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Expression of the Cell Surface Proteoglycan Glypican-5 Is Developmentally Regulated in Kidney, Limb, and Brain

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Heparan sulfate is ubiquitous at the cell surface, where it is expressed predominantly on proteoglycans of either the transmembrane syndecan family or the glycosylphosphatidylinositol (GPI)-anchored glypican family, and has been proposed to function as a ''coreceptor'' for a number of ''heparin-binding'' growth factors. Although little is known about functional differences between individual members of the glypican gene family, mutations in both the *Drosophila* gene *dally* and the human gene for glypican-3 strongly suggest that at least some glypicans do function in cellular growth control and morphogenesis. In particular, deletion of the human glypican-3 gene is responsible for Simpson–Golabi–Behmel syndrome, and its associated pre- and postnatal tissue overgrowth, increased risk of embryonal tumors during early childhood, and numerous visceral and skeletal anomalies. We have identified and characterized, by sequencing of EST clones and products of rapid amplification of cDNA ends (RACE), an mRNA that encodes a 572-amino-acid member of the glypican gene family (glypican-5) that is most related (50% amino acid similarity, 39% identity) to glypican-3. Glypican-5 mRNA is detected as a 3.9- and 4.4-kb transcript in adult and neonatal mouse brain total RNA, and *in situ* hybridization results localize transcript primarily to restricted regions of the developing central nervous system, limb, and kidney in patterns consistent with a role in the control of cell growth or differentiation. Interestingly, *glypican-5* localizes to 13q31-32 of the human genome, deletions of which are associated with human 13q– syndrome, a developmental disorder with a pattern of defects that shows significant overlap with the pattern of glypican-5 expression.   © 1997 Academic Press

INTRODUCTION

Proteoglycans are a distinct class of glycoprotein found widely on cell surfaces and in extracellular matrices. Heparan sulfate proteoglycans are present on the surfaces of nearly all adherent cells, where they bind a number of ''heparin-binding'' proteins, including growth factors, extracellular matrix molecules, cell–cell adhesion molecules, and molecules involved in several degradative pathways (Jackson *et al.,* 1991; Lander, 1994). It has been proposed that heparan sulfate proteoglycans function as ''coreceptors'' for these molecules, facilitating their interactions with more conventional cell surface receptors (Bernfield *et al.,* 1992; Schlessinger *et al.,* 1995). Strong support for this model comes from

studies of the fibroblast growth factor family: Apparently, heparan sulfate is required at or near the cell surface for fibroblast growth factors to bind to and activate their receptor protein tyrosine kinases (Rapraeger *et al.,* 1991; Yayon *et al.,* 1991). More recently, experiments have suggested a similar requirement for cell surface heparan sulfate in signaling mediated by heparin-binding epidermal growth factor (Aviezer and Yayon, 1994), hepatocyte growth factor (Zioncheck *et al.,* 1995), and Wnts (Reichsman *et al.,* 1996).

Cell surface heparan sulfate is carried predominantly by proteoglycans belonging to two gene families: the syndecans, which are transmembrane proteins (Saunders *et al.,* 1989), and the glypicans, which are glycosylphosphatidylinositol (GPI)-anchored (David *et al.,* 1990). Little is known about functional differences among family members, or even about functional differences between syndecans and glypicans. A unique and distinguishing feature of the glypicans is a high degree of conservation of polypeptide sequence among family members, including conservation of

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a pattern of 14 cysteine residues, at least some of which are involved in disulfide bonds that help maintain the polypeptide in a compact, folded state (Herndon and Lander, 1990). In vertebrates, four glypican family members have so far been characterized: glypican-1 (glypican) (David *et al.,* 1990), glypican-2 (cerebroglycan) (Stipp *et al.,* 1994), glypican-3 (OCI-5) (Filmus *et al.,* 1988, 1995), and glypican-4 (K-glypican) (Watanabe *et al.,* 1995). Recently, these were joined by a *Drosophila* homolog, the product of the *dally* gene (Nakato *et al.,* 1995).

The effects of mutations in both the *dally* gene, and in the human gene for glypican-3, strongly suggest that at least some glypicans function in cellular growth control and morphogenesis. Deletion of human glypican-3 results in pre- and postnatal tissue overgrowth, increased risk of embryonal tumors during early childhood, and numerous visceral and skeletal anomalies (Pilia *et al.,* 1996). Loss of *dally* function in *Drosophila* leads to abnormalities in certain patterned cell divisions (Nakato *et al.,* 1995). Genetic interactions between *dally* and *decapentaplegic* (*dpp*) point to a role for glypicans in aiding signaling mediated by growth factors of the dpp/bone morphogenetic protein (BMP) subgroup of the transforming growth factor-β superfamily (Jackson *et al.,* 1997).

In an attempt to further understand the structural and potential functional diversity of the glypican gene family, we sought to identify additional vertebrate homologues. The current explosion of partial cDNA sequences available in the expressed sequence-tagged databases (dbEST) has provided a powerful tool for such endeavors. In particular, one may detect members of gene families that might otherwise be missed by standard biochemical or molecular biological methods due to low abundance of protein or mRNA transcripts. Using this strategy, we identified and characterized a new proteoglycan, which we have named glypican-5 (Saunders *et al.,* 1996), that shows all the structural features characteristic of glypicans, and is most closely related to glypican-3. Veuglers and colleagues, using the same strategy as ours, also recently identified this novel member of the glypican gene family (Veugelers *et al.,* 1997). We report here that glypican-5 mRNA expression is developmentally regulated in tissues including the kidney, limbs, and brain, in patterns consistent with a role in the control of cell growth or differentiation. Interestingly, the human glypican-5 gene localizes to chromosomal region 13q31-32, deletion of which is associated with 13q− syndrome, a developmental disorder with a pattern of defects that shows significant overlap with the pattern of glypican-5 expression.

MATERIALS AND METHODS

cDNA Cloning and Analysis

cDNA clones 52758, 52759, and 23723 were obtained (Research Genetics, Huntsville, AL) and sequenced as described below. A series of nested PCR primers based on sequences derived from clone 52758 (H29705-62R, 5′-GACGTTTGAAGAAACTGCTGC-3′; H29705-30R, 5′-GAGCCGCAATCTGATATC-3′; and H29705-15R, 5′-TCTCTCCTCCATCTT-3′) were used in 5′ RACE (5′ RACE System, Life Technologies, Gaithersburg, MD), from human 13-week fetal brain RNA, to isolate nucleotides 1–309 of human glypican-5 (GenBank Accession No. AF001462).

A partial murine glypican-5 cDNA probe was isolated by RT–PCR followed by sequential PCR with nested primers on one end of the fragment. First-strand cDNA synthesis was carried out on 1 μg newborn mouse brain RNA using a degenerate primer H29705-1055DR: 5′-TC(C/T)TC(A/C/G/T)CC(A/G)TTCCA(A/G)CA-3′. This first strand reaction was subjected to primary PCR with degenerate primers H29705-1055DR, and H29705-646D: 5′-GA(A/G)(C/T)TTAA(C/T)CC(A/C/G/T)CA(C/T)TGG-3′. A band corresponding to approximately 410 bp was purified by agarose gel electrophoresis from a complex mixture, and subjected to secondary PCR using primer H29705-1055DR and primer H29705-705: 5′-GCATGGAAC-ATACGACATTGG-3′. The resulting 351-bp cDNA fragment (GenBank Accession No. AF001463), encoding the partial murine glypican-5 amino acid sequence as shown in Fig. 1, was subcloned into Bluescript (SK⁻) (Stratagene, La Jolla, CA) to yield pMuGlyp5.

Gel-purified cDNA fragments were subcloned into either pPCR-Script or pBluescript (SK⁻) (Stratagene) and inserts were sequenced by the dideoxy chain termination method using a modified T7 DNA polymerase (Sequenase, United States Biochemical, Cleveland, OH) and denatured, double-stranded template. Both T7 and T3 primers, as well as custom synthesized primers, were used for sequencing. Compression sequencing artifacts were resolved by the use of both dGTP and dITP.

Biochemical Analysis

Mammalian expression vector pSP-6His was created by insertion of a series of complementary oligonucleotides encoding the signal peptide and cleavage site of an immunoglobulin heavy chain (Stern *et al.,* 1987) directly followed by an N-terminal six histidine sequence (MLCSWVIFFLMAVVNSEVQNHHHHHHAA), between the *Bam*HI and *Not*I sites of pcDNA3 (Invitrogen, San Diego, CA). A cDNA for human glypican-5, deleted for its putative signal peptide, was created by combining the *Eco*RV–*Bst*Y1 fragment of clone 52758 and a *Hin*dIII/*Not*I–*Eco*RV PCR fragment generated using primers EXP-F, 5′-CCCAAGCTTGCGGCCGCAGACCTACGA-AGAAGTT-3′, and EXP-R, 5′-CCCCTGACAGCTCCCAGCAG-3′, into the *Hin*dIII–*Bam*HI site of Bluescript (Stratagene). The *Not*I–*Xba*I fragment of this intermediate vector (pBS-Glyp5), encoding the truncated human glypican-5 cDNA, was then inserted into the *Not*I–*Xba*I site of pSP-6His to create the final expression vector pSP-6His-glypican-5.

COS-7 cells were transiently transfected with pSP-6His-glypican-5 or control plasmid (pSP-6His) using lipofectamine (Life Technologies) and Optimem (Life Technologies) reduced serum media, following manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced with DMEM (Life Technologies) containing 10% FCS and the cells were cultured for an additional 48 hr. Conditioned medium from four 100-mm plates was collected for each transfection and made 6 *M* in guanidinium chloride, 10 m*M* in 2-mercaptoethanol, and 50 m*M* in Tris–HCl, pH 8.0, and bound batchwise to 200 μl of preequilibrated Ni-NTA resin (Qiagen, Santa Clarita, CA) overnight at 4°C. Resin was transferred to Poly-Prep columns (Bio-Rad, Hercules, CA) and washed with 25 ml of 50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 6 *M* guanidinium

chloride, 10 m*M* 2-mercaptoethanol followed by 25 ml 50 m*M* Tris–HCl, pH 8.0. The resin from each sample was divided into four equal aliquots and briefly centrifuged, and the supernatant was discarded. The aliquots of resin were each resuspended in 50 μl of digestion buffer (50 m*M* Tris–HCl, 15 m*M* phosphoric acid (pH 7.1), 1 μg/ml pepstatin, 1 m*M* PMSF, and 250 μ*M* NEM) containing chondroitinase ABC (0.05 U/ml) (Sigma, St. Louis, MO), heparitinase (4 μg/ml of enzyme prepared from flavobacterium heparinum by hydroxyapatite chromatography as described by Linker and Hovingh (1972)), neither enzyme, or both enzymes together. Samples were allowed to digest for 2 hr at 37°C; then they were boiled in SDS-containing sample buffer (final concentrations: 50 m*M* Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 150 μg/ml bromphenol blue, 50 m*M* dithiothreitol), separated on a 7.5% SDS–PAGE reducing gel, and electrophoretically transferred to nitrocellulose. Blots were probed with an anti-6His-specific monoclonal (Clontech, Palo Alto, CA) and enhanced chemiluminescence detection reagents (Amersham, Arlington Heights, IL).

Northern/Dot Blot Analysis

A mouse multiple tissue Northern blot (Clontech) was analyzed for glypican-5 mRNA. This blot contains approximately 2 μg of poly(A)$^+$ RNA prepared from seven distinct adult BALB/c tissues, subjected to electrophoresis on a denaturing formaldehyde 1.2% agarose gel, transferred to a cationic nylon membrane, and immobilized by UV irradiation (Sambrook *et al.,* 1989). The filter was hybridized in ExpressHyb solution (Clontech), initially with the murine glypican-5 cDNA probe labeled with [α-^{32}P]dCTP by the random priming method (Feinberg and Vogelstein, 1983), and then subsequently with a β-actin cDNA probe to verify similar loading and quality of RNA in each lane.

A human multiple tissue RNA blot (Human RNA Master Blot, Clontech) was also analyzed for glypican-5 mRNA. This dot blot contains normalized loadings of 100–500 ng of each poly(A)$^+$ RNA per dot from 50 different human tissues and 6 different control RNAs and DNAs. The filter was hybridized in ExpressHyb solution (Clontech) with the human glypican-5 *Not*I–*Xba*I fragment of pBS-Glyp5 labeled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). Signals were detected and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In Situ Hybridization

Random-bred Swiss mice (CD-1, Charles River Laboratories) were naturally mated. The day of identification of a vaginal plug was considered E0.5, and the day of birth was considered P0. For embryonic tissue, pregnant animals were sacrificed by cervical dislocation and embryos were removed. Neonatal and early postnatal animals were anesthetized by hypothermia and decapitated, and brains were immediately removed into ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 7.83 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$). Nonpregnant adult animals (>6 weeks old) were also sacrificed by cervical dislocation and their brains were removed into ice-cold PBS. Freshly dissected mouse embryos and postnatal brains were embedded in OCT (Miles, Elkhart, IN) and quick frozen in isopentane on dry ice, while adult brains were quick frozen directly in isopentane without embedding. All samples were temporarily stored at −80°C and then equilibrated to −18°C, and 12- to 14-μm cryostat sections were collected on Probe-On Plus slides

(Fisher Biotech, Pittsburgh, PA) which were stored at −80°C with desiccant prior to hybridization. Sections were equilibrated to room temperature, fixed in 4% paraformaldehyde in PBS at room temperature for 20 min, rinsed twice in PBS with 0.1 *M* glycine and once in PBS, acetylated in 0.1 *M* triethanolamine (pH 8.0) and 0.25% acetic anhydride for 10 min, rinsed twice more in PBS, and then dehydrated through an ethanol series. Hybridization was carried out using ^{35}S-labeled murine glypican-5 antisense and control sense probes, generated by *in vitro* transcription of plasmid pMuGlyp5, and a hybridization and posthybridization protocol as described by Gall *et al.* (1995). Slides were dipped in Kodak NTB-2 liquid emulsion diluted 1:1 with distilled water, and exposed for 21–28 days at −80°C. Developed sections were stained in Hoechst 33258 (bisbenzimide) at 20 μg/ml or toluidine blue (0.005% in water). In some cases, adjacent slides were also hybridized with 4EX, a probe for glypican-1 (Litwack *et al.,* 1994).

Radiation Hybrid Mapping

The chromosomal localization of human glypican-5 was determined by PCR screening of the 93 clones of the GeneBridge 4 radiation hybrid panel (Walter *et al.,* 1994) (Research Genetics). PCR was performed using two pairs of primers. The first pair, Gen-Glyp5-F1 (F1), 5′-TGGAATGAAGACCACCAC-3′, and Gen-Glyp5-R1 (R1), 5′-TGTCCATATTGAATTTTATTCG-3′, was derived from the exon and intron sequence, respectively, that flank a splice donor site (data not shown) following the sequence encoding amino acid residue 385 (identifiable in the fifth line of human glypican-5 sequence in Fig. 1A as the amino acid ''K'' in the sequence EETLANRRKERINSLR). The second pair, Gen-Glyp5-F2 (F2), 5′-TGGTGATGTTACTTCCCG-3′, and Gen-Glyp5-R2 (R2), 5′-TTGGCTTCTGGAAGTTAGTG-3′, was derived from the C-terminal coding sequence and 3′ untranslated region, respectively. PCR conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, in a buffer containing 100 ng template DNA, 500 n*M* each primer, 200 μ*M* dNTP, 3 m*M* MgCl$_2$, 50 m*M* KCl, 10 m*M* Tris–HCl, pH 9.0, and 5% DMSO in a total volume of 20 μl. Primer pair F1/R1 and F2/R2 amplified a 157- and 167-bp fragment, respectively, from human genomic DNA, but not from hamster DNA. Placement of the glypican-5 locus onto the STS-based map of the human genome was carried out using the RHMAPPER program available through the World Wide Web server of the Whitehead Institute (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl).

RESULTS

Cloning of Human and Murine cDNAs Encoding a Novel Glypican Family Member

To identify potential new members of the glypican gene family, we performed a BLAST search of the National Center for Biotechnology Information database of expressed sequence tags (dbEST), using the full-length amino acid sequence of the known family members as query sequences. In addition to identifying multiple cDNA sequences corresponding to murine and human clones of the known vertebrate glypicans, we identified five partial cDNA sequences (GenBank Accession Nos. F11477, H29705, H29706, T77270, and F13387 de-

rived from clones designated c-2x12, 52758, 52759, 23723, and c-3mf05, respectively, from a normalized human infant brain cDNA library) that represented apparently novel glypican-like sequences. Three of these cDNA clones (52758, 52759, and 23723) were obtained from a commercial supplier (Research Genetics) and further sequenced, confirming by sequence identity that cDNA clone c-2x12 was also derived from the same mRNA species, which we named glypican-5. Because all of these cDNA's lacked 5′ sequences that could encode amino acids homologous to the 69–75 most N-terminal amino acids of other glypicans, we cloned the 5′ end of the glypican-5 mRNA from 13-week human fetal brain RNA using rapid amplification of cDNA ends (RACE). The composite cDNA sequence for glypican-5 is available as GenBank Accession No. AF001462.

To facilitate analysis of glypican-5 mRNA expression in mice, we also derived a murine cDNA probe. Forward (H29705-646D) and reverse (H29705-1055DR) degenerate PCR primers were designed based on amino acid residues 287–292 and 418–423, respectively, of the deduced human glypican-5 sequence. These represent sequences that have a relatively low level of degeneracy, and that occur at positions at which there is high conservation among other glypican family members (Fig. 1). RT–PCR was performed on P0 murine brain RNA and the predicted 410-bp size range purified from a complex mixture. Secondary PCR amplification of this template DNA, using the same degenerate reverse primer and a nondegenerate human-specific forward primer (H29705-705), resulted in a single PCR fragment of the predicted 351-bp size. Subcloning and sequencing of this fragment confirmed it to be murine glypican-5, with 85% amino acid sequence identity to human glypican-5 (Fig. 1). The partial murine glypican-5 sequence is available as GenBank Accession No. AF001463.

Sequence Analysis

Our composite human glypican-5 cDNA is 1938 bp in length. The sequence agrees at most positions with that obtained by Veuglers *et al.* (1997; GenBank Accession No U66033) and includes an additional 67 nt of 5′ untranslated sequence. We noted sequence discrepancies between U66033 and our sequence at nucleotides positions 694 and 815 of our sequence, which was surprising since we both used the identical EST cDNA clone (clone ID, 52758) to obtain sequence in those regions. Since these differences resulted in alternative amino acid assignments of Gly^{205} to Cys^{205} and Ser^{245} to Phe^{245} in their and our sequences, respectively, we used synthetic sequencing primers positioned approximately 45 nt from the residues in question to unequivocally confirm that our cDNA sequence reflects the correct cDNA sequence of clone ID, 52758, and therefore the correct conceptual amino acid sequence for human glypican-5. The open reading frame predicts a 572-amino-acid polypeptide containing both an amino and carboxy terminal stretch of hydrophobic residues, consistent with a

potential leader sequence and GPI-attachment signal, respectively. Application of the algorithm of Nielsen *et al.* (1997) to the N-terminal hydrophobic stretch predicts a signal peptide with cleavage at Ser^{24}, while the C-terminal sequence is typical of GPI-attachment signals in that a stretch of hydrophobic amino acids is preceded by a potential ''hinge,'' in this case the charged amino acid Glu^{555}. Application of the ω, $\omega + 2$ rule (Kodukula *et al.,* 1993) identifies two sites with equal probability for cleavage and GPI attachment: residues Gly^{549} and Ala^{551}. Removal of both the N- and C-terminal signal sequences from glypican-5 would result in a mature protein with a molecular mass of 58.5 kDa.

Figure 1 aligns the predicted human and partial murine glypican-5 protein sequence with the sequences of other members of the glypican gene family. Mature human glypican-5 is predicted to contain structural features consistent with other members of the glypican gene family, including the 14 cysteine residues that are conserved among all glypican family members (as well as the additional pair of cysteines that is present in glypican-1, -3, and -4), in addition to five serine–glycine dipeptide sequences (Ser^{441}, Ser^{486}, Ser^{495}, Ser^{507}, and Ser^{509}) that, taken in the context of neighboring amino acid sequences, are potentially consistent with glycosaminoglycan attachment sites (Bourdon *et al.,* 1987; Zhang and Esko, 1994).

Biochemical Characterization of Glypican-5 Protein

To test whether glypican-5 is indeed a proteoglycan we expressed it in COS-7 cells by transient transfection. A mammalian expression vector, pSP-6His-glypican-5, which encodes human glypican-5 substituted with a 6-histidine tag at the N-terminus of the mature protein, was devised to permit purification on Ni-NTA agarose (Janknecht *et al.,* 1991). Conditioned media from pSP-6His-glypican-5-transfected and control-transfected cultures were subjected to Ni-NTA affinity chromatography and the bound material was treated with glycosaminoglycan lyases, followed by SDS–PAGE, and Western blotting with an anti-6His monoclonal antibody. No signal was detected in material isolated from pSP-6His-glypican-5 transfectants that was untreated (Fig. 2, lane 1), treated with chondroitinase ABC alone (Fig. 2, lane 2), or treated with heparitinase alone (Fig. 2, lane 3). However, combination of heparitinase and chondroitinase ABC resulted in the detection of a single protein band of approximately 65 kDa by reducing SDS–PAGE (Fig. 2, lane 4) In material derived from control transfections, no bands were detected under any digestion condition (data not shown). These results indicate that glypican-5 is modified with glycosaminoglycan chains, and that, at least under conditions of transient transfection in COS-7 cells (but perhaps not in other cells—see Discussion), can be a hybrid proteoglycan, possessing both heparan and chondroitin sulfate. Our inability to detect glypican-5 by Western blotting prior to removal
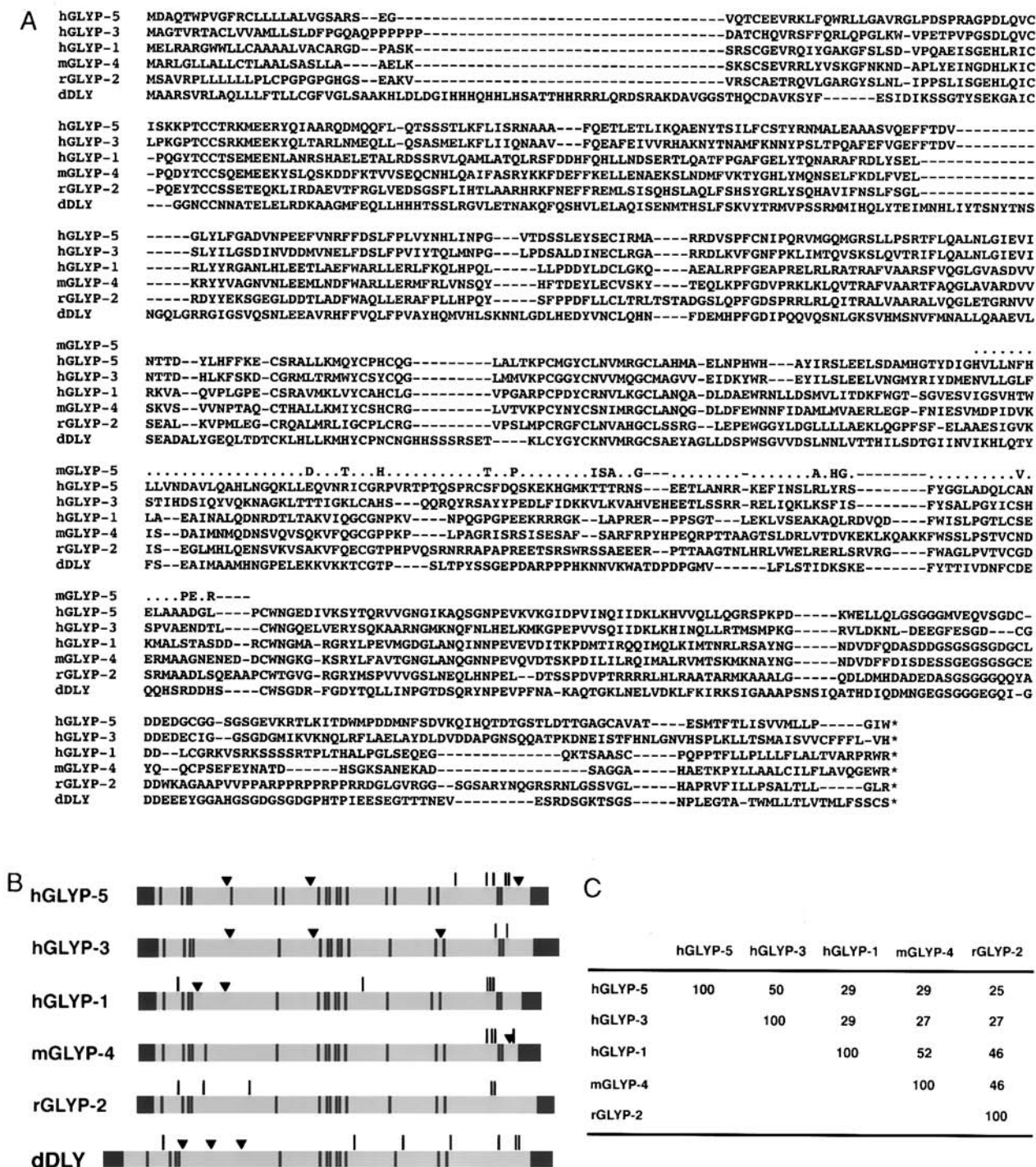
FIG. 1. Sequence and structural similarities of the glypican family. (A) Multiple sequence alignment of vertebrate and invertebrate glypican gene products. Dots represent identities between murine and human glypican-5. Dashes denote gaps in the alignments. (B) Schematic alignment of glypican core proteins, revealing the high degree of structural conservation between family members. Filled boxes correspond to signal sequences, gray areas correspond to predicted mature core proteins. Black lines within the gray areas denote cysteine residues, while black lines extending up from the gray areas denote potential glycosaminoglycan attachment sites. Potential N-linked sites of glycosylation are indicated by black triangles. (C) Sequence similarities between the predicted mature core proteins of vertebrate glypicans as predicted by the GCG software Distances and the multiple sequence alignment shown in A. hGLYP-1, human glypican; rGLYP-2, rat cerebroglycan; hGLYP-3, human OCI-5; mGLYP-4, mouse K-glypican; hGLYP-5, human glypican-5; dDLY, *Drosophila dally.* The human and murine glypican-5 cDNA sequences are available as GenBank Accession Nos. AF001462 and AF001463, respectively.
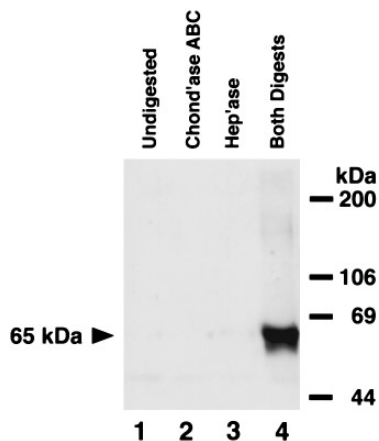
FIG. 2. Glycosaminoglycan composition of glypican-5 transiently expressed in COS-7 cells. An N-terminal 6-histidine-tagged glypican-5 construct (pSP-6His-glypican-5) and control plasmid were transfected and transiently expressed in COS-7 cells. Expressed product, purified by Ni-NTA, was either not treated with any enzymes (lane 1), or digested with chondroitinase ABC (lane 2), heparitinase (lane 3), or both enzymes (lane 4). Samples were subjected to SDS–PAGE under reducing conditions, followed by Western blotting using an anti-6His monoclonal antibody for detection. A single ~65-kDa core protein band was detected in material from pSP-6His-glypican-5-transfected cells after enzymatic digestion with both heparitinase and chondroitinase ABC. No bands were detected in material isolated from cells transfected with a control plasmid (not shown).

of both types of glycosaminoglycans is not surprising, given the often poor transfer efficiency of large proteoglycans and the generally poor binding of intact proteoglycans to nitrocellulose membranes (Rapraeger *et al.,* 1985).

Expression Pattern of Glypican-5 mRNA

To investigate the tissue distribution of glypican-5 mRNA in human and murine tissues, we carried out a series of membrane (Northern and dot blot) and *in situ* hybridizations. Hybridization of the murine glypican-5 cDNA probe to a murine adult tissue Northern blot detected a major 3.9-kb mRNA and a minor 4.4-kb species predominantly in adult brain (Fig. 3, lane 7). As seen in Fig. 3, lane 2, a low level of glypican-5 mRNA was also detected in adult kidney. In very long exposures (not shown), a very weak signal was detected in testis as well, whereas adult skeletal muscle, liver, lung, spleen and heart remained negative (Fig. 4, lanes 3,4,5,6,and 8). Hybridization of the same blot with a cDNA probe corresponding to the full-length coding region of human glypican-5 revealed essentially the same pattern of hybridization (data not shown).

We next examined the tissue distribution of glypican-5 mRNA in a wide range of human adult, as well as fetal

tissues, with special emphasis on regional distribution within the brain (the organ in which, in mice, the highest levels of glypican-5 mRNA had been seen). For this purpose a cDNA probe, corresponding to the full-length human glypican-5 cDNA, was hybridized to a dot blot containing poly(A)$^+$ RNA from multiple human tissues (Fig. 4). The quantity of mRNA loaded on each spot of this membrane had been previously normalized relative to a battery of eight constitutive genes, thus allowing meaningful comparisons of mRNA levels between tissues (Spanakis, 1993; Spanakis and Brouty-Boyé, 1994). As seen in Fig. 4, glypican-5 message was detected widely throughout the central nervous system, including within the pituitary gland. Consistent with the data in mouse, glypican-5 mRNA was also detected in the kidney and testis. A wide range of tissues, including several abdominal visceral, cardiovascular, pulmonary, glandular/endocrine, and immune system structures revealed only background levels of hybridization. Glypican-5 mRNA was also detected in a number of human fetal tissues, including brain, kidney, liver, and lung, but was not detected in others including heart, spleen, and thymus. This suggested that glypican-5 expression may be developmentally regulated.
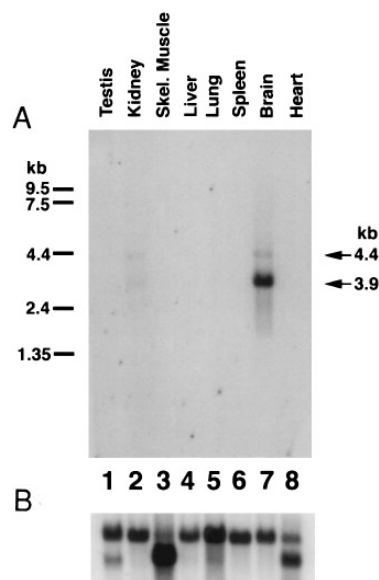

FIG. 3. Northern blot analysis of glypican-5 expression in adult mouse tissues. Each lane contains 2 μg of poly(A)$^+$ RNA from adult BALB/c testis (1), kidney (2), skeletal muscle (3), liver (4), lung (5), spleen (6), brain (7), and heart (8). (A) Hybridization with the murine glypican-5 cDNA probe (pMuGlyp5) identifies a 3.9- and 4.4-kb message prominently in brain and at lower levels in kidney. (B) Control hybridization with a β-actin cDNA probe to confirm equivalency of loading and quality of mRNA, identifies a 2.0-kb band in all lanes. The 1.8-kb band detected in cardiac and skeletal muscle, and to a lesser extent testis, reflects alternative actin isoforms that are normally found in these tissues.
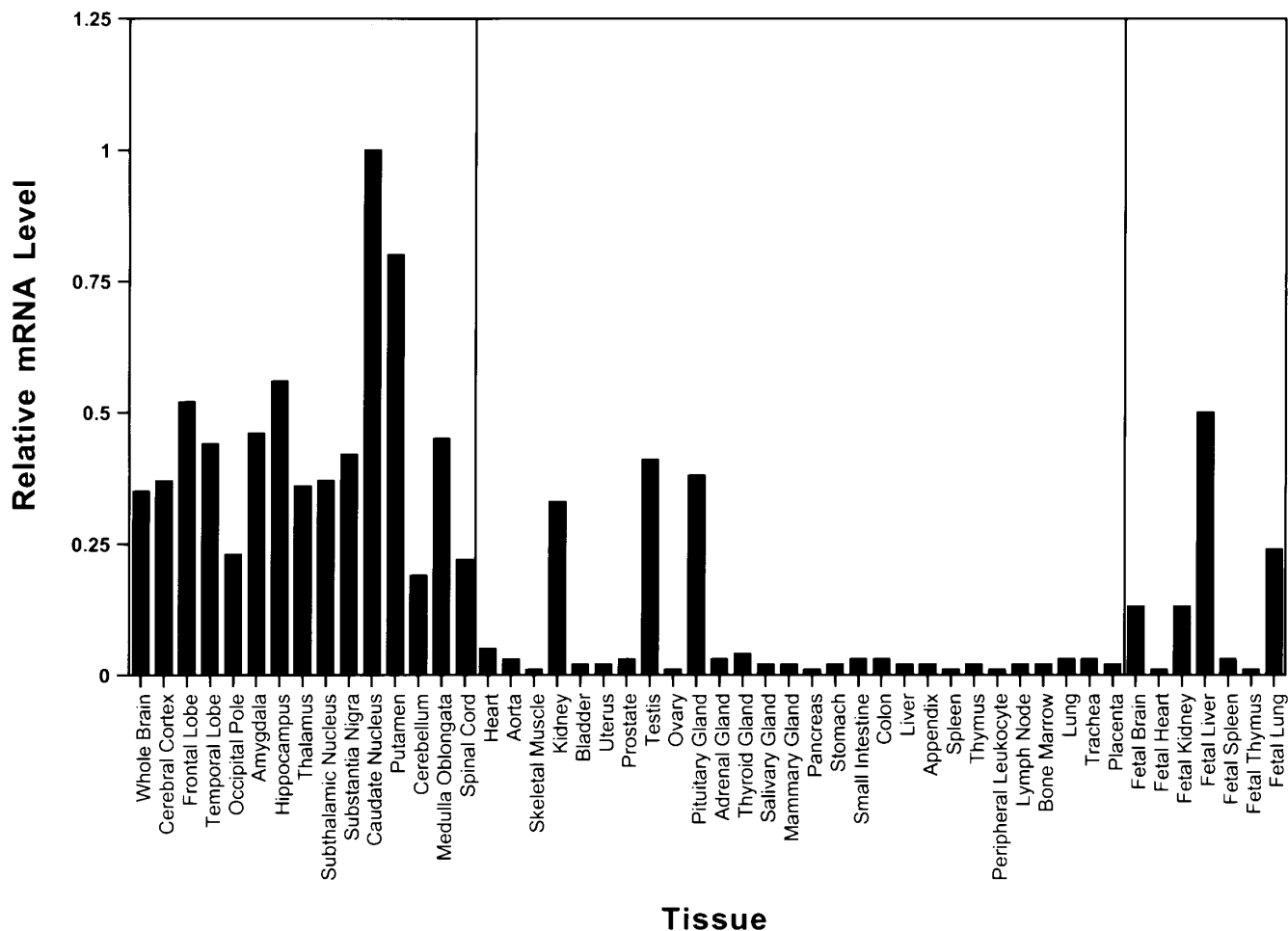
FIG. 4. Analysis of glypican-5 expression in human adult and fetal tissues. Human RNA Master Blot (Clontech) containing 100–500 ng of each poly(A)$^+$ RNA per dot, loaded in normalized quantities relative to eight constitutive genes, was hybridized with a [α-^{32}P]dCTP-labeled human glypican-5 *Not*I–*Xba*I fragment of pBS-Glyp5. Signals were detected and quantified using the PhosphorImager and IMAGEQUANT, and plotted as relative mRNA level for each tissue.

The cellular localization of glypican-5 mRNA in the developing and adult mouse was evaluated by *in situ* hybridization. [^{35}S]UTP-labeled, single-stranded, murine glypican-5 antisense and sense control probes were hybridized to fresh frozen tissue sections from animals at E10.5, E12.5, E14.5, E16.5, postnatal day 0 (P0), and at adulthood (>6 weeks). In general, glypican-5 mRNA was found in restricted subsets of cells within a few discrete organ systems. For example, at E10.5, glypican-5 is expressed in the limb buds, urogenital ridge, ventral wall of the otic vesicle, dorsal root ganglia (DRG), parts of the maxillary process (Fig. 5), and the most ventral surface of the midbrain tegmentum (not shown).

Glypican-5 hybridization within the limb bud was especially strong, with labeling throughout the mesenchyme but not in the overlying ectoderm (Figs. 5A and 5B). Exami-

nation of multiple sections did not suggest any apparent gradation of hybridization along the anteroposterior or dorsoventral axes of the limb; however, there did appear to be reduced hybridization over the chondrogenic core. Examination of the urogenital ridge at high magnification (Figs. 5D and 5E) indicated that glypican-5 mRNA is expressed in the mesonephros. Hybridization was consistently weakest in the rostral nephric duct and strongest in the caudal nephric duct. The latter gives rise to both the most newly generated mesonephric vesicles and ultimately the ureteric buds of the metanephric kidney (Saxén, 1987).

At later embryonic ages, glypican-5 mRNA was found in many of the same organ systems as were seen at E10.5, although not always in the same cellular distribution. For example, at E14.5, glypican-5 mRNA was still found in the limb, but expression was now restricted to condensing mes-
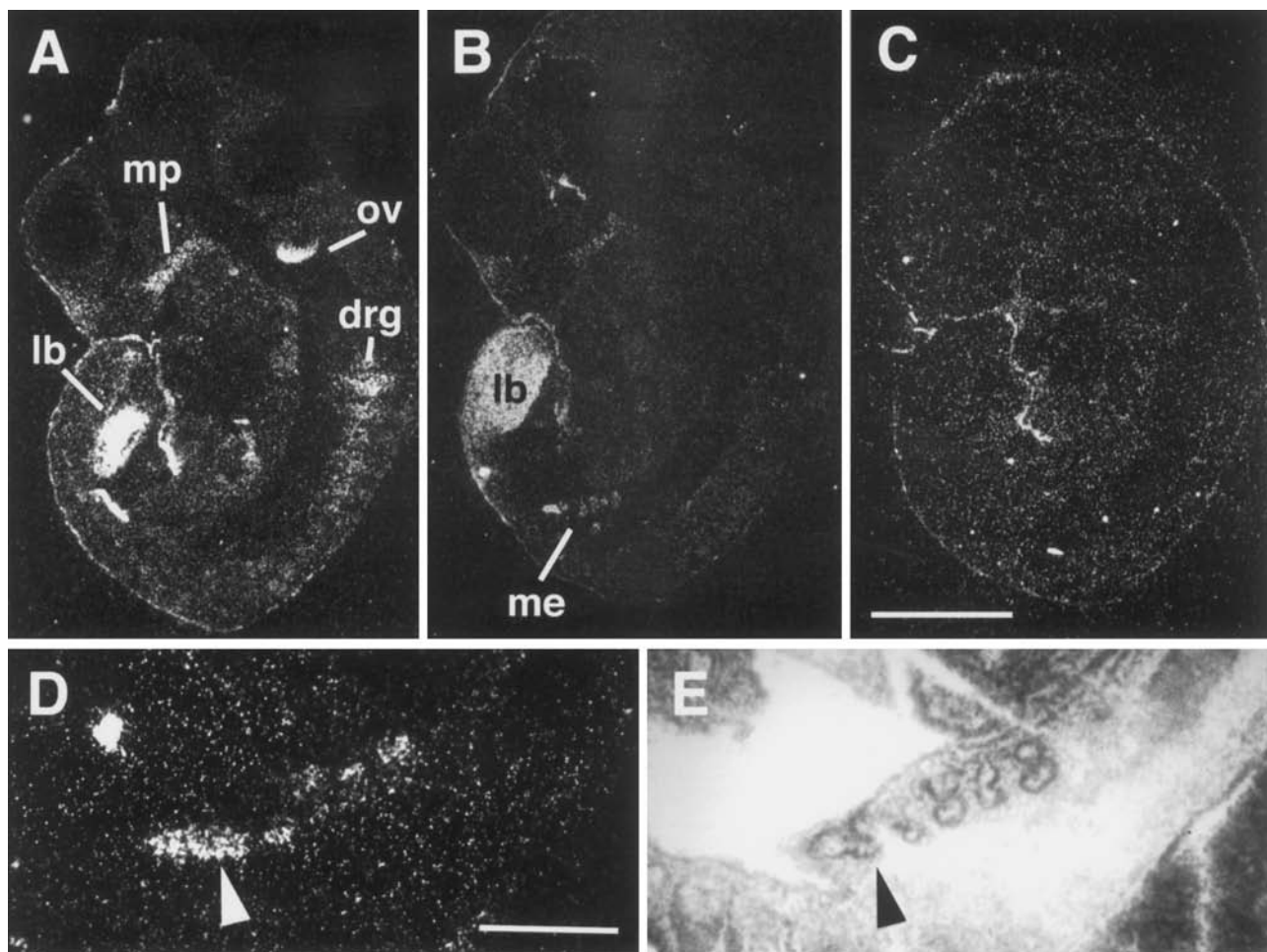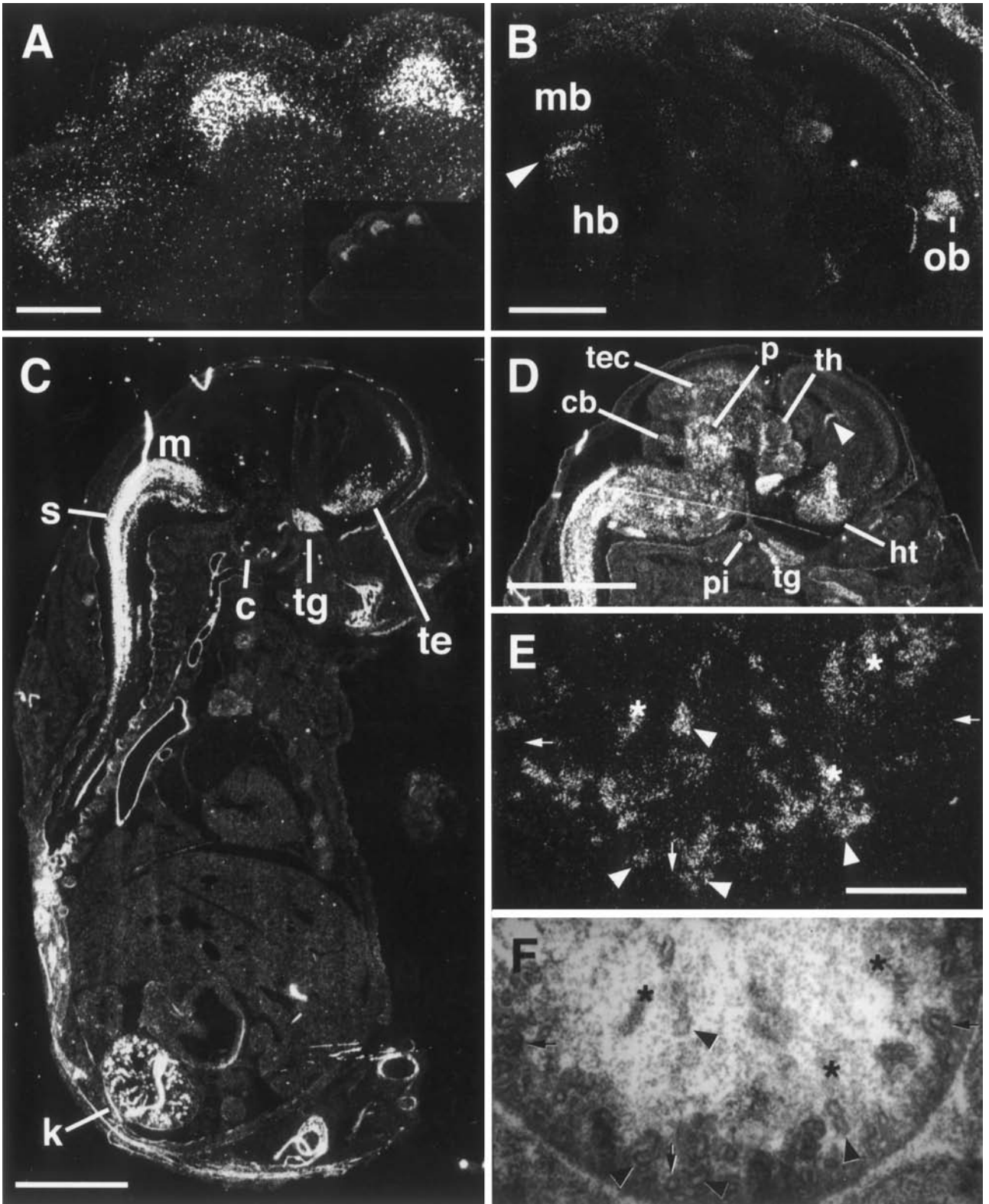
FIG. 5. Expression of glypican-5 mRNA in Embryonic Day 10.5 mouse. (A and B) Autoradiography of two parasagittal sections (B more lateral than A) hybridized with a ^{35}S-labeled murine glypican-5 antisense probe. (C) Adjacent section hybridized with sense control probe. (D and E) High magnification view of mesonephros hybridized with ^{35}S-labeled glypican-5 probe (D) and the same section stained with toluidine blue (E). Arrowhead indicates hybridization signal in caudal nephric duct (the bright spot adjacent to the letter D is due to light scattering by a dust particle). Abbreviations: (drg) dorsal root ganglion, (lb) limb bud, (ov) otic vesicle, (me) mesonephros, (mp) maxillary process. Sections in A–D were exposed for 6 weeks and photographed using dark-field illumination. A–C are shown at the same magnification, as are D and E. Bar in C is 1 mm; in D, 300 μm.

enchyme at the distal tips of the developing digits (Fig. 6A). In the urogenital system, glypican-5 mRNA was readily detected in the developing permanent kidney (metanephros) at E14.5 (not shown) and E16.5 (Fig. 6C), where it was localized to condensing mesenchymal aggregates and the epithelial tubules derived from these aggregates (Figs. 6E and 6F). It was consistently observed that the most highly differentiated tubular structures, as defined by a well-formed tubular architecture containing a central lumen, lacked hybridization signal. This suggested that glypican-5 mRNA expression may decline as renal tubules mature. Glypican-5 hybridization was also detected at E16.5 in another part of the urogenital system, the developing vas deferens, which is derived from the mesonephric duct.

Although, at E10.5, the distribution of glypican-5 mRNA in the central nervous system was seen to be quite limited, expression becomes more widespread as the nervous system matures. In the anterior telencelphalon, for example, collections of cells just dorsal and ventral to the evaginating olfactory bulb express glypