Pergamon

Biochemical Pharmacology, Vol. 47, No. 1, pp. 111–115, 1994.

0006-2952/94 $6.00 + 0.00

FIBROBLAST GROWTH FACTOR-2 (FGF-2) IN THE NUCLEUS: TRANSLOCATION PROCESS AND TARGETS

FRANÇOIS AMALRIC, GERARD BOUCHE,* HELENE BONNET, PHILIPPE BRETHENOU, ANA MARIA ROMAN, ISABELLE TRUCHET and NATALINA QUARTO

Laboratoire de Biologie Moleculaire Eucaryote, Institut de Biologie Cellulaire et de Genetique du CNRS, 118 Route de Narbonne, 31062 Toulouse Cedex, France

Abstract—FGF-2 (basic fibroblast growth factor) was recently detected in the nucleus of a variety of cell types. The large isoforms contain a functional nuclear localization signal that allows their nuclear accumulation in producing cells, while a small amount of FGF-2 added exogenously to target cells is translocated to the nucleus in phase G1 of the cell cycle according to an unknown process. We report here using Chinese hamster ovary cell mutants bearing deficiency in heparan sulfate proteoglycans (HSPGs) synthesis that HSPGs are required for transport of exogenous FGF-2 to the nucleus. Furthermore a co-transport was suggested since an active complex containing FGF-2 and HSPGs was isolated from nuclei of treated cells. Several FGF-2 nuclear targets were described. *In vivo* as *in vitro*, it activates rDNA transcription and it binds to a specific DNA sequence that is present in the non-transcribed spacer of ribosomal genes. *In vitro*, FGF-2 has a strong affinity for histone H1 and it activates the protein kinase CKII. In the nucleus FGF-2 could regulate gene expression through modulation of chromatin structure.

The FGF† family constitutes a family of nine structurally related polypeptides sharing 30–55% sequence homology and heparin-binding affinity [1, 2]. Acidic FGF (FGF-1) and basic (FGF-2) are the prototypes. Other members of the FGF family include the products of the oncogenes int-2 (FGF-3), hst/K-fgf (FGF-4), FGF-5, the keratinocyte growth factor KGF (FGF-7) and the more recently identified androgen induced growth factor, AIGF (FGF-8) [3] and glial-activating factor, GAF (FGF-9) [4].

FGF-2 is a pleiotropic factor with a broad range of biological activities, both *in vitro* and *in vivo*. These activities include mitogenesis, chemiotaxis, neuronal survival, neurite extension, inhibition of terminal differentiation, induction of mesoderm formation, angiogenesis and wound-healing. FGF-2 is endowed with a peculiar structure that distinguishes it from other growth factors. One of these is the lack of a consensus hydrophobic signal peptide for secretion [5]. In addition, the presence and utilization of alternative CUG codons of translation [6, 7], account for the expression of multiple FGF-2 isoforms which localize to different subcellular compartments [8–10].

Various isoforms are recovered into the nucleus. In producing cells, only the large forms (24.0, 22.5 and 22 kDa) are detected in the nucleoplasm while in cells grown in the presence of the growth factor all the isoforms are internalized, a part being recovered into the nuclear fraction [11]. The distribution of FGF-2 into the nucleus has been studied by different immunocytochemical approaches including ultrastructural ones. A variety of data have been obtained depending on the origin of FGF-2 (endogenous or exogenous), cell growth conditions, position in the cell cycle, differentiation state etc. For example, exogenous FGF-2 is associated with the nucleolus while endogenous FGF-2 is mainly associated with the non-nucleolar chromatin. These apparently discording data must result from differences in properties induced by the N-terminal domain of the large forms, or from the presence or absence of specific FGF-2 targets that allow the accumulation of the factor in nuclear subdomains [12, 13]. Characterization of the targets will allow us to gain an insight into the functions of the different FGF-2 isoforms.

The biological response of target cells to FGF-2 is mediated through a dual receptor system [14] consisting of high affinity cell-surface receptors that possess intrinsic tyrosine kinase activity [15] and low affinity receptors which are cell-surface and extracellular-matrix associated HSPGs [16].

This article will focus on two different aspects of FGF-2: internalization and nuclear translocation of FGF-2 mediated through its low affinity receptors HSPGs and nuclear targets of FGF-2.

