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Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor

(expression cloning/low-affinity receptor/virus and growth factor attachment site)

MICHAEL C. KIEFER, JAMES C. STEPHANS, KEVIN CRAWFORD, KEN OKINO, AND PHILIP J. BARR

Chiron Corporation, 4560 Horton Street, Emeryville CA 94608

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ABSTRACT **Expression cloning of cDNAs encoding a basic fibroblast growth factor (FGF) binding protein confirms previous hypotheses that this molecule is a cell-surface heparan sulfate proteoglycan. A cDNA library constructed from a hamster kidney cell line rich in FGF receptor activity was transfected into a human lymphoblastoid cell line. Clones expressing functional basic FGF binding proteins at their surfaces were enriched by panning on plastic dishes coated with human basic FGF. The amino acid sequence deduced from the isolated cDNAs revealed several interesting features, including hydrophobic signal and transmembrane domains that flank an extracellular region containing six potential attachment sites for glycosaminoglycan side chains. The structure also contains a short hydrophilic cytoplasmic tail sequence homologous to previously reported actin binding domains. Binding of basic FGF to cells expressing the binding protein could be inhibited by heparin and heparan sulfate but not by chondroitin sulfate, dermatan sulfate, or keratan sulfate. In addition to binding basic FGF, this protein or related surface proteins may function as an initial cellular attachment site for other growth factors and for viruses, such as herpes simplex virus.**

Structural characterization of the fibroblast growth factors (FGFs) and molecular cloning of their cDNAs and genes has led to the identification of a family of related genes encoding several homologous proteins. Currently, the FGF family includes the prototypic acidic and basic FGFs (1–3), the product of the *int-2* proto-oncogene (4, 5), a factor isolated from sarcoma tissue DNA (*hst* or KS-FGF) (6, 7), FGF-5 (8), FGF-6 (9), and the keratinocyte growth factor (10). In addition to their primary amino acid sequence homology, each member of the family is typified by a strong affinity for heparin and heparan sulfate. This *in vitro* affinity is clearly related to the attachment of the FGFs to cell surfaces and extracellular matrices *in vivo* (11). Many biological roles for each FGF and their extreme biomedical significance have been established. For example, acidic and basic FGFs are mitogenic for a number of cell types including those of mesenchymal, epithelial, or neural origin (12, 13). Consequently, studies directed toward *in vivo* wound healing and nerve regeneration have been initiated (14, 15).

The multiplicity of characterized FGFs and their diverse spectrum of activities has suggested the possibility that several receptors might exist for this growth factor family. Indeed, for the acidic and basic FGFs themselves, two classes of receptor have been well-documented (16–19). These receptor classes are distinguished by their differing affinities for FGF. For example, binding of basic FGF to a high-affinity site on baby hamster kidney (BHK) cells occurs with a dissociation constant in the range of 20 pM, whereas basic FGF binds to the low-affinity site with a dissociation constant in the range of 2 nM and is released with 2 M sodium chloride (19). Much progress in the molecular characterization of the high-affinity FGF receptors has been reported. Molecular cloning of cDNAs for the chicken high-affinity basic FGF receptor (20, 21) has revealed a structure that includes three extracellular immunoglobulin-like domains, an unusual acidic region, a membrane-spanning region, and an intracellular tyrosine kinase domain. A highly related protein, encoded by the human *FLG* gene (*fms*-like gene) was also isolated and shown to exhibit high-affinity binding to acidic FGF (22, 23). The availability of these expressed cDNAs should allow rigorous analysis of binding properties for each member of the FGF family and correlation with their *in vivo* receptor interactions. In contrast, however, the molecular characterization of low-affinity receptors for the FGFs has not been reported. Here, we describe the cDNA cloning and structural analysis* of a membrane-bound heparan sulfate proteoglycan (HSPG) that has the binding characteristics of the low-affinity receptor for basic FGF (19).

MATERIALS AND METHODS

cDNA Expression Vector EBO-pcD-XN. The vector was constructed from the component parts of pcD (24), the Epstein–Barr virus-based (EBO) DNA segment of EBO-pSV2-neo (25), and a synthetic polylinker that contains *Pst* I, *Xba* I, *Sac* I, *Bcl* I, *Not* I, *Kpn* I, and two *Bst*XI (26) restriction endonuclease sites. The synthetic polylinker was ligated to a *Pst* I/*Kpn* I-digested pcD vector that had been modified (27) to yield pcD-XN. *Sfi* I-linearized pcD-XN and EBO DNA were ligated as described (25) to yield EBO-pcD-XN.

