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Fibroblast growth factor 3, a protein with a dual subcellular fate, is interacting with human ribosomal protein S2

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Abstract

The secreted isoform of fibroblast growth factor 3 (FGF3) induces a mitogenic cell response, while the nuclear form inhibits cell proliferation. Recently, we identified a nucleolar FGF3-binding protein which is implicated in processing of pre-rRNA as a possible target of nuclear FGF3 signalling. Here, we report a second candidate protein identified by a yeast two-hybrid screen for nuclear FGF3 action, ribosomal protein S2, rpS2. Recombinant rpS2 binds to in vitro translated FGF3 and to nuclear FGF3 extracted from transfected COS-1 cells. Characterization of the FGF3 binding domain of rpS2 showed that both the Arg-Gly-rich N-terminal region and a short carboxyl-terminal sequence of rpS2 are necessary for FGF3 binding. Mapping the S2 binding domains of FGF3 revealed that these domains are important for both NoBP and rpS2 interaction. Transient co-expression of rpS2 and nuclear FGF3 resulted in a reduced nucleolar localization of the FGF. These findings suggest that the nuclear form of FGF3 inhibits cell proliferation by interfering with ribosomal biogenesis.

Keywords: Fibroblast growth factor; Nuclear targeting; Ribosomal proteins; Ribosomal biogenesis; FGF3; Nuclear localization; Ribosomal protein S2; Ribosomal biosynthesis

Fibroblast growth factors comprise one of the largest polypeptide growth factor families with currently 23 structurally related proteins. Members of this protein family display a broad range of biological activities that are vital in both the embryo and adult [1]. FGF signals have proved to be crucial for organogenesis involved in the epithelial–mesenchymal induction processes. In vitro, FGFs have the ability to induce mitogenicity, cell growth, differentiation, motility, and survival, depending on the experimental conditions. In vivo and in the adult FGF activities are important in physiological conditions including wound healing, tissue repair, and maintenance of the cell homeostasis, while inappropriate expression has been implicated in tumorigenesis and metastasis [2–8]. Most of the diverse biological actions by FGFs appear to be mediated by binding to and activation of high- and low-affinity cell surface receptors. Heparan sulfate proteoglycans function as low-affinity receptors and seem to be essential to sustain binding and dimerization of the high-affinity tyrosine kinase receptors under physiological conditions [9–12]. There is now good evidence that at least some members of the FGF protein family, including FGF1, FGF2, FGF3, and the FGF homologous factors (FHFs) 1–4 (FGF11–14), can signal by interacting with intracellular binding proteins [13,14]. FHFs lacking a classical secretory signal sequence appear to remain intracellular where they bind to cytoplasmic targets including the mitogen-activated protein kinase scaffolding protein islet brain2 (IB2) and voltage-gated sodium channels [15,16]. Despite their structural homology to FGFs, FHFs seem not to bind and activate any of the

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tyrosine kinase receptors [17]. In contrast, FGF1 and FGF3 bind to intracellular target proteins but in addition are released from cells. In case of FGF3 through the classical secretory pathway [18].

FGF3 was originally identified as a proto-oncogene in virally induced mouse mammary tumors. In situ hybridization analysis revealed that normal expression of FGF3 is primarily restricted to prenatal mouse development. FGF3 together with FGF8 is required for formation of the otic placode induction and for early development of the otocyst in mouse and fish [19]. Both factors are important during the development of posterior brain regions in mouse and patterning of the forebrain in Zebrafish. Translation of FGF3 starts at a single CUG initiation codon as the major start site and gives rise to a protein which is directed into the secretory pathway and into the cell nucleus at similar proportions. The dual subcellular localization of FGF3 which is unique amongst the FGFs is achieved by a balanced composition of classical import signals at the N-terminus allowing competition between different trafficking pathways [20,21]. While mutant FGF3 designed to be exclusively secreted interacts with cell surface FGF receptors, induces DNA synthesis, and has the ability to transform cells, mutant FGF3 exclusively targeted to the nucleus has an inhibitory effect on the cell proliferation [22]. Recently, we identified a nucleolar protein, we first named NoBP for nucleolar binding protein, as a binding partner for nucleolar FGF3 using FGF3 as bait in a yeast two-hybrid system [14]. The nucleolar protein was found to be identical with Ebp2p, a protein required for pre-rRNA processing and therefore ribosomal biogenesis [23,24]. The NoBP/Ebp2p binding domains for FGF3 exactly match the sequence motifs we earlier identified as essential for FGF3 translocation into the nucleoli. NoBP/Ebp2p transcription is upregulated during the late G1/early S phase in proliferating cells and is down-regulated in the promyelocytic leukemia cell line HL60 when induced to differentiate, suggesting that NoBP/Ebp2p is associated with a proliferation cell status and FGF3 interacting exerts an inhibitory effect via interfering with the function of NoBP/Ebp2p in the biosynthesis of ribosomes.