Internalization and nuclear translocation of exogenous FGF-2 through HSPGs-mediated pathway

Several findings suggest that HSPGs function as modulators of FGF-2 activity. For example, the binding of FGF-2 to heparan sulfate chain of proteoglycan appears to protect the factor from degradation or partial proteolysis and increase the radius of FGF-2 diffusion [17–19]. Moreover, HSPGs binding is thought to be important in providing a matrix bound or cell-surface bound reservoir of FGF-2, and indeed, to promote long-term availability of FGF-2 to the cells, as well as, to ensure long-term stimulation even after a short exposure to the growth factor [20]. More recently, the role of HSPGs binding in the function of FGF-2 has been taken a step further. It has been shown that the binding of

* Corresponding author.

† Abbreviations: FGFs, fibroblast growth factors; HSPGs, heparan sulfate proteoglycans; PA, urokinase plasminogen activity; CHO, Chinese hamster ovary.

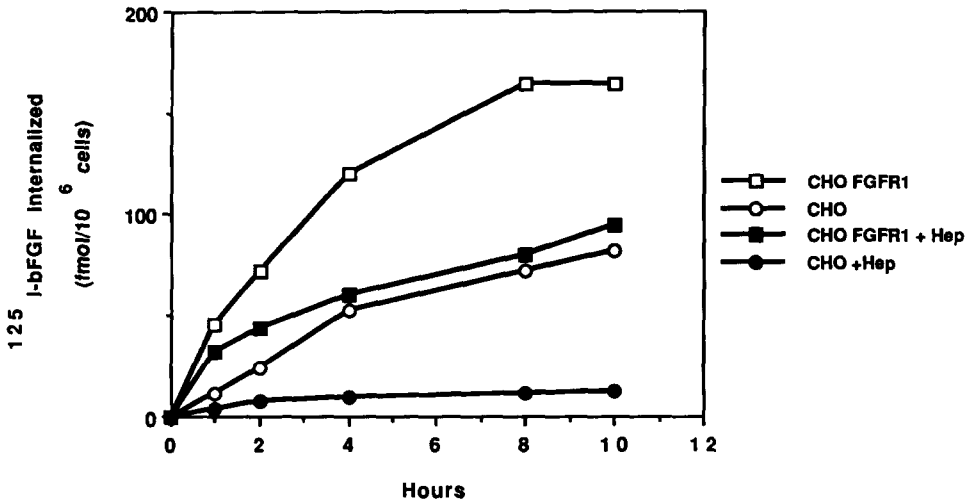

Fig. 1. Parental CHO and CHO-FGFR1 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 0.15% gelatin, 30 ng/mL ^{125}I-bFGF and with or without 10 μg/mL heparin at 37° for the indicated period. At the end of the incubation period the cells were washed three times with PBS, twice with 2 M NaCl in 20 mM Hepes pH 7.5 and twice with 2 M NaCl in 20 mM Na-acetate pH 4.0. The cells were then extracted with 0.5% SDS in PBS and the radioactivity determined.

FGF-2 to the heparan sulfate chains of a membrane HSPGs or free HS (heparin) is a "crucial prerequisite" for high affinity binding and biological activities of FGF-2 [21, 22]. So far, little attention has been focused on the potential role of HSPGs in the FGF-2 internalization process. We present here evidence that internalization of FGF-2 may also occur through an HSPGs-mediated pathway. Furthermore, the fraction of internalized FGF-2 that is translocated to the nucleus, is found associated with heparinase sensitive molecules.

FGF-2 uptake was followed in CHO cells that expressed insignificant levels of high affinity receptors and in CHO-FGFR1 transfectants, expressing at least 10 times more FGF high affinity receptor 1 (FGFR1) than parental CHO. When CHO cells expressing FGFR1 were incubated in medium containing ^{125}I-FGF-2, FGF-2 was internalized at a linear rate for up to 8 hr. Surprisingly, the parental CHO cells with very few high affinity receptors also internalized FGF-2, and accumulated about 50% as much FGF-2 after 4 hr and 80% as much after 10 hr as cells expressing FGFR1. In the presence of 10 μg/mL heparin the internalization of FGF-2 was inhibited by 40% in CHO-FGFR1 transfectants and by 80% in parental CHO cells (Fig. 1). Thus, these results suggest the existence of two different FGF-2 internalization pathways: a heparin-sensitive pathway and a heparin-resistant pathway related to the presence of FGFR1. In addition, heparin inhibits FGF-2 internalization in a dose-dependent manner mimicing its inhibitory effect on binding of FGF-2 to HSPGs. This observation further supports a direct role of HSPGs in the FGF-2 internalization process. Further evidence for internalization of FGF-2 through HSPGs is provided by CHO mutants bearing deficiency in HSPGs synthesis [23]. Internalization of ^{125}I-FGF-2 was followed in CHO 745 mutant, a cell line xylosil-transferase deficient that lacks cell-surface HSPGs, and in CHO 606 mutant that express undersulfated HSPGs. Time-course experiments demonstrated that CHO 745 mutant cells did not internalize significant amount of FGF-2 up to 10 hr, but mutant cells CHO 606, producing undersulfated HSPGs, still internalized ^{125}I-FGF-2 (Fig. 2). In these mutants, the amount of internalized FGF-2 after 4 and 10 hr, was, respectively, 50 and 60% compared to that internalized by the wild-type CHO-KI cells. Addition of 10 μg/mL of heparin inhibited internalization in CHO 606 mutant, as well as, in wild-type cells.