Construction of the cDNA Library. Total RNA was isolated from BHK-21 cells by the guanidinium isothiocyanate method (28) and poly(A)$^+$ RNA was purified by a single fractionation over oligo(dT)-cellulose (28). First-strand cDNA was synthesized as described (24) using 10 μg of poly(A)$^+$ RNA and 2.5 μg of a *Not* I–d(T)$_{16}$ primer–adapter [5′-d(AAGGTTCCAAGGTTTGCGGCCGCTTTGTCGAC-GGT$_{16}$-3′]. Double-stranded cDNA was generated and ligated to *Xba* I–*Bgl* II adaptors [5′-d(pCCTGAAGATC-TCCT)-3′ and 5′-d(CTAGAGGAGATCTTCAGG)-3′], as described (26). The resulting cDNA was digested with *Not* I and fractionated on Bio-Gel A-15m, and the DNA larger than 400 base pairs was further size-selected by agarose gel electrophoresis. cDNA molecules larger than 2.2 kilobases (kb) were excised, electroeluted, and purified on an Elutip-d column (Schleicher & Schuell). The size-selected double-stranded cDNA was ligated to gel-purified *Xba* I/*Not* I-digested EBO-pcD-XN and introduced into *Escherichia coli*

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Abbreviations: FGF, fibroblast growth factor; HSPG, heparan sulfate proteoglycan; BHK, baby hamster kidney; GAG, glycosaminoglycan; HSV, herpes simplex virus.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29967).

MC1061 cells by electroporation using a Bio-Rad Gene-Pulser. A library of 1.2×10^6 recombinants was obtained with an average-size cDNA insert of 3.3 kb.

Transfection of Human Lymphoblastoid Cells. The cDNA library was stably introduced into the WI-L2-729 HF_2 cell line (ATCC CRL 8062) by electroporation and selection with hygromycin B (25) and was stored at −80°C.

Selection of cDNA Clones by Panning. Tissue culture dishes (Falcon 3003) were incubated overnight at 4°C with recombinant human basic FGF (30 μg/ml in water). The dishes were aspirated, rinsed with isotonic phosphate-buffered saline (PBS), and then blocked by incubating (1 hr, 25°C) with PBS/2% (vol/vol) fetal calf serum. Approximately 2×10^7 stably transfected WI-L2-729 HF_2 cells were thawed, grown for 48 hr, and detached by incubation in PBS/1 mM EDTA (15 min, 25°C). The cells were centrifuged, washed in PBS, and resuspended at $1–2 \times 10^6$ cells per ml in PBS/2% fetal calf serum. Cells (5 ml) were applied to each of the dishes and allowed to attach for 3 min at 25°C. The dishes were washed three times with PBS and then selective medium was added. The bound cells were expanded to approximately 1×10^7 cells and then panned as before. After a third round of panning, the bound cells were washed three times with PBS and lysed, and episomal DNA was isolated (29). The isolated DNA was transformed into *E. coli* MC1061 cells by electroporation.

Subcloning and DNA Sequencing. Plasmid DNA was isolated by the alkaline lysis method (28). cDNA fragments were subcloned into M13 and both strands were sequenced by the dideoxynucleotide chain-termination method (30) using M13 primers as well as specific internal primers.

RNA and DNA Blot Analyses. Nucleic acid blots were performed as described (refs. 28, 31, and 32; see Fig. 4). Probe A was labeled according to Feinberg and Vogelstein (33). Probe B was labeled similarly but without the random oligonucleotide primers.

RESULTS AND DISCUSSION

The ligand-affinity or panning method for expression cloning of cDNAs for cell surface proteins is now well-established (26, 34). To isolate cDNAs encoding the high- and low-affinity FGF receptors, we chose a cloning strategy that relied on functional stable expression of a cDNA library in mammalian cells and selection of cells expressing FGF binding proteins by panning on plastic dishes coated with recombinant human basic FGF (35). Anti-FGF receptor antibodies were not available. To determine the feasibility of cloning FGF receptors by direct panning on basic FGF, we first showed that basic FGF-coated plastic could bind BHK-21 cells and that free basic FGF could strongly compete with the BHK-21 cells in binding to immobilized basic FGF (data not shown). The BHK-21 cell line has been shown (16, 19) to express high levels of both high- and low-affinity FGF receptors.

