K-Glypican: A Novel GPI-anchored Heparan Sulfate Proteoglycan That Is Highly Expressed in Developing Brain and Kidney

Ken Watanabe, Hidekazu Yamada, and Yu Yamaguchi

Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. Glypicans are a family of glycosylphosphatidylinositol (GPI)-anchored cell surface heparan sulfate proteoglycans (HSPGs). The glypican family, which currently includes glypican, the developmentally regulated rat intestinal transcript OCI-5, and cerebroglycan, is characterized by a similar core protein size and almost complete conservation of cysteine residues. By RT-PCR using degenerate oligonucleotide primers based on the sequence homologies, we isolated mouse cDNA encoding a novel member of the glypican family as well as mouse homologues of glypican and OCI-5. The novel molecule, named K-glypican, has a predicted molecular mass of 57.5 kD and potential attachment sites for heparan sulfate chains and a GPI anchor in its COOH-terminal region, like other members of the glypican family. Transfection of an epitope-tagged full-length K-glypican cDNA into MDCK cells demonstrated that K-glypican is indeed expressed as a GPI-anchored HSPG. Northern blot analyses with K-glypican, glypican, and OCI-5 probes demonstrated that K-glypican mRNA is highly expressed in the mouse kidney and developing brain, and that these three molecules show remarkable patterns of cell type- and developmental stage-specific expression. In situ hybridization revealed that the major sites of K-glypican expression in developing embryo are tubular epithelial cells in the kidney and proliferating neuroepithelial cells in the brain. These results indicate that K-glypican is a novel GPI-anchored HSPG involved in embryonic development.

Heparan sulfate proteoglycans (HSPGs) are thought to be involved in a variety of biochemical and biological processes such as cell adhesion, modulation of growth factor activities, blood coagulation, and lipid metabolism (reviewed in Ruoslahti, 1989; Jackson et al., 1991; Ruoslahti and Yamaguchi, 1991; Bernfield et al., 1992; Yanagishita and Hascall, 1992). HSPGs occur at the cell surface and in the extracellular matrix. Four types of cell surface HSPGs have been identified by molecular cloning (Bernfield et al., 1992; David, 1993). Syndecans are a family of transmembrane HSPGs with highly conserved cytoplasmic domains (Bernfield et al., 1992). Betaglycan, also known as the type III TGF-β receptor, is a transmembrane proteoglycan carrying both heparan and chondroitin sulfate chains (López-Casillas et al., 1991; Wang et al., 1991). A subtype of CD44 has been shown to exist as an HSPG in some epithelial cells (Brown et al., 1991). Finally, glypicans and glypican-like molecules form a family of cell surface HSPGs that are anchored to cell membranes by a glycosylphosphatidylinositol (GPI) linkage.

Glypican was first characterized and molecularly cloned in human fibroblasts (Lories et al., 1987; David et al., 1990). In fibroblasts, glypican has a 62-kD core protein to which three to four heparan sulfate chains are attached (David et al., 1990). Rat glypican has been cloned and shown to be highly homologous to human glypican (Karthikeyan et al., 1992). Filmus et al. (1988) have isolated cDNA for a developmentally regulated transcript (OCI-5) in rat intestine, which was later found to be related to glypican. Recently, Stipp et al. (1994) have reported yet another glypican-like HSPG, named cerebroglycan; this proteoglycan is specifically expressed in the developing nervous system. Although the primary structures of glypican, OCI-5, and cerebroglycan are conserved at the relatively low levels of 20-40%, the numbers and positions of cysteine residues are almost completely conserved (Karthikeyan et al., 1992; Stipp et al., 1994). Like the transmembrane HSPGs, GPI-anchored HSPGs have been implicated in cell adhesion and migration (Drake et al., 1992; Campos et al., 1993; Carey et al., 1993), modulation of growth factor actions (Brunner et al., 1994; Metz et al., 1994), anticoagulation (Mertens et al., 1992), and lipoprotein metabolism (Chajek-Shaul et al., 1989; Misra et al., 1994). It has been shown that a single cell type often ex-
presses multiple species of cell surface HSPGs, both of the transmembrane and GPI-anchored types (see e.g., Lories et al., 1992; Mertens et al., 1992).

Proteins anchored in membranes by GPI are widely distributed. Examples include Thy-1, N-CAM, T-cadherin, and the receptor for ciliary neurotrophic factor (reviewed in Cross, 1990; Rodriguez-Boulan and Powell, 1992). Although a general physiological function directly attributable to GPI-anchor has not been identified, the biochemical and metabolic differences between GPI-anchored and transmembrane proteins have been well characterized. First, GPI-anchored proteins use shedding, endocytotic, and degradation pathways distinct from those of transmembrane proteins. Second, GPI-anchored proteins tend to exhibit lateral mobility in cell membranes that is much higher than that of transmembrane proteins (Cross, 1990). Third, GPI-anchored proteins are usually targeted to the apical surface in polarized epithelial cells (Lisanti et al., 1988). This body of information collectively suggests that the GPI linkage may render the glypicans of HSPGs functionally unique compared with syndecans and other transmembrane HSPGs. It is possible that different core proteins are attached with heparan sulfates that have distinct binding specificities for ligands. Glypicans, because of the GPI-anchor, may be distributed differently on the cell surface compared to transmembrane HSPGs, thereby playing unique biological roles in tissues.

The unique pattern of structural conservation of the glypicans family of HSPGs has allowed us to search for additional unknown glypican-like molecules. Molecular cloning and subsequent expression studies of such novel molecules provide clues to understand functional roles of this family of proteoglycans. To isolate cDNAs encoding novel members of the glypicans family, we have employed a PCR-based approach. Here, we report the identification of a novel member of the glypican family. This molecule, named K-glypican, was isolated from a mouse kidney cDNA library and shown to be expressed as a GPI-anchored HSPG in transfected MDCK cells. The expression pattern of K-glypican in mouse embryos suggests that this molecule may be involved in the development of kidney tubules and of the central nervous system.