After internalization, a fraction of exogenous FGF-2 is translocated to the nucleus [24]. Independently, Hishihara *et al.* [25] have reported that a subpopulation of cell-surface HSPGs is also found in the nucleus. Since the data strongly suggested that FGF-2 and HSPGs are concomitantly internalized, we investigated whether an HSPG–FGF complex can be detected in the nucleus of CHO cells. HSPGs of CHO cells were labeled with $Na^{35}SO_4$/mL for 16 hr in the presence of FGF-2. A nuclear extract was prepared and fractionated on a DE-52 anion exchange column. A $^{35}SO_4$-labeled peak was eluted at 0.5 M NaCl, salt concentration at which HSPGs are usually eluted. Subsequent digestion with chondroitinase ABC and heparinase demonstrated that the $^{35}SO_4$-labeled peak was heparinase-sensitive. Thus, HSPG-like molecules are present in the nucleus of CHO cells. The presence of associated FGF-2 activity was demonstrated using

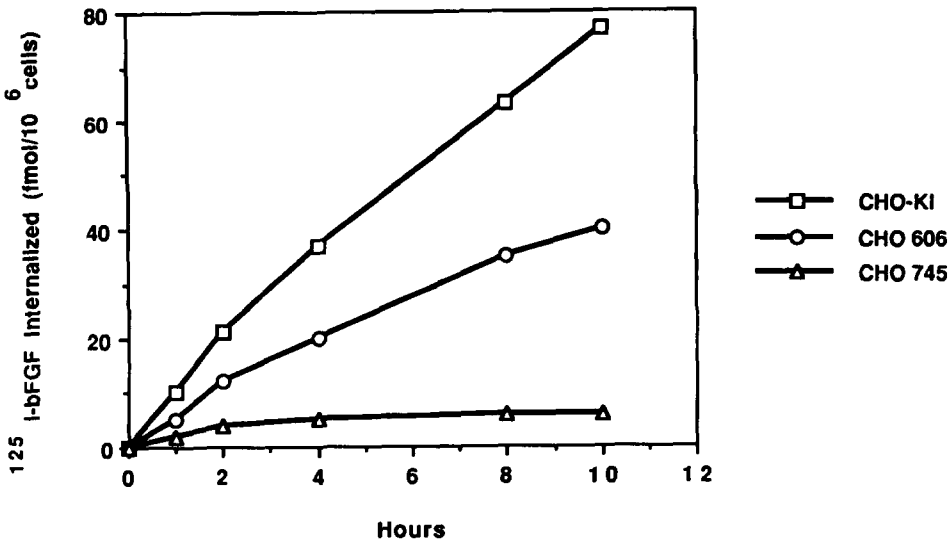

Fig. 2. Wild-type CHO-KI, CHO mutant 745 and CHO mutant 606 were incubated in DMEM containing 0.15% heparin plus 30 ng/mL ^{125}I-bFGF for the indicated periods at 37°. At the end of the incubation period cells were processed as described in Fig. 3, and the radioactivity in the extracts assessed.

different biological tests, CHO cells being negative for endogenous FGF-2 (N. Quarto). In particular, among the biological activities described for FGF-2, this fraction has the capacity to induce PA on bovine capillary endothelial (BCE) cells [26]. The DE-52 heparinase sensitive peak stimulated PA production in BCE cells 6-fold compared to control cells. This activation process was inhibited by immune anti-FGF-2 IgG. A similar stimulation of PA-activity was obtained when BCE cells were stimulated with recombinant FGF-2.

In conclusion, exogenous FGF-2 is internalized according to different pathways: endocytosis involving high affinity receptors and direct transport to the nucleus with HSPG. In addition, the large isoforms of endogenous FGF-2 are translocated to the nucleus through a nuclear localization signal.

Nuclear targets of FGF-2

Nuclear targeting of FGF-2 represents a very attractive topic that could provide crucial progress in the knowledge of mechanisms of action of this growth factor. An initial study of the action of exogenous FGF-2 on target cells, as adult bovine aortic endothelial (ABAE) and Swiss 3T3 cells, has provided attractive ideas suggesting that FGF-2 might also have other functions beside its growth factor activity. These earlier experiments correlated the cell-cycle dependent nuclear uptake of FGF-2 with an increased transcription of genes encoding ribosomal RNA (rDNA) [24]. In fact, the addition of FGF-2 to sparse quiescent ABAE or 3T3 cells induced the transition G1 to S phase of the cell-cycle after 2 and 8 hr, respectively. Iodinated FGF-2, exogenously added to these cells, was detected in the cytoplasm (90%) and in the nucleus (10%) of the cells undergoing G1–S transition. FGF-2 uptake was continuous in the cytoplasm through the cell-cycle with a maximum in G2, while nuclear uptake occurred only in late G1 [27].

Indirect immunofluorescence with affinity purified polyclonal anti-FGF-2, and electron microscopy analysis after immunoreaction using colloidal gold labeled anti-rabbit IgG showed a nucleolar localization of FGF-2 [27]. In run-on experiments carried out on nuclei purified from quiescent ABAE and 3T3 cells, addition of FGF-2 also resulted in an increased transcription of rDNA.

The observation that cell-cycle dependent uptake of FGF-2 correlated, both *in vivo* and *in vitro* to an increase of transcription of genes coding for ribosomal RNA (rDNA) raised the possibility that FGF-2 could act on rDNA transcription, either by binding directly to DNA regulatory sequences, or as part of a *trans*-acting multimeric complex. To verify this hypothesis a strategy was developed based on the purification of putative DNA–FGF complexes resulting from *in vivo* cross-linking with the reversible *trans*-dichlorodiam platinum (DDP) cross-linker. Under standard FGF-2 purification protocol, cross-linked DNA co-purified. DNA fragments were cloned and the corresponding inserts were sequenced. One of them (among 15) corresponds to a part of rDNA non-transcribed spacer. Subsequently *in vitro* experiments were carried out such as gel retardation, DNA footprinting and exonuclease III protection assays, with this DNA fragment. Two interaction sites were mapped in close vicinity suggesting that FGF-2 binds to DNA as a dimer. It is noteworthy that the binding sequence is also present in the

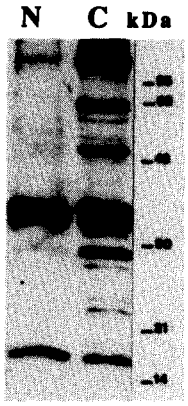

Fig. 3. ABAE cells were fractionated in cytoplasmic and nuclear fractions. After fractionation by electrophoresis, the corresponding western blot was probed with bFGF then incubated with anti FGF-2 serum and revealed by anti rabbit IgG.

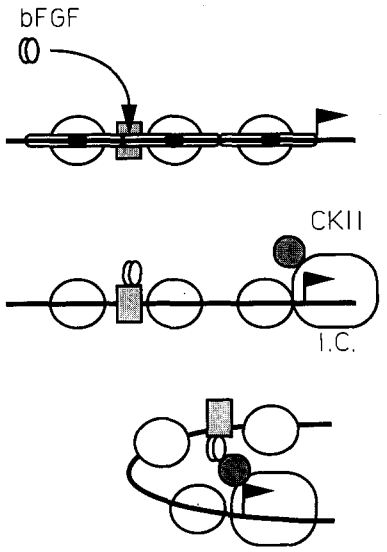

Fig. 4. Hypothetical model for FGF-2 transactivation of RNA polymerase I machinery (I.C., initiation complex).

other cross-linked DNA fragments (G. Bouche; unpublished data). This suggests that FGF-2 could bind in different parts of the genome and thus could participate in the modulation of several gene expression.

In parallel to transcription activation, addition of FGF-2 to nuclei purified from quiescent ABAE cells results in an increased phosphorylation of a subset of proteins [24]. Among them, nucleolin a substrate for CKII kinase was particularly affected. In *in vitro* experiments, using purified CKII kinase and nucleolin, FGF-2 acts as an activator of the protein kinase activity. From cells grown in the presence of biotinylated FGF-2, a complex FGF-2-kinase CKII was isolated, suggesting that these two proteins interact *in vivo*. It is known that the protein kinase CKII regulates the activity of several factors involved in rDNA transcription, as well as, transactivators of RNA polymerase.

By the same approach and by ligand blotting experiments, it appeared that FGF-2 presented strong affinity and is in direct contact with two different classes of proteins in the cytoplasm and in the nucleus (Fig. 3). In the nucleus the protein that presented the highest affinity for FGF-2 was the histone H1. Several findings suggested that histone H1 plays an important role in the repression of basal transcription. These data suggest that FGF-2 through interactions both with CKII and histone H1 could behave as a modulator of gene expression.

Indeed, a model that takes into account all the above data can be proposed for transactivation of RNA polymerase I machinery by FGF-2. Figure 4 illustrates schematically the hypothetical proposed model. It can be speculated that: (1) FGF-2 interacts with sequences located upstream to the start site of rRNA gene, counteracting the inhibition of H1 repressed promoter, by an antirepression mechanism. (2) Subsequently, FGF-2 interacts and activates CK II kinase, which regulates substrates such as nucleolin, RNA polymerase I and topoisomerase I, leading to transcriptional activation of rRNA genes.

Conclusion

FGF-2 is a signalling polypeptide involved in several biological processes. Besides its growth factor activity transduced by tyrosine kinase receptors, the factor is internalized and interacts with intracellular targets. Targets cells, such as endothelial cells, also synthesized different isoforms of FGF-2 that remained stable in the nucleus and in the cytoplasm. Thus it must be considered that a growth factor could have direct roles inside the cell.

First, it may be important to consider that FGF-2 is internalized through two pathways: (1) a high affinity receptor-mediated pathway; and (2) a low affinity receptor-mediated pathway. The studies carried out on CHO cells expressing few high affinity receptors, as well as on genetic HSPG-deficient mutants, reveal the existence of a direct role of HSPGs to internalize FGF-2, and more interestingly, their ability to translocate the exogenous FGF-2 to the nucleus. Thus, the existence of an HSPG-mediated pathway, governing internalization and nuclear translocation of FGF-2 might represent a novel and attractive concept of FGF-signal transduction. Therefore, it will be of interest to study in detail this internalization mechanism and investigate the potential signal transduction mediated by, in order to identify other important components of the cellular response, this pleiotropic factor.

Second, the study of the action of exogenous FGF-2 on responsive cells, such as ABAE and 3T3 cells, provides fascinating ideas, suggesting that FGF-2 might play other function(s) beside its growth factor activity. For example, the close relationship existing

between the appearance of FGF-2 in the nucleus and the start of DNA replication argues in favor of a direct involvement of FGF-2 in DNA-replication. Moreover, the stimulation *in vitro*, as well as *in vivo* of rDNA transcription exerted by FGF-2, and its interaction with specific rDNA sequences, strongly suggests that FGF-2 regulates directly or indirectly the transcription of some genes. The observation that CKII and histone H1 are potential targets of FGF-2 further argues in favor of this hypothesis.

Acknowledgements—We kindly thank Dr Jeffry Esko for providing us with CHO mutant cells, and Dr Claudio Basilico for the murine FGFR-1. This study was supported by grants from the Centre National de la Recherche Scientifique, Fondation pour la Recherche Médicale and Association pour le Développement de le Recherche sur le Cancer to François Amalric; Natalina Quarto was supported by fellowships from the Ministère des Affaires Etrangères and La Ligue Nationale contre le Cancer.

REFERENCES

1. Burgess WH and Maciag T, The heparin-binding (fibroblast) growth factor family of proteins. *Annu Rev Biochem* **58**: 575–606, 1989.
2. Rifkin DB and Moscatelli D, Recent developments in the cell biology of basic fibroblast growth factor. *J Cell Biol* **109**: 1–6, 1989.
3. Tanaka AK, Miyamoto N, Minamino M, Takeda M, Sato M, Matsuo H and Matsumoto K, Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. *Proc Natl Acad Sci USA* **89**: 8928–8932, 1992.
4. Miyamoto M, Naruo KH, Seko C, Matsumoto S, Kondo T and Kurokawa T, Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Mol Cell Biol* **13**: 4251–4259, 1993.
5. Abraham JA, Mergia A, Whang JL, Tumolo J, Friedman D, Gospodarowicz D and Fiddes JC, Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**: 543–546, 1986.
6. Florkiewicz RZ and Sommer A, The human bFGF gene encodes four polypeptides: three initiate translation from non ATG-codons. *Proc Natl Acad Sci USA* **86**: 3978–3982, 1989.
7. Prats H, Kaghad H, Prats AC, Klagsbrun M, Lelias JM, Liazun P, Chalon P, Tauber P, Amalric F, Smith JA and Caput D, High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc Natl Acad Sci USA* **85**: 1836–1840, 1989.
8. Renko M, Quarto N, Morimoto T and Rifkin DB, Nuclear and cytoplasmic localization of basic fibroblast growth factor species. *J Cell Physiol* **144**: 108–114, 1989.
9. Quarto N, Finger PF and Rifkin DB, The amino-terminal extention of high molecular weight bFGF is a nuclear targeting signal. *J Cell Physiol* **147**: 311–318, 1991a.
10. Bugler B, Amalric F and Prats H, Alternative initiation of translation determines cytoplasmic or nuclear localization of bFGF. *Mol Cell Biol* **11**: 543–547, 1991.
11. Amalric F, Baldin V, Bosc-Bierne I, Bugler B, Couderc B, Guyader M, Patry V, Prats H, Roman AM and Bouche G, Nuclear translocation of basic fibroblast growth factor. *Ann NY Acad Sci* **638**: 127–138, 1991.
12. Quarto N, Bouche G, Bugler B, Chailleux C, Prats AC, Prats H, Roman AM, Truchet I and Amalric F, The basic fibroblast growth factor isoforms endogenous and exogenous behavior. In: *Growth Factors, Peptides and Receptors* (Ed. Moody T), pp. 147–185. Plenum Press, NY, 1993.
13. Quarto N, Talarico D, Florkiewicz RZ and Rifkin DB, Selective expression of high molecular weight basic fibroblast growth factor confers a unique phenotype to NIH3T3 cells. *Cell Reg* **2**: 699–708, 1991b.
14. Klagsbrun M and Baird A, A dual receptor system is required for basic fibroblast growth factor activity. *Cell* **67**: 229–231, 1991.
15. Jaye M, Schlessinger J and Dionne CA, Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochim Biophys Acta* **1135**: 185–199, 1992.
16. Moscatelli D, High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J Cell Physiol* **131**: 123–130, 1987.
17. Gospodarowicz D and Cheng J, Heparin protects basic and acidic FGF from inactivation. *J Cell Physiol* **128**: 475–484, 1986.
18. Sommer A and Rifkin DB, Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J Cell Physiol* **138**: 215–220, 1989.
19. Flaumenhaft R, Moscatelli D and Rifkin DB, Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor. *J Cell Biol* **111**: 1651–1659, 1990.
20. Flaumenhaft R, Moscatelli D, Saksela O and Rifkin DB, Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth factor for long term stimulation of plasminogen activator production and DNA synthesis. *J Cell Physiol* **140**: 75–81, 1989.
21. Yayon A, Klagsbrun M, Esko JD, Leder P and Ornitz DM, Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**: 841–848, 1991.
22. Rapraeger AC, Krufka A and Olwin BB, Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**: 1705–1708, 1991.
23. Esko JD, Rostand KS and Weinke JL, Tumor formation dependent on proteoglycan biosynthesis. *Science* **241**: 1092–1096, 1988.
24. Bouche G, Gas N, Prats H, Baldin V, Tauber JP, Teissie J and Amalric F, Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing Go-G1 transition. *Proc Natl Acad Sci USA* **84**: 6770–6774, 1987.
25. Hishihara M, Fedarko NS and Conrad HE, Transport of heparan sulfate into the nuclei of hepatocytes. *J Biol Chem* **261**: 13575–13580, 1986.
26. Moscatelli D, Presta M and Rifkin DB, Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis and migration. *Proc Natl Acad Sci USA* **83**: 2091–2095, 1986b.
27. Baldin V, Roman AM, Bosc-Bierne I, Amalric F and Bouche G, Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. *EMBO J* **9**: 1511–1517, 1990.