We constructed a cDNA expression vector, EBO-pcD-XN (Fig. 1), that combined sequences derived from the Okayama–Berg mammalian expression vector pcD (24) and the Epstein–Barr virus elements (36) from EBO-pSV2neo (25). The Epstein–Barr virus–based DNA segment, which also contains the hygromycin phosphotransferase gene, allows for selection of transformed mammalian cells that replicate the plasmid as stable extrachromosomal elements. In addition, a synthetic polylinker containing unique *Xba* I and *Not* I sites allowed for unidirectional cloning of double-stranded cDNAs by using a synthetic *Not* I site-containing oligo(dT) primer adapter. Isolated mRNA from the BHK-21 cell line was used to construct the cDNA expression library.

The cDNA library was stably introduced into the human lymphoblastoid cell line W1-L2-729 HF_2 (ATCC CRL 8062)

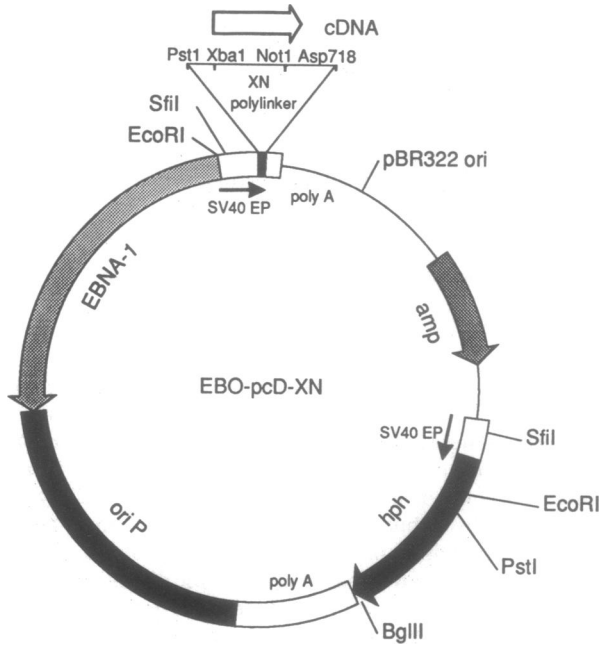

FIG. 1. General structure of the EBO-pcD-XN cDNA cloning and expression vector. The principle elements of the vector are identified and have been described (25). The direction of transcription is indicated by arrows. SV40 EP, simian virus 40 early region promoter; amp, ampicillin-resistance hygromycin phosphotransferase gene; ori, origin.

by electroporation and selection on hygromycin (25). The W1-L2-729 HF_2 cell line does not bind significantly to basic FGF-coated culture dishes (Fig. 2). Repeated panning (three times) of the stably transfected WI-L2-729 HF_2 cells on basic FGF-coated culture dishes allowed for the enrichment of cells that contained two distinct families of recombinant plasmids with similar insert restriction patterns. WI-L2-729 HF_2 cells stably transfected with purified plasmids from each family were found to bind efficiently to basic FGF-coated culture dishes (Fig. 2) whereas no binding was observed in areas of the dish not coated with basic FGF (data not shown). The binding was markedly reduced in the presence of free basic FGF, heparin, and heparan sulfate, whereas epidermal growth factor, platelet-derived growth factor, acidic FGF, chondroitin sulfate, dermatan sulfate, and keratan sulfate had little effect. Treatment of the transfected cells with heparinase abolished 98% of the binding (Fig. 2*E*). These results are highly consistent with those described by Moscatelli (19) for characterization of the endogenous low-affinity FGF receptor of BHK-21 cells and further suggests a specific glycosaminoglycan (GAG)–basic FGF interaction.