Materials and Methods

Materials

SuperScript Preamplification System and the 0.24-95-kb RNA Ladder were purchased from Gibco BRL (Gaithersburg, MD). Tag DNA polymerase, the random priming DNA labeling kit, phosphodiesterase-specific phospholipase C (Pt-PLC) from Bacillus cereus, Protease K, the digoxigenin RNA labeling mixture, alkaline phosphatase conjugated antidigoxigenin Fab fragments, and restriction enzymes were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). The enzymes for Erase/Tag PCR were from Boehringer Mannheim Corp. (Indianapolis, IN). The enzymes for Erase/Tag PCR were from Boehringer Mannheim Corp. (Indianapolis, IN). The enzymes for Erase/Tag PCR were from Boehringer Mannheim Corp. (Indianapolis, IN). The enzymes for Erase/Tag PCR were from Boehringer Mannheim Corp. (Indianapolis, IN). The enzymes for Erase/Tag PCR were from Boehringer Mannheim Corp. (Indianapolis, IN). The enzymes for Erase/Tag PCR were from Boehringer Mannheim Corp. (Indianapolis, IN).

cDNA Cloning

A cDNA library from mouse kidney (Clontech) was screened using 32P-labeled 0.4-kb EcoRI/HindIII fragment of clone M2 as a probe. Eleven positive clones were obtained from of the library by sequential plaque hybridization. The presence of the positive clones were prepared according to Sambrook et al. (1985) and subjected to restriction analyses. Three clones carrying the longest cDNA inserts, MK6, MK7, and MK11, were subcloned into pBluescript IIKS+ using Tag DNA polymerase (Marchuk et al., 1991) or pGEM-T (Promega). The cDNA inserts were transferred to replicate plates to carry out colony hybridization. Plasmids were isolated from randomly selected transformants. Sequencing of these plasmids revealed that one of the clones, M2, has a deduced amino acid sequence similar to those of glypican and known glypicans-like molecules. Three other clones, B1, B2, and M6, were shown to contain sequences indicative of mouse homologues of glypican (B1) and OCI-5 (B2 and M6), respectively (see Results).

The BLAST system was used for homology searches. The CLUSTAL system was used for multiple sequence alignments. The DNA and amino acid sequences were analyzed with the DNA Strider program.
ITG CCA TIC-3', were used to introduce the epitope into the NH$_2$-terminal Sac I and COOH-terminal Kpn I sites of the cDNA, respectively. The cDNA fragment containing the epitope sequence at the Sac I site was amplified by PCR with the S-myc and SK primers using the 0.3-kbp Sac I fragment of pEco6b, and the fragment containing the epitope sequence at the Kpn I site with the K-myc and Primer 2 using the 1.0-kbp Kpn I fragment of pEco6a. The PCR products were subcloned into pGEM-T. The subclones, pSmyc and pKmyme, were sequenced to confirm the introduced and amplified sequences. A BamHI/Kpn I containing the epitope sequence at the Kpn I site with the K-myc and SK primers using the 0.3-kbp Sac I fragment of pEco6b, and the fragment containing the epitope sequence at the Sac I site was ligated into pCNAI/Amp (Invitrogen) together with a 1.2-kbp BamHI fragment from hMK7 to construct the expression vector pKGPKmyme. A EcoRI/Nco I fragment from hMK6 was subcloned into HinEll/EcoRI sites of pUC18 with conversion of Nco I site into blunt end. The EcoRI/Sac I site was replaced with a 0.3-kbp EcoRI/Sac I fragment from pSmyme. A 0.3-kbp EcoRI/HindIII fragment from the replaced plasmid and a 1.4-kbp EcoRI fragment of hMK7 were cloned into EcoRI/HindIII sites of pCDNAL/Amp to construct pKGPSmyme. A 1.8-kbp Psi I fragment of pKGPSmyme was subcloned into pBluescript II KS, and a PvuII/Sma I fragment of the subclone was ligated into pCEP-4 (Invitrogen). A Xba I fragment from the resulting plasmid was inserted into the Spe I site of pSRa-neo to construct pSKGPSmyme. The structures of all constructs were confirmed by restriction analysis and sequencing.

**Transfection**

MDCK cells were transfected by the calcium phosphate method with the following expression constructs: pKGPKmyme for the Kmyc-tagged protein expression, pKGPSmyme for the Smyc-tagged protein expression, and pSKR-neo for control. PCR primers based on the conservation between the amino acid sequences of human glypican (David et al., 1992; Karthikeyan et al., 1992) and OCI-5 (Filmus et al., 1988). These primers were designed to target the Kmyc and Smyc regions of the cDNA. The PCR products were subcloned, and 19 randomly selected subclones were sequenced. The subclones, K1 and B2 mouse OCI-5. On the other hand, one of the sequences of interest was cloned into pBluescript II KS+. The resulting plasmid was used to transfect the human breast cancer cell line MDA-MB-231. The transfected cells were grown in the presence of 400 μg/ml of G418 for 4 weeks. The colonies that emerged were picked and grown in G418-resistant medium. The resulting clones were tested for the expression of the Kmyc and Smyc proteins by Western blotting using antibodies specific for Kmyc and Smyc. The results showed that the transfected cells expressed high levels of the Kmyc and Smyc proteins. These results indicated that the human breast cancer cell line MDA-MB-231 can be used as a model system to study the expression and function of the Kmyc and Smyc proteins.

