the three major immunoreactive bFGF forms from the values determined by densitometric scanning: CUG1-initiated form, 210-aa bFGF ($\blacklozenge$); CUG2/3-initiated forms, 201- and 196-aa bFGF ($\blacksquare$) (these two forms were not resolved by SDS/PAGE); AUG-initiated form, 155-aa bFGF ($\square$). Expression of the results is the same as in Figure 3. The results are a typical determination from three separate analyses.

Figure 5 Co-immunoprecipitation of cellular proteins with bFGF-CAT fusion proteins

(A) After COS transfections with constructs coding for different bFGF-CAT fusion proteins, cells were labelled with [35 S]methionine/cysteine, then co-immunoprecipitated with anti-CAT antibodies. Total proteins were resolved by SDS/PAGE, then autoradiographed for 48–72 h. The first two lanes correspond to transfected COS cells encoding the CAT protein alone (CAT) and untransfected COS cells (NT) as controls of non-specific immunoprecipitation. Lanes 4, 5, 6 and 70 represent immunoprecipitation of transfected COS cells encoding respectively the pSCT4, 5, 6 and 70 CAT fusion proteins. Standard molecular sizes are shown on the left and co-immunoprecipitated proteins are arrowed on the right. (B) Diagram of each bFGF-CAT fusion protein with CUGs and AUG as natural initiation codons, and AUG* as artificial initiation codons to synthesize pSCT5 and pSCT6 bFGF-CAT proteins.

Co-immunoprecipitation of specific proteins with bFGF

To confirm *in vivo* the presence of specific complexes depending on the bFGF form, we carried out co-immunoprecipitation assays after COS transfections with different bFGF-CAT fusion proteins (see Figure 5B and the Materials and methods section). One such fusion of 43 kDa encoded by pSCT70 allowing the expression of the whole AUG-initiated form (155 aas) in fusion with CAT was used to detect proteins specifically associated with the common part of the four bFGFs. Other fusion proteins were constructed to determine whether the N-terminus of the CUG-initiated forms is involved in specific complex-formation. In the pSCT4 plasmid, the 5' end of bFGF (*Apa*I site, codon 52) was fused to the entire CAT open reading frame. This plasmid encoded three chimaeric FGF-CAT proteins (29, 28, 27.5 kDa) initiated at the three native CUG codons (Figure 5B). In the pSCT5 plasmid, the first CUG-initiation codon was mutated into an AUG, allowing the translation of the first 36 bFGF N-terminal residues fused to CAT. The protein encoded by pSCT6 comprised the bFGF residues 35–52 in frame with CAT. Immunoprecipitations were completed after [35 S]methionine/cysteine labelling *in vivo* of transfected COS cells using anti-CAT antibodies. Each chimaeric protein was overexpressed and migrated on SDS/PAGE at its predicted size (Figure 5A). In

comparison with the immunocomplex with the control CAT protein-transfected cells or the untransfected cell extracts (lanes CAT and NT), two additional labelled bands migrating at about 76 and 34 kDa could be detected in the pSCT70-CAT immune complex (Figure 5A, bands e and d). A major and two minor proteins of respectively 36, 32 and 31 kDa would be specifically associated with the pSCT4 FGF-CAT protein (Figure 5A, bands c, b and a). Meanwhile the N-terminus-truncated pSCT5-CAT and pSCT6-CAT chimaeric proteins presented an immunoprecipitation pattern similar to the controls. These results suggest that 155-aa and 210-aa bFGF are specifically associated with different kinds of protein and confirm the above results obtained by TS analysis.

DISCUSSION

Given the increasing importance of endogenous isoforms in bFGF biological activity, in addition to classical signalling through cell-surface receptors, we aimed to study the molecular state of the different bFGF isoforms *in vivo* using the technique of radiation fragmentation.

In its simplest form, the target theory of radiation fragmentation predicts an exponential decrease in complex integrity with the radiation dose. The TS is the mass of 1 mol of polypeptide, the backbone chain(s) of which has been broken down as a result of a single hit by an ionizing radiation. Energy may be transferred between subunits in an oligomeric protein complex. In this case, the TS is the sum of the sizes of all the proteins in which the energy has been transferred because they are physically associated. This technique applied to protein *in situ* has allowed the detection of native complexes in their cellular environment, thus avoiding purification protocols [18].