Inserts from each subgroup (RO-12 and RO-5) were excised and sequenced. The two families were shown to encode identical proteins and differed only in the site of attachment of poly(A) sequences within their 3′-untranslated regions. RO-12 and RO-5 cDNA sequences differed only in that RO-5 was polyadenylylated 14 residues beyond the internal polyadenylylation signal. Schematic representations of the cDNAs are shown in Fig. 3*A*. The sequences of the cDNAs and their encoded protein are shown in Fig. 3*B*.

The longest open reading frame encodes a polypeptide containing 309 amino acids with a calculated molecular weight of 32,683 and shares the characteristic features of cell surface proteoglycans. The proposed methionine initiator is preceded by an acceptable ribosome binding site (38) and is followed by a hydrophobic amino terminus with a putative signal peptidase cleavage site after amino acid 22 (39). A second hydrophobic region of 25 amino acids (residues

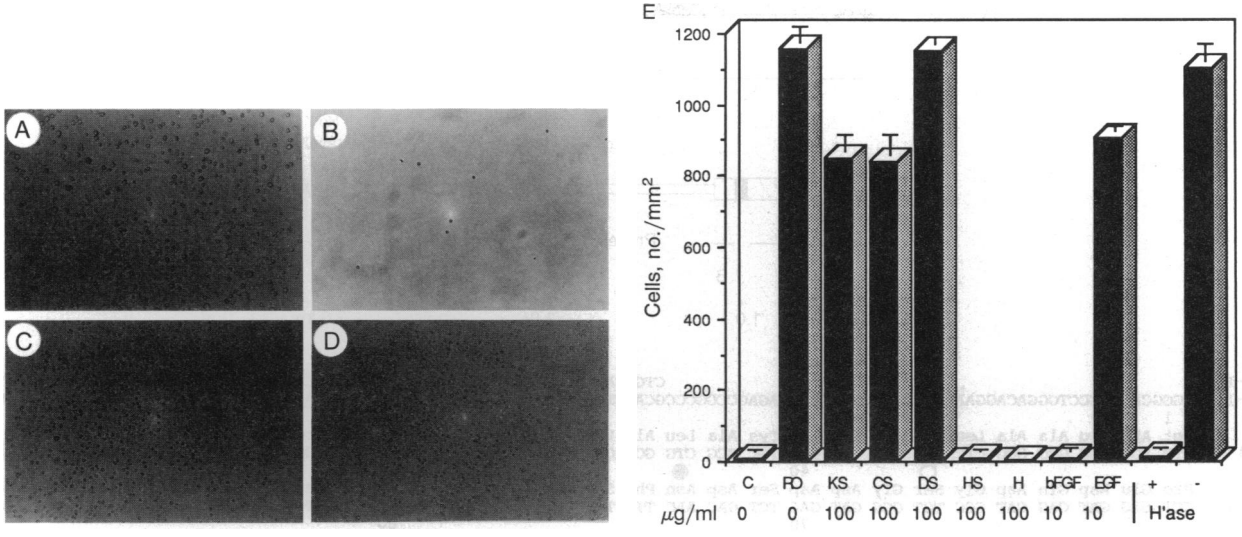

FIG. 2. Binding of FGF-HSPG-transformed W1-L2-729 HF2 cells to basic FGF. Binding assays were performed essentially as described for panning, except that cells were photographed (*A–D*) or counted (*E*) after the first panning cycle. Photographs are of BHK-21 cells (*A*), W1-L2-729 HF2 cells (*B*), RO-12-transformed W1-L2-729 HF2 cells (*C*), RO-5-transformed W1-L2-729 HF2 cells (*D*). (*E*) Binding of control (bar C) and RO-12-transformed W1-L2-729 HF2 cells with no additions (bar RO) and in the presence of keratan sulfate (bar KS), chondroitin sulfate (bar CS), dermatan sulfate (bar DS), heparan sulfate (bar HS), heparin (bar H), basic FGF (bar bFGF), or epidermal growth factor (bar EGF) at the indicated concentrations (μg/ml). Similar binding experiments were performed in the presence of acidic FGF and platelet-derived growth factor (see text). Also shown are transformed cells pretreated with heparinase (H'ase; bar +) or heparinase buffer (bar −), respectively, as described (19). The values shown represent the mean of three independent experiments.

251–275) followed by several charged residues defines a putative transmembrane region. The transmembrane region divides the polypeptide into a proportionally large extracellular domain (228 amino acids) and a short cytoplasmic region (34 amino acids). The extracellular domain contains six serine-glycine dipeptides that are potential GAG attachment sites (40) and one potential asparagine-linked glycosylation site. There are no cysteine residues in the extracellular region, although the signal peptide contains a single cysteine. The only cysteine in the predicted mature molecule is found in the transmembrane region. The extracellular domain also contains paired basic amino acid residues adjacent to the transmembrane region that may serve as a processing site for a membrane protease. A cleavage at this site could yield an extrinsic membrane-bound form of the molecule (41–43) or an extracellular form that may be retained by the matrix. The small cytoplasmic region (residues 276–309) contains three of the four tyrosines found in the entire molecule. The cytoplasmic domain, however, shows no similarity to tyrosine kinase domains, such as those found in the high-affinity FGF receptor, but rather, is homologous to cytoplasmic domains of proteins that are thought to associate with the cytoskeleton through binding of actin (43, 44). The structural features of the protein encoded by the cDNA and the binding characteristics of the expressed product suggest a close relationship or identity to the low-affinity FGF receptor (19). Herein, we refer to the GAG-substituted core protein encoded by our cDNA clones as cell-surface FGF-binding heparan sulfate proteoglycan (FGF-HSPG).

Northern blot analysis of BHK-21 poly(A)$^+$ RNA with probe A (see Fig. 3*A*) identified two hybridizing mRNA species of approximately 3.2 kb and 2.4 kb (Fig. 4*A*). Similarly, two hybridizing bands were seen with RO-12-transformed WI-L2-729 HF_2 cells, supporting the conclusion that the shorter transcript was indeed generated by use of the internal polyadenylylation signal found in RO-12. Southern blot analysis of *Eco*RI-digested hamster genomic DNA using the extracellular region of the RO-12 clone as a probe (probe A) identified one strongly hybridizing band at 10 kb and four or five weaker smaller hybridizing bands under stringent washing conditions (Fig. 4*B*). When a synthetic oligonucleotide duplex representing the entire cytoplasmic domain of the protein, probe B (Fig. 3*A*), was used, a similar pattern was seen. When murine DNA was examined, two bands of 15 kb and 8 kb hybridized to each probe with equal intensity. These results suggest that the mouse genome contains two closely related FGF-HSPG genes and that the hamster genome contains a small family of genes related to the FGF-HSPG gene.

The hamster FGF-HSPG is homologous to the proteins encoded by two cDNA clones (43, 44). First, although overall sequence similarity to a 48-kDa integral membrane proteoglycan core protein from human lung fibroblasts (44) is low, the cytoplasmic domains exhibit high sequence similarity (68%). Also, the hamster FGF-HSPG very closely resembles the murine integral membrane proteoglycan syndecan (43), a polypeptide that bears both heparan sulfate and chondroitin sulfate and links the cytoskeleton to the interstitial matrix. Overall sequence similarity between the two proteins is 85% with the highest regions of similarity again occurring in the cytoplasmic domain (100%), the transmembrane domain (96%), and the signal peptide (95%). Syndecan contains five potential attachment sites for GAG side chains whereas the hamster FGF-HSPG contains six. Despite this difference, however, the high degree of amino acid sequence similarity suggests that syndecan may be the murine homolog of the FGF-HSPG. In further support of this proposal, the additional serine-glycine pair of the hamster sequence is not surrounded by the acidic residues typically found in well-characterized GAG attachment sites (40) and, therefore, may not be utilized for such modification. Also, the recently determined sequence of a cDNA encoding the human FGF-HSPG shows no serine-glycine pair in the corresponding position of the protein (unpublished results) but retains the other serine-glycine pairs. Our Southern blot analysis suggests that the murine genome contains two closely related FGF-HSPG genes. The variability in the number of serine-glycine pairs between syndecan and the FGF-HSPG may, therefore, reflect a gene difference rather than a species difference.

The availability of large quantities of cloned FGF-HSPG will now allow further studies on the role of FGF binding in

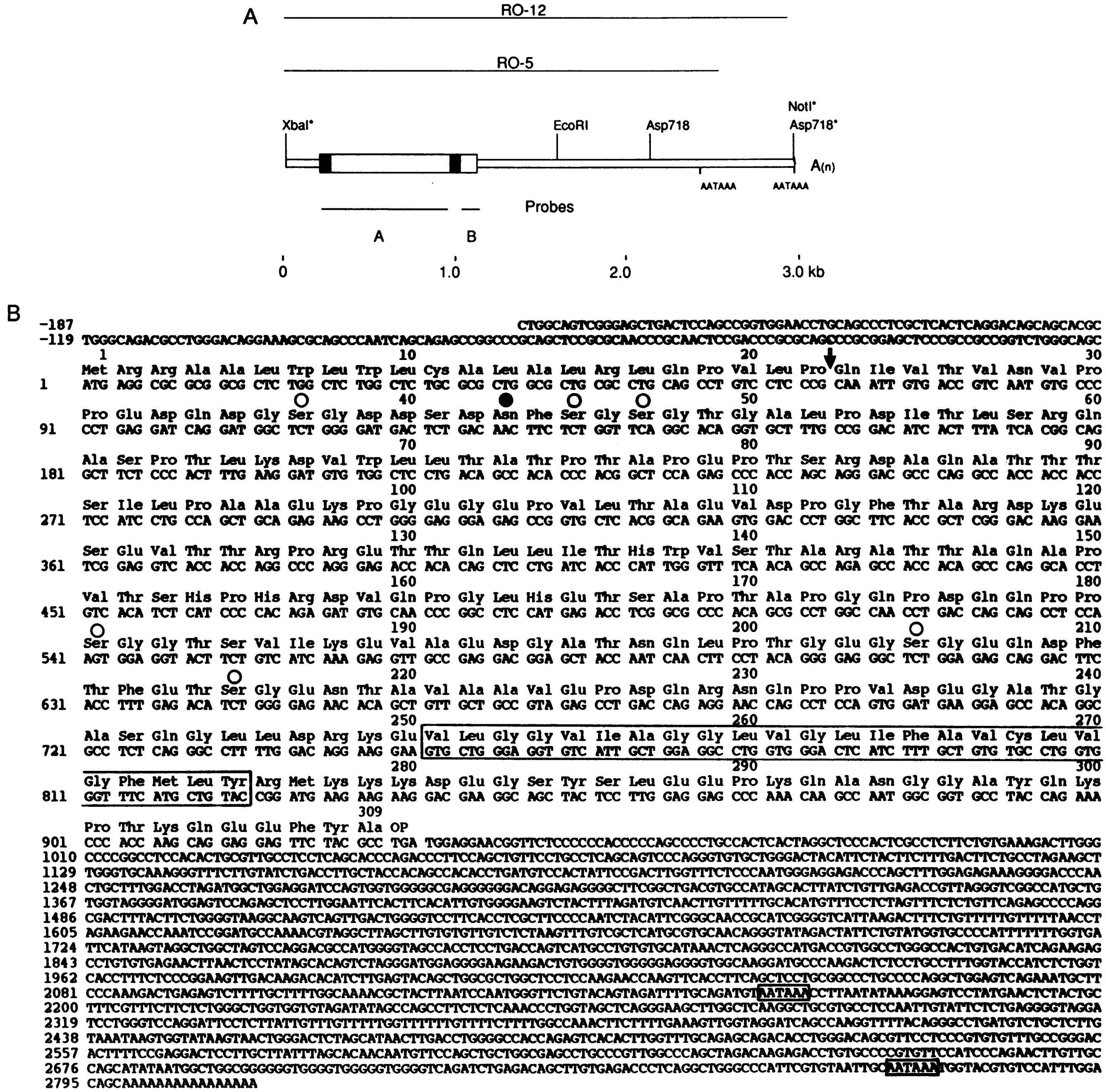

FIG. 3. (A) Representation of hamster FGF-HSPG cDNA clones. Plasmid DNA preparations were digested with *Xba* I and *Asp* 718 to excise the cDNA inserts and were analyzed by agarose gel electrophoresis. Fourteen of 24 clones contained 2.1-kb and 0.9-kb DNA fragments and 5 clones contained 2.1-kb and 0.25-kb fragments. Representative clones from each family (RO-12 and RO-5, respectively) are shown (thin lines) above a diagram of the complete coding sequence (boxed) and adjacent untranslated regions. The cross-hatched region represents the putative signal peptide and the solid region represents the transmembrane region. Polyadenylation signal sequences are noted (AATAAA). Several internal and flanking (*) restriction sites are shown as well as the location of two probes used for Northern and Southern blot analysis. Probe A is a cloned truncated cDNA generated from RO-12 by the polymerase chain reaction (37). Probe B consists of two partly overlapping synthetic oligonucleotides spanning the entire sequence of the cytoplasmic domain of RO-12. (B) The cDNA and derived amino acid sequence of hamster FGF-HSPG. The putative amino-terminal signal peptidase cleavage site (↓) is shown. The hydrophobic transmembrane region is boxed. Potential GAG attachment sites (○) and an asparagine-linked glycosylation site (●) are denoted. Two polyadenylylation signal sequences (AATAAA) are boxed.

extracellular matrix interactions and mitogenesis and will also allow the analysis of low-affinity binding of the newly identified members of the FGF family. A role for cell surface HSPGs in the binding of other growth and differentiation factors has been described. For example, hemopoietic growth factors, granulocyte/macrophage colony-stimulating factor and interleukin 3, are clearly bound by a sulfated GAG of mouse marrow stroma (45). Also, osteogenin, a bone morphogenetic protein (46, 47), and other bone-derived growth factors (48) have strong affinity for heparin *in vitro*. Furthermore, certain viruses, and primarily herpes simplex virus 1 (HSV-1), are capable of not only attaching to cells through the cell surface HSPGs but might subvert the entire FGF receptor system for entry into target cells (49, 50). The initial attachment of HSV-1 and HSV-2 to cell surface HSPGs has been well documented (50) and may involve the viral envelope glycoproteins gB and gD. We have shown using our cell attachment assay that both recombinant gB and gD of

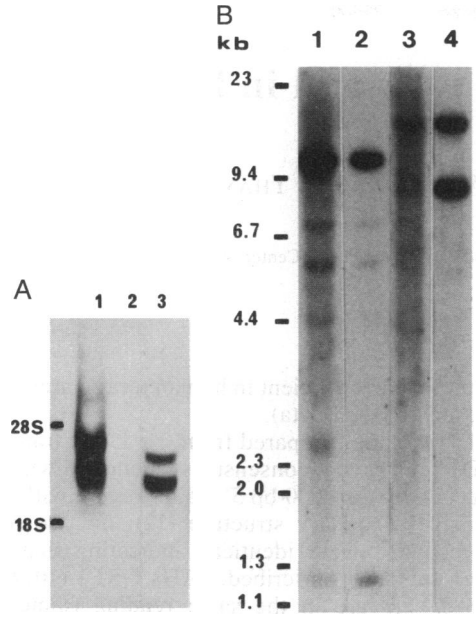

FIG. 4. RNA and genomic DNA blot analysis of the FGF-HSPG. (*A*) Poly(A)$^+$ RNA (2 μg) from RO-12-transformed W1-L2-729 HF_2 cells (lane 1), WI-L2-729 HF_2 cells (lane 2), and BHK-21 cells (lane 3) was fractionated on a 2.2 M formaldehyde/1.4% agarose gel, blotted, and hybridized with ^{32}P-labeled probe A. (*B*) *Eco*RI-digested hamster DNA (lanes 1 and 2; 10 μg) and mouse DNA (lanes 3 and 4; 10 μg) were fractionated on a 0.7% agarose gel, blotted, and hybridized with ^{32}P-labeled probe A (lanes 1 and 3) and probe B (lanes 2 and 4). The filters were washed at 65°C in 15 mM NaCl/1.5 mM sodium citrate (0.1 × SSC)/0.1% $NaDodSO_4$.

HSV-2 are capable of inhibiting the binding of FGF-HSPG-transfected cells to bFGF-coated plates (M.C.K., J.C.S., P.J.B., and Rae Lyn Burke, unpublished observations). Also Hajjar *et al.* (49) have shown that penetration and infection of cells by HSV-1 can be inhibited by both FGF and FGF peptide analogs that bind the high-affinity FGF receptor. The cell types that are infectable by HSV-1 closely parallel their responsiveness to FGF, further supporting the proposal that HSV utilizes the FGF receptor system for cell entry. These observations may help in the design of specific therapeutic approaches for the treatment of HSV infection.

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