As interfering with the biogenesis of ribosomes may have a significant effect on the dual function of FGF3 in development, we further characterized possible target proteins of nuclear FGF3. Specifically, we demonstrate that FGF3 binds to rpS2, a ribosomal protein of the small ribosomal subunit often associated with upregulated cell proliferation and tumor cell growth [25–27]. In addition to FGF3, mammalian and Yeast rpS2 also interact with FGF1, another FGF with a known nuclear localization. Characterization of the FGF3 binding domains of rpS2 revealed that both the arginine glycine-rich N-terminus and a small C-terminal sequence are necessary for optimal FGF3 binding. Interestingly, the rpS2 interacting domain of FGF3 exactly matched the NoBP/Ebp2p binding domain.

Materials and methods

Cell culture. COS-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). For transient DNA transfections, 20 μg of purified plasmid DNA was introduced into 5×10^5 COS-1 cells by electroporation (450 V/250 μF) using a Bio-Rad Gene-Pulser. Between 48 and 72 h after transfection, the cells were harvested for immunoblot analysis or processed for immunofluorescence.

Immunofluorescence. COS-1 cells grown on glass coverslips were transfected with the appropriate plasmids, and 48 h later the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 4 min, and treated with 3% bovine serum albumin in PBS to block nonspecific binding of the antibodies. The coverslips were exposed to primary antibodies and fluorescently tagged secondary antibodies were diluted in 3% bovine serum albumin, PBS. After washing in PBS, the stained cells were mounted in 90% glycerol containing *p*-phenylenediamine and viewed with a 100× oil immersion lens on a Zeiss microscope equipped with the appropriate barrier filters for Texas red optics. Mouse FGF3 carboxyl-terminal antipeptide rabbit polyclonal serum were diluted 1 in 200 in PBS. β-Galactosidase was detected using a monoclonal antibody, kindly supplied by H. Durban, Imperial Cancer Research Fund, and rpS2 was detected using a mouse monoclonal antibody against the $RGS(his)_6$ tag (Qiagen).

Plasmid constructions. pS2-1.1 was constructed by inserting the anti-$RGS(his)_6$ epitope upstream and in-frame of the coding region of human ribosomal protein S2 (rpS2) cDNA. The modified human rpS2 cDNA was then inserted into the expression vector pKC4 under control of the early simian virus 40 (SV40) promoter. The vectors pKC4.16 and pKC4.18, which express mutant FGF3 proteins lacking the signal peptide, have been described previously [21].

Immunoblot analysis, immunoprecipitation, and in vitro translation. The procedures used for preparing cell lysates have been described in detail elsewhere [21]. Samples from equivalent numbers of cells were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 12.5% or 15% polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher & Schuell), and then probed with rabbit polyclonal antibody to FGF3 or a mouse monoclonal antibody against the $RGS(his)_6$ tag (Qiagen). The immunoreactive proteins were detected by enhanced chemiluminescence using horseradish peroxidase-coupled anti-rabbit immunoglobulin antibodies as described by the manufacturer (Amersham International). Mouse FGF3 and mouse FGF1 cDNAs in pGem4Z were used in an in vitro translation system (Promega) to generate ^{35}S-labelled products for use in binding assays as described in the text. Immunoprecipitation of His-tagged rpS2 and of FGF3 from transfected COS-1 cells. Transfected COS-1 cells were lysed by homogenization in ice-cold lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, and 10 mM imidazole).The lysates were incubated at 4 °C overnight with a monoclonal anti-$RGS(his)_6$ (Qiagen, Hilden, Germany) or with an anti-FGF3 peptide polyclonal rabbit antibody preabsorbed onto protein G–agarose and protein A–agarose, respectively. The precipitates were washed three times with sodium phosphate buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl), eluted in Laemmli loading buffer, and subjected to SDS–PAGE and Western blot analysis with anti-$RGS(his)_6$ monoclonal antibody or anti-FGF3 serum.

GST fusion proteins. To obtain the glutathione *S*-transferase (GST)-rpS2 constructs, DNA sequences encoding the appropriate amino acids were amplified by PCR using *Pfu* polymerase (Promega) and oligonucleotides with *Sma*I and *Eco*RI recognition sequences. The PCR products were cloned into *Sma*I–*Eco*RI-digested pGEX (Pharmacia). All expression constructs were verified by DNA sequence analysis and transformed into *Escherichia coli* DH5α for the expression of the fusion proteins.

GST-rpS2 fusion protein affinity chromatography. An overnight 30-ml *E. coli* culture containing pGEX-rpS2 or a control GST plasmid was diluted 10-fold into Luria–Bertani ampicillin medium and grown at 37 °C to an optical density at 600 nm of 1.0 before induction with 0.5 mM iso-