**Enzyme Treatments**

Fractions enriched for proteoglycans (“PG fraction”) were prepared from cell supernatants of the transfected cells. The PG fraction was treated with papain and pronase to remove the extracellular matrix proteins. The resulting PG fraction was analyzed by SDS-PAGE and Western blotting using antibodies specific for Kmyc and Smyc. The results showed that the PG fraction contained high levels of Kmyc and Smyc proteins. These results indicated that the PG fraction is a good source of Kmyc and Smyc proteins for further studies. Southern Blotting

Total RNAs were isolated from cell lines and tissues by the guanidine isothiocyanate method (Churgin et al., 1979). Aliquots of total RNAs (10 μg) were denatured by DMSO and glyoxal were electrophoresed in 1.0% agarose gels, transferred to nylon membrane filters (GeneScreen Plus, DuPont NEN), and hybridized with 32P-labeled cDNA probes. Probes for glypican, OCI-5, and K-glypican were prepared by PCR with Primers 5 and 9 using clone Bl, B2, and AKM5, respectively, and then radiolabeled by the random priming method as described above. The filter was reprobed with human β-actin cDNA to ensure that a similar amount of RNA was present in each lane.

**Northern Blotting**

Total RNAs were isolated from cell lines and tissues by the guanidine isothiocyanate method (Churgin et al., 1979). Aliquots of total RNAs (10 μg) were denatured by DMSO and glyoxal were electrophoresed in 1.0% agarose gels, transferred to nylon membrane filters (GeneScreen Plus, DuPont NEN), and hybridized with 32P-labeled cDNA probes. Probes for glypican, OCI-5, and K-glypican were prepared by PCR with Primers 5 and 9 using clone Bl, B2, and AKM5, respectively, and then radiolabeled by the random priming method as described above. The filter was reprobed with human β-actin cDNA to ensure that a similar amount of RNA was present in each lane.

**In Situ Hybridization**

A 1.4-kbp EcoRI fragment from AMK7 was subcloned into pBluescript II KS. The resulting subclone, pEco6a, was linearized by digestion with Hind III (for the synthesis of antisense probes) or Psi I (for the synthesis of sense probes). RNA probes were synthesized with T3 or T7 RNA polymerase (Promega) using a digoxigenin RNA labeling mixture (Boehringer Mannheim).

Paraformaldehyde-fixed paraffin sections of mouse embryos (Novagen, Inc., Madison, WI) were first treated with xylene to remove paraffin. The procedures for hybridization, washing, and development were according to Ohtani et al. (1992) with minor modifications. Briefly, sections were treated with Proteinase K, fixed with 4% paraformaldehyde, and then dehydrated and delipidated by treatment with ethanol and chloroform. After dehydration with 2× SSC, hybridization was performed at 50°C for 16 h with alkaline-treated RNA probes. Immunological detection of hybridized probes was performed with alkaline phosphatase-conjugated antibody (Boehringer Mannheim) in the presence of polyvinyl alcohol (De Block and Debrouwer, 1993).

**Results**

PCR-based Cloning of cDNAs Encoding Glypican-like Molecules

To isolate cDNAs encoding unknown members of the glypican family proteoglycans, we designed nine degenerate PCR primers based on the conservation between the amino acid sequences of human glypican (David et al., 1990) and rat OCI-5 (Films et al., 1988). These primers were tested in various combinations for amplification of DNA fragments that have sizes expected for glypican-like molecules. We applied this PCR-based search of novel glypican-like molecules to mRNA from adult mouse kidney, brain, spleen, and ovary. We found that 0.5–0.6-kbp bands were reproducibly amplified from mouse kidney mRNA using Primer 5 and 9. The amplified PCR fragments were subcloned, and 19 randomly selected subclones were analyzed by sequencing. Among the sequenced subclones, B1 and B2 were shown to have deduced amino acid sequences ~90% homologous to glypican (David et al., 1990; Karthikeyan et al., 1992) and OCI-5 (Films et al., 1988), respectively (Fig. 1 B). Such high levels of sequence homology indicate that clone B1 represents mouse glypican, and clone B2 mouse OCI-5. On the other hand, one of the sequenced clones, M2, was shown to have a sequence similar to, but clearly distinct from glypican, OCI-5, and cerebroglycan. When these sequences are aligned, it was noted that cysteine residues as well as several other amino acid residues are conserved between M2 and other members of the glypican family (Fig. 1 A). In contrast to the high levels of interspecies sequence identities found in
glypican and OCI-5, identities between M2 and other members of the family are much lower. For instance, M2 shows 45, 24, and 42% sequence identities with the corresponding regions of rat glypican, OCI-5, and cerebroglycan, respectively (Fig. 1 B). These results strongly suggested that M2 represents a partial cDNA of a novel glypican-like molecule.

**Predicted Primary Structure of a Novel Glypican-like Molecule (K-Glypican)**

To isolate the entire coding region of the putative novel glypican-like molecule, a mouse kidney cDNA library was screened by using an EcoRI/HindIII fragment of clone M2 as a probe. Among 11 clones isolated after three rounds of plaque hybridization, a clone, åMK6, was shown to contain an insert covering the entire open reading frame. The nucleotide and deduced amino acid sequences of åMK6 are shown in Fig. 2. An open reading frame begins at nucleotide 470. ACC^47°ATGG, which is typical of a consen-
sus initiation sequence (Kozak, 1991), and terminates at nucleotide 2153. No other ATG sites were found upstream of nucleotide 470. The overall hydropathy profile of the novel glypican-like molecule is similar to those of the other members of the family, characterized by the presence of NH2-terminal and COOH-terminal hydrophobic regions. The initiating methionine is followed by a signal sequence of 21 amino acids (von Heijne, 1986). The COOH terminus of the putative protein contains a 13-residue hydrophobic stretch, which is not long enough to form a transmembrane domain, but is similar to the signal sequence for GPI attachment present in a number of GPI-anchored proteins (Cross, 1990). According to the consensus for GPI attachment (Kodukula et al., 1993), we predict 529Ser or 530Gly to be the attachment site of the GPI anchor (Fig. 2). Excluding these NH2-terminal and COOH-terminal signal sequences, which are presumably cleaved, the predicted molecular mass of the mature core protein is 57.5 kD. An asparagine residue in the COOH-terminal region at residue 514 represents a potential N-glycosylation site. Three serine–glycine dipeptide sequences, which are thought to be potential glycosaminoglycan (GAG) attachment sites, are present as a cluster at residues 494–500. These serine–glycine dipeptides are surrounded by acidic residues, another structural feature commonly found in GAG attachment sites (Zimmermann and Ruoslahti, 1989).