Under these conditions of direct complex analysis, we found that the different bFGF isoforms belong to different protein complexes, of which bFGF is a member. In transfected CHO cells expressing the 210- or 155-aa bFGF form independently, the 210-aa bFGF was involved in a complex of nearly 320 kDa whereas the 155-aa bFGF was involved in a complex of 130 kDa.

The next interesting result was that the TS analysis of bFGF isoforms naturally expressed in human hepatoma cells gave similar results: the sizes of the molecular protein complexes were of the same order of magnitude, CUG1-initiated protein being involved in a complex of nearly 270 kDa and AUG-initiated protein in a complex of 80 kDa. This cell model also revealed that the CUG2/3-initiated proteins were involved in another complex of 180 kDa.

The subcellular localization of the different bFGF isoforms was confirmed in CHO cell lines and has previously been described for the SK-Hep1 cell [9]. We showed that the 210-aa bFGF in the transfected CHO cells was nuclear whereas the 155-aa form was mainly cytoplasmic. Several studies of endogenous bFGF in SK-Hep1 cells revealed that the 18 kDa form is found primarily in the cytosolic fraction, whereas the 22 and 24 kDa forms occur preferentially in the ribosomal and nuclear fractions [9]. A recent report has shown a tight association of each bFGF isoform with polyribosomes but the distinct targets, rRNA or proteins, were not characterized [28]. All bFGF isoforms were also found associated with the membranes [29].

Thus the bFGF isoforms are part of different molecular protein complexes depending on their subcellular localization and potential biological function. The results (summarized in Table 1) suggest that the various isoforms of bFGF exist as relatively large protein complexes. One possibility is that they represent aggregates of a single monomer. The second possibility is that bFGF forms complexes with one or more protein species.

Table 1 TS of the different forms of bFGF in the two cell lines studied

nd, Not determined.

bFGF isoforms		TS (kDa)	
(aa)	(kDa)	SK-Hep1 cells	CHO cells
210	22.6	270 ± 60	317 ± 32
201/196	21.7/21.2	180 ± 20	nd
155	17.2	80 ± 11	132 ± 11

Immunoprecipitation assays appear to confirm this possibility, since FGF binding involves different proteins depending on the nature of the isoform. Additional proteins co-immunoprecipitated with the form that included only the complete N-terminal sequence (Figure 5A, lane 4) were not identical with those associated with the smaller 155-aa chimaeric protein (Figure 5A, lane 70). This terminal extension is indeed organized in one glycine-arginine-rich domain encoded by the pSCT5 plasmid and another including the nuclear localization sequence encoded by pSCT6 [8]. The fact that no unusual molecules were found associated with these N-terminal-truncated forms (pSCT5 and pSCT6) under these immunoprecipitation conditions may reflect the required presence of an integral N-terminus as a protein-binding domain. Nevertheless these results suggest several binding domains involving both the N-terminal part and the common part of all the bFGF isoforms. They also confirm the radiation-fragmentation results, with differential involvement of each bFGF form depending probably on its specific cellular localization and consequently on the presence of the N-terminal extension.

Except for the high-affinity receptors (bFGF binding with low-affinity receptors by sugar-protein interaction cannot be detected with this approach), only one report of protein able to interact with the bFGF protein has been published and concerns *in vitro* binding casein kinase 2 leading to stimulation of its kinase activity [30]. Unfortunately the β -subunit contained in the heterotetramer casein kinase 2, which is involved in the bFGF complex, cannot be distinguished from one of the FGF-CAT forms on account of its similar molecular mass of 28 kDa. Casein kinase 2 could be putative nuclear target of bFGF and it would be interesting to confirm this interaction *in vivo*, particularly so because casein kinase 2 is heavily involved in mitogenic signalling. These studies are in progress.

High-affinity receptors do not appear to be involved in endogenous bFGF molecular complexes, since there is little or no FGF receptor in the CHO cell line [6], and the molecular masses of complexes that contain bFGF in transfected CHO and SK-Hep1 cells (expressing FGF receptor) are comparable. It would be of major interest to identify the endogenous bFGF-associated proteins. Characterization of these proteins may indicate the

mechanisms by which the different forms of bFGF in their distinct subcellular locations have specific functions in producing cells.

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