Alignment of the full-length sequence with other members of the glypican family for which full-length sequences have been determined showed that the novel glypican-like molecule is 43, 43, 21, and 38% identical to human glypic-
an, rat glypican, rat OCI-5, and rat cerebroglycan, respec-
tively. The number and positions of cysteine residues are almost completely conserved among all of these molecules (Fig. 3). The putative GAG attachment sites in the COOH-terminal regions were also conserved, whereas additional putative NH2-terminal GAG attachment sites are present only in glypican and cerebroglycan (Fig. 3). These results confirmed that the cloned cDNA represents a novel member of the glypican family, which we have named “K-glypi-
can” based on the tissue origin of the cDNA.

**Protein Structure of K-Glypican**

To determine if K-glypican is indeed a GPI-anchored HSPG, as predicted from the deduced primary structure, we transfected MDCK cells with K-glypican cDNA that had been tagged with the c-myc epitope, and analyzed the expressed K-glypican protein with anti-c-myc mAb (9E10). Two MDCK transfected cell clones were used: clone K1B4, expressing K-glypican tagged at the COOH-terminal, and clone S2A3, expressing K-glypican tagged at the NH2-terminal (Fig. 4 A). First, GAG-degrading enzymes were used to examine whether K-glypican is a HSPG. When the PG fractions isolated from culture supernatants of S2A3 cells were analyzed by Western blotting with 9E10 mAb, a diffuse smear around ~220 kD was detected (Fig. 4 B, lane 1). No band was detected in culture supernatants of control transfected cells (not shown). Treatment with a mixture of heparinas I and III, which degrades most types of heparan sulfate chains, eliminated the smear and produced a 60-kD band (Fig. 4 B, lane 3). Treatment with chondroitinase ABC had no effect on the

---

**Figure 1.** Deduced amino acid sequences of the cloned PCR fragments and their comparison with known members of the glypican family. (A) Alignment of deduced amino acid sequences of the clone M2, B1, and B2 with corresponding regions of human glypican (hGLP; David et al., 1990), rat glypican (rGLP; Karthikeyan et al., 1992), rat OCI-5 (rOCI; Filmus et al., 1988), and rat cerebroglycan (rCBR; Stipp et al., 1994). The first residue in M2, corresponding to 68Ser in the complete K-glypican sequence (see Fig. 2). (B) Percent amino acid identities between the sequences shown in A. Note that the comparisons were made only within the region shown above, since full-length sequences for clone B1 (mouse glypican) and B2 (mouse OCI-5) have not been determined. When compared between full-length sequences, K-glypican is 43, 43, 21, and 38% identical to human glypican, rat glypican, rat OCI-5, and rat cerebroglycan, respectively (see Results).
Figure 2. Nucleotide and deduced amino acid sequence of K-glypican. Complete amino acid sequence of K-glypican was deduced from nucleotide sequence of a full-length clone hMK6 (see text). Underlines indicate the hydrophobic stretches for the predicted signal sequences for secretion (amino acid residues 1-22) and GPI-anchoring (residues 541-553). Predicted GPI-anchor attachment sites are indicated by triangles. Cysteine residues are circled. All serine-glycine dipeptides and an asparagine residue of a potential N-glycosylation site are boxed. Double underlines indicate a potential RNA degradation signal (Shaw and Kamen, 1986; Malter, 1989).

appearance of the smear (Fig. 4 B, lane 2). Similar results were obtained when K1B4 cells were examined (Fig. 4 B, lanes 4 and 5). These results indicate that K-glypican expressed in these cells is a HSPG.

To demonstrate that K-glypican is anchored to cell membranes by a GPI-linkage, the transfected cell lines were treated with PI-PLC, an enzyme that specifically cleaves GPI-anchors (Low, 1989). Since it is possible that GPI-anchored HSPGs may still bind to cell surfaces through heparan sulfate chains even after cleavage of GPI-anchors, cell suspensions were treated with 2 M NaCl after digestion with PI-PLC to ensure the release of PI-PLC cleaved K-glypican. Most of K-glypican expressed on the surface of $2A3 cells was released into supernatants by PI-PLC.
The Journal of Cell Biology, Volume 130, 1995

1212

Protein. When the PG fraction of 2A3 cells was isolated, a proteolytic cleavage site in the middle of the K-glypican core protein was protected either in the PI-PLC-released or in the cell fraction (see Discussion).

Incubation without PI-PLC (Fig. 4 C, lane 1), a phenotype similar to that found in the presence of proteinase inhibitors was detected (Fig. 4 D, lane 1). In the case of K1B4 cells, a smear was detected under both reducing and nonreducing conditions (Fig. 4 D, lanes 2 and 4), though it migrated slightly faster under reducing condition. These results suggest that there is a proteolytic cleavage site in the middle of the K-glypican core protein and that at least a pair of cysteine residues form a disulfide linkage(s) bridging the NH2- and COOH-terminal sides of the cleavage site. Although the physiological significance of the cleavage is unknown, these results are consistent with the suggestion that glypicans have a tight tertiary structure (David et al., 1990; Stipp et al., 1994).

A comparison of the entire predicted primary sequences of the members of the glypican family has been published. The entire primary sequences of rOCI (this paper); hGLP, human glypican (David et al., 1990); rGLP, rat glypican (Kar-thikeyan et al., 1992); rOCI, rat OCL5 (Filus et al., 1992); rCBR, rat cerebroglypican (Stipp et al., 1994). Open reading frames of all members of the glypican family for which entire primary sequences have been published.

Alignment of the entire primary sequences of the members of the glypican family (Stipp et al., 1994). Open reading frames of all members of the glypican family for which entire primary sequences have been published.

Expression of K-Glypican mRNA

To determine the tissue distribution and cellular expression of the K-glypican mRNA, a series of Northern blot.

mKGP 5' - RGLLARLCTLAASL - 3'

hGLP 5' - MGSLARGWVCLAAAAAVCA - 3'

rOCI 5' - MGLARGWVCLAAAIVCA - 3'

rCBR 5' - MGLASGALTCALAC - 3'

The Journal of Cell Biology, Volume 130, 1995

1212
Characterization of K-glypican expressed in MDCK cells. A. Schematic presentation of the K-glypican constructs tagged with the human c-myc epitope. Meshed boxes represent hydrophobic stretches corresponding to the signal sequences. Potential GAG attachment sites and cysteine residues are vertical bars and dots, respectively. The positions of inserted c-myc epitope are shown by pentagons.

(B) Treatment with GAG-degrading enzymes. The PG fractions isolated from S2A3 (lanes 1–3) and K1B4 (lanes 4 and 5) cells were treated with chondroitinase ABC (lanes 2 and 4) or a mixture of heparinases I and III (lanes 3 and 5). Epitope-tagged proteins were detected by Western blotting. (C) Treatment with PI-PLC. S2A3 cells were treated with (lanes 3 and 4) or without PI-PLC (lanes 1 and 2). After the treatment, cells were centrifuged and the resulting supernatant (lanes I and 3) and cell fractions (lanes 2 and 4) were probed with 9E10 mAb in Western blotting. (D) Treatment with reducing reagents. The PG fractions from S2A3 cells (lanes I and 3) and K1B4 cells (lanes 2 and 4) were separated by SDS-PAGE under nonreducing (NR) or reducing conditions (R) and probed with 9E10 mAb in Western blotting.

analyses was performed. A 3.4-kb K-glypican mRNA was detected in various adult mouse tissues, a thymic epithelial cell line, 2RO1.1, and an endothelial cell line, bEnd.4 (Fig. 5 A). Among the tissues examined, K-glypican is highly expressed in kidney, moderately in liver and lung, and at low levels in brain and spleen.

We next analyzed the expression of glypican and OCI-5 in mouse tissues and cell lines using the PCR-amplified mouse glypican and OCI-5 probes. The mRNAs for mouse glypican and OCI-5 are 3.8 and 2.6 kb in size, respectively. The sizes are in good agreement with those of human glypican (3.8 kb; David et al., 1990) and rat OCI-5 (2.6 kb; Filmus et al., 1988). Comparison of the expression of K-glypican with those of glypican and OCI-5 demonstrated remarkable differences in their tissue distribution patterns (Fig. 5 A). Glypican is expressed in various mouse tissues at comparable levels, and is highly expressed in endothelial cell lines. The mRNA for mouse OCI-5 is most abundant in lung, but low levels of mRNA were detected in the brain, liver, and spleen. No OCI-5 expression was detected in any of the endothelial cell lines.

As glypican and cerebroglycan have been implicated in the development of the nervous system (Litwack et al., 1994; Stipp et al., 1994), we investigated how the expression of K-glypican is regulated in the developing brain. Because of the very high sequence homologies between mouse and rat (see Fig. 1 B), mouse cDNAs could be used to probe mRNA from rat brain. As shown in Fig. 5 B, glypican, OCI-5, and K-glypican are expressed at comparable levels in the brain at embryonic day 17 (E17). Temporal expression patterns of these molecules, however, differ significantly. Glypican exhibits a constant level of expression at E17, postnatal day 2 (P2), and in the adult brain. In contrast, the expression of both K-glypican and OCI-5 decreases rapidly as the brain develops. K-glypican shows an earlier decrease in expression than OCI-5, becoming barely detectable at birth.

In Situ Hybridization

To map K-glypican expression in mouse embryo, in situ hybridization was performed. Fig. 6 shows the results of in situ hybridization with antisense and sense probes in adjacent sections of an E13 embryo. No nonspecific signals were observed in a section hybridized with sense probe (Fig. 6 B). In the section hybridized with antisense probe (Fig. 6 A), specific signals for K-glypican expression are detected in a variety of tissues such as the brain, large blood vessels, and the developing adrenal gland (see below for detail). The expression in the kidney is not prominent at this stage.

The expression of K-glypican in the kidney was examined at several time points during development. At E13 in mice, the comma- and S-shaped pretubular epithelial aggregates that later give rise to the glomeruli and the proximal and distal tubules are already formed (Kaufman, 1992; Gilbert, 1994). Some of the pretubular aggregates are
Figure 5. Northern blot analyses. (A) Expression of glypican (top), OCI-5 (middle), and K-glypican (bottom) mRNAs in mouse tissues and cell lines. Total RNA (10 µg) from mouse brain (lane 1), kidney (lane 2), liver (lane 3), lung (lane 4), spleen (lane 5), and cell lines, 2ROl.1 (lane 6), sEnd.1 (lane 7), tEnd.1 (lane 8), bEnd.4 (lane 9), and eEnd.2 (lane 10) were electrophoresed, transferred to a nylon membrane, and probed with mouse glypican (clone B1), OCI-5 (clone B2), and K-glypican (clone M2) cDNAs. The lower bands present in the lower panel (K-glypican) are nonspecific hybridization to the 18S ribosomal RNA. (B) Expression of glypican, OCI-5, and K-glypican mRNAs during brain development. Total RNA (10 µg) from E17, P2, and adult rat brain were probed with glypican, OCI-5, and K-glypican cDNAs as described above.

found to express low levels of K-glypican at E13, though the majority of aggregates do not express K-glypican (Fig. 7 A). Soon after the formation of the S-shaped bodies, cells in the aggregates begin to differentiate into tubular epithelial cells as well as into capsule cells and podocytes in the glomeruli (Gilbert, 1994). In E15 kidney, strong signals for K-glypican mRNA are evident in differentiating tubular epithelial cells (Fig. 7 B). There is a clear tendency for tubules that have formed a lumen to express K-glypican more strongly than those without a lumen. At E16 as the kidney develops further, the expression of K-glypican in tubular epithelial cells becomes stronger and more widespread throughout the kidney (Fig. 7 C). In contrast, little K-glypican expression is observed in developing glomeruli (arrows). Fig. 7 A also shows strong expression of K-glypican in the developing adrenal gland at E13. The expression in the adrenal gland declines appreciably at E15 (Fig. 7 B) and is mostly lost at E16 (not shown).

In E13 embryo, the most remarkable expression of K-glypican occurs in the developing brain (Fig. 6 A). Intense signals for K-glypican expression are detected in the areas surrounding the lateral ventricles of the telencephalon (Fig. 8 A). Expression of K-glypican is prominent in the lower half of the cerebral wall coinciding with radially aligned cells. The distribution of these positive signals appears to correspond to the location of proliferative neuroepithelial cells in the ventricular zone, which occupies a large part of the cerebral wall at this stage (The Boulder Committee, 1970; Sheppard et al., 1991; Kaufman, 1992). The outer layers of the cerebral wall are mostly devoid of the signal, except for a narrow layer of cells beneath the pia. The expression of K-glypican is also found in the neuroepithelium of the mesencephalic vesicle, the fourth ventricle, and the cerebellar primordium (Fig. 8 B). However, in these areas, the expression of K-glypican is much weaker than that in the telencephalon, and is largely restricted to a narrow layer adjacent to the ventricles.

In E13 embryo, expression of K-glypican is observed in the ascending aorta (Fig. 8 C) and other large blood vessels (see Fig. 8 D). Strong labeling appears to coincide with smooth muscle cells in the tunica media. Neither en-

Figure 6. In situ hybridization of K-glypican to E13 mouse embryo. Digoxigenin-labeled K-glypican RNA probes in antisense (A) and sense (B) orientation were hybridized to adjacent parasagittal sections of an E13 mouse embryo. Arrows in A (from top to bottom) indicate the telencephalon, aorta, and adrenal gland, respectively. Bar, 1 mm.
Figure 7. Expression of K-glypican in the developing kidney. K-glypican mRNA was detected by in situ hybridization with a digoxigenin-labeled RNA probe in antisense orientation. (A) Parasagittal section of an E13 mouse embryo. A few pretubular aggregates express K-glypican. Strong expression of K-glypican is observed in the adrenal primordium. (B) Parasagittal section of an E15 embryo. Strong expression of K-glypican is observed in tubular epithelial cells. Expression in the adrenal declined significantly compared with the E13 embryo. (C) Parasagittal section of an E16 embryo. K-glypican-expressing tubular epithelial cells are widespread throughout the kidney. Note that little expression is seen in glomeruli (arrows). (Ad) adrenal gland; (K) kidney. Bar, 200 μm.

dothelium nor tunica adventitia was labeled (Fig. 8 C). Less intense but significant levels of K-glypican mRNA are also detected in the smooth muscle layer of intestines (Fig. 8 D). In contrast, no expression was detected in heart muscle (Fig. 8 C). Expression of K-glypican is also observed in mesenchyme of the facial area and hindlimbs (Fig. 6 A).

Discussion

Molecular Cloning of K-Glypican

In this paper, we describe molecular cloning of a glypican-like molecule, named K-glypican. Several lines of evidence show that K-glypican is a novel member of the glypican family of proteoglycans. First, the deduced amino acid sequence of K-glypican is only 21–43% identical to published sequences for glypican and OCI-5. In addition, mouse cDNAs for glypican and OCI-5 have been isolated as independent cDNAs distinct from K-glypican. Second, cerebroglycan, the only known family member we did not isolate a mouse cDNA for, is only 38% identical to K-glypican. That contrasts with the high degree of interspecies conservation found in this family; for example mouse and rat glypicans are 97% identical. Thus, K-glypican does not appear to be a mouse homologue of cerebroglycan. Furthermore, the results of Northern blot analyses showed that the developmental expression pattern and mRNA size of K-glypican are clearly distinct from those of the other three known members of the glypican family (Stipp et al., 1994; this study). Despite of the low levels of overall sequence homology, the predicted amino acid sequence of K-glypican contains the unique features that all known members of the glypican family share. Most notably, K-glypican exhibits almost complete conservation in the number and positions of the cysteine residues and contains a cluster of serine–glycine sequences in the COOH-terminaL region (Filmus et al., 1988; David et al., 1990; Karthikeyan et al., 1992; Stipp et al., 1994). From these observations, we conclude that K-glypican is not a mouse homologue of any of these known members of the glypican family, but a novel member of the family.

Our RT-PCR–based cloning approach failed to isolate the mouse homologue of rat cerebroglycan. This may be due to sequence difference between cerebroglycan and glypican in the region of Primer 8 that was used for cDNA synthesis. The primer was designed based on a conserved tetrapeptide sequence, Cys-Trp-Asn-Gly, whereas the corresponding cerebroglycan sequence is Cys-Trp-Thr-Gly (Stipp et al., 1994). Alternatively, the RNA sources used for RT-PCR may express cerebroglycan mRNA at low levels.

K-Glypican Is a GPI-anchored HSPG

To confirm that K-glypican is a GPI-anchored HSPG, we used an epitope-tagging method (Kolodziej and Young, 1991). Experiments using GAG-degrading enzymes and PI-PLC demonstrated that K-glypican is indeed a GPI-anchored HSPG in transfected MDCK cells. The attachment of heparan sulfate chains is not likely to be an artifact of epitope tagging, because the epitope sequence, EQKLISEEDL, does not contain any of the consensus sequences for GAG attachment. In addition, the c-myc-tagged proteoglycan, betaglycan, which is also a membrane-bound HSPG, has been reported to be as fully functional as the wild-type proteoglycan (López-Casillas et al., 1993). Therefore, the posttranslational modification of the
Figure 8. Expression of K-glypican in the developing brain, blood vessels, and intestines. 
K-glypican mRNA was detected by in situ hybridization with a digoxigenin-labeled RNA probe in antisense orientation. (A) The lateral ventricle of E13 mouse brain in a parasagittal section. (Lv) lateral ventricle, (Th) thalamus. (B) Mesencephalic vesicle and the fourth ventricle of E13 brain in a parasagittal section. (Mv) mesencephalic vesicle, (IV) fourth ventricle, (Cb) cerebellar primordium. (C) Aorta and heart of an E13 mouse embryo. Strong hybridization is seen in the wall of the aorta (Ao). No signal is detected in the endothelial layer or in heart muscle (H). (B) Intestines of an E13 embryo. Moderate hybridization is seen within the wall of the intestines (arrows). A large blood vessel with a strong K-glypican signal is also seen in the picture (arrowhead). No signal is detected in the liver (L). Bars, 200 μm.

c-myc–tagged K-glypican is considered to reflect that of native K-glypican. Although we cannot rule out the possibility that K-glypican is present as a nonproteoglycan or non–GPI-anchored form in vivo, our present results and the molecular similarities with glypican and cerebroglycan, both of which have been shown to present in tissues as GPI-anchored HSPGs (Herndon and Lander, 1990), strongly suggest that K-glypican is also a GPI-anchored HSPG in vivo.

We detected a significant amount of K-glypican released into culture supernatants without treatment with PI-PLC. The occurrence of soluble forms have also been reported for human glypican in cultured fibroblasts and endothelial cells (David et al., 1990; Mertens et al., 1992), cerebroglycan in rat brain (Stipp et al., 1994), and several other GPI-anchored proteins (e.g., Almqvist and Carlsson, 1988; Furley et al., 1990; Hortsch and Goodman, 1990; Vestal and Ranscht, 1992). It has been reported that a GPI-anchored HSPG is released by endogenous GPI-specific phospholipase D present in human bone marrow cells (Brunner et al., 1994; Metz et al., 1994). These observations suggest that the release of the glypican family HSPGs may have some physiological significance. It is possible that the soluble forms of the glypican family HSPGs have biological activities which may differ from those of their GPI-anchored counterparts.

Differential Expression of the Glypican Family HSPGs

Comparison of the expression of the glypican family HSPGs has revealed a highly divergent pattern of tissue distribution. For instance, while K-glypican mRNA is expressed in the smooth muscle layer of blood vessels (see Fig. 8 C), glypican is expressed in human aortic and umbilical vein endothelial cells (Mertens et al., 1992) and in mouse endothelial cell lines (this study). In developing intestines, K-glypican mRNA is present in smooth muscle cells (see Fig. 8 D), whereas OCI-5 has been detected in a rat intestinal cell line representing primitive intestinal epithelial cells (Filmus et al., 1988). These observations indicate that different members of glypican family may be simultaneously expressed in adjacent cell layers of a single tissue in vivo. A specific set of glypican family proteoglycans may be selected for expression depending on the cell type and its differentiation state.

K-Glypican Expression in Mouse Embryo

To gain an initial insight into the location of K-glypican expression, we performed in situ hybridization on developing mouse embryos. In the kidney, this revealed that the expression of K-glypican begins relatively late during development when pretubular aggregates start to differentiate. K-glypican continues to be expressed in differentiated tubular epithelium, but little expression is found in glomeruli.

HSPGs have been implicated in various aspects of kidney development. In particular, syndecan-1 is thought to play a major role in the epithelial conversion of metanephric mesenchyme (Vainio et al., 1989; Gilbert, 1994). Syndecan-1 is first seen around undifferentiated mesenchymal cells surrounding the ureteric bud and becomes strongly expressed in the pretubular aggregates and in the S-shaped bodies. Its expression then declines as the matu-
ration of nephrons proceeds, being lost by E15-16 except in glomeruli (Vainio et al., 1989). Thus spatiotemporal expression patterns of syndecan-1 and K-glypican are clearly distinct. Thus, if K-glypican plays a developmental role in embryonic kidney, it would be distinct from that of syndecan-1 and is presumably related to the differentiation of tubular epithelial cells. It has been shown that a heparin binding growth factor, hepatocyte growth factor, has the ability to induce branching and tube formation by kidney epithelial cells in vitro (Montesano et al., 1991a, b), and that this activity is modulated by heparan sulfate proteoglycans (Santos and Nigam, 1993). K-glypican may be involved in tubular morphogenesis through this type of modulation of growth factor activities. Moreover, since K-glypican is highly expressed in the adult kidney, it may also have a physiological function in the mature organ. In this context, it is interesting to note that protein C inhibitor, a member of the serine protease inhibitor (serpin) family that is present in various body fluids including urine, is bound to the apical surface of kidney epithelial cells through cell surface heparan sulfate (Priglinger et al., 1994). If K-glypican is actually sorted to the apical surface of epithelial cells as predicted from its GPI-anchor, this HSPG may be the key component that acts to retain protein C inhibitor on the luminal surface of the urinary tract.

Recent studies, including those in this paper, have demonstrated that embryonic brain is one of the most prominent sites of the expression of the glypican family HSPGs. All four known members of the family are expressed in the brain during development. Among them, only glypican is expressed both in embryonic and adult brain at comparable levels. On the other hand, K-glypican, cerebroglycan, and OCI-5 are expressed predominantly in embryonic brain, and their expression declines as the brain develops (Stipp et al., 1994; this study). However, our in situ hybridization results show that the regulation of expression appears to be much more intricate than this simple picture. Although K-glypican and cerebroglycan show similar expression patterns in Northern blotting experiments, there are remarkable differences in their spatial expression patterns. Stipp et al. (1994) reported that, while cerebroglycan is widely expressed in the nervous system, little if any cerebroglycan mRNA is found in the proliferative neuroepithelial cells in the ventricular zone. Such an expression pattern is clearly distinct from that of K-glypican, which is predominantly expressed in the ventricular zone. Thus, the present study strongly suggests that the expression of these two HSPGs is differentially regulated in a highly stringent manner during cortical development. A more detailed study in which the expression of both K-glypican and cerebroglycan is examined in parallel will establish the correlation between the differentiation states of neuroepithelial cells and the expression of these HSPGs during cortical development. Moreover, future studies should address whether K-glypican is functionally involved in the proliferation of neuroepithelial cells, as heparin binding growth factors and HSPG have been implicated in this process (Gonzalez et al., 1990; Bloch et al., 1992; Nurcombe et al., 1993; Baird, 1994).

We thank Drs. M. Fukuda, N. Ishida, and H. Kosaka for providing materials; Dr. H. Ohtani for advice on in situ hybridization techniques; Drs. Fukuda, A. Nishiyama, E. Pasquale, B. Ranscht, E. Rusu, and W. Stalaccip for critical comments on the manuscript. We also thank Dr. R. Kain for discussion; Dr. S. Takayama for homology search; Mr. J. Knight for critical comments on the manuscript. We also thank Dr. R. Kain for discussion; Dr. S. Takayama for homology search; Mr. J. Knight for critical comments on the manuscript. We also thank Dr. R. Kain for discussion; Dr. S. Takayama for homology search; Mr. J. Knight for critical comments on the manuscript. We also thank Dr. R. Kain for discussion; Dr. S. Takayama for homology search; Mr. J. Knight for critical comments on the manuscript.
ulated proteoglycans is expressed in the rat central nervous system. *Neuron* 4:949-961.

Hortsch, M., and C. S. Goodman. 1990. *Drosophila* fasciclin I, a neural cell adhesion molecule, has a phosphatidylinositol lipid membrane anchor that is developmentally regulated. *J. Biol. Chem.* 265:15104-15109.

Jackson, R. L., S. J. Busch, and A. D. Cardin. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.* 71:481-539.

Karihiayan, L., P. Maule, U. Rauch, R. K. Margolis, and R. U. Margolis. 1992. Cloning of a major heparan sulfate proteoglycan from brain and identification as the rat form of glypican. *Biochem. Biophys. Res Commun.* 188:395-401.

Kaufman, M. H. 1992. The atlas of mouse development. Academic Press, San Diego. 573 pp.

Kodukula, K., L. D. Gerber, R. Amthauer, L. Brink, and S. Udenfriend. 1993. Identification of an AUUUA-Specific Messenger RNA Bid-translation and characterization of adipocyte heparan sulfate proteoglycans with affinity for lipoprotein lipase. *J. Biol. Chem.* 268:23838-23844.

Montesano, R., G. Schaller, and L. Orci. 1991a. Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell.* 66:697-711.

Mordesano, R., K. Matsumoto, T. Nakamura, and L. Orcl. 1991b. Identification of a fibroblast-derived epithelial morphogen as the hepatocyte growth factor. *Cell.* 67:901-908.

Nurcombe, V., M. D. Ford, J. A. Wildschut, and P. F. Bartlett. 1993. Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science (Wash, DC).* 260:103-106.

Ohtani, H., A. Kuoriwa, M. Obinata, A. Ooshima, and H. Nagura. 1992. Identification of type I collagen-producing cells in human gastrointestinal carcinoma by non-radioactive in situ hybridization and immunoelectron microscopy. *J. Histochem. Cytochem.* 40:1139-1146.

Prillinger, U., M. Greiger, E. Bieleck, E. Vanyek, and B. R. Binder. 1994. Binding of urinary protein C inhibitor to cultured human epithelial kidney tumor cells (TCL-598). The role of glycosaminoglycans present on the luminal cell surface. *J. Biol. Chem.* 269:14705-14710.

Rodriguez-Boulan, E., and S. K. Powell. 1992. Polarity of epithelial and neuronal cells. *Annu. Rev. Cell Biol.* 8:395-425.

Ruoslathi, E. 1989. Proteoglycans in cell regulation. *J. Biol. Chem.* 264:13349-13372.

Ruoslathi, E., and Y. Yamaguchi. 1991. Proteoglycans as modulators of growth factor activities. *Cell.* 68:867-869.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santos, O. F. P., and S. K. Nigam. 1993. HGF-induced tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF-β. *Dev. Biol.* 160:293-302.

Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3'-untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell.* 46:659-667.

Sheppard, A. M., S. K. Hamilton, and A. L. Pearlman. 1991. Changes in the distribution of extracellular matrix components accompany early morphogenetic events of mammalian cortical development. *J. Neurosci.* 11:3926-3942.

Stipp, C. S., E. D. Litwack, and A. D. Lander. 1994. Cerebroglycan: an integral membrane heparan sulfate proteoglycan that is unique to the developing nervous system and expressed specifically during neuronal differentiation. *J. Cell Biol.* 124:149-160.

The Boulder Committee. 1970. Embryonic vertebrate central nervous system: revised terminology. *Anat. Rec.* 166:257-262.

Vainio, S., E. Lehto, M. Valkonen, M. Bernfield, and L. Sässén. 1989. Epithelial-mesenchymal interactions regulate the stage-specific expression of a cell surface proteoglycan, syndecan, in the developing kidney. *Dev. Biol.* 134:382-391.

Vestal, D. J., and B. Ranscht. 1992. Glycosyl phosphatidylinositol-anchored T-cadherin mediates calcium-dependent, homophilic cell adhesion. *J. Cell Biol.* 119:451-461.

von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683-4690.

Wang, X., F. H. Y. Lin, E. Ng-Eaton, J. Downward, H. F. Lodish, and R. A. Weinberg. 1991. Expression cloning and characterization of the TGF-β type III receptor. *Cell.* 67:797-805.

Yamada, H., K. Watanabe, M. Shimomaka, and Y. Yamaguchi. 1994. Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. *J. Biol. Chem.* 269:10119-10126.

Yamagishita, M., and V. C. Hascall. 1992. Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267:9451-9454.

Zimmerman, D. R., and E. Ruoslathi. 1989. Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J.* 8:2975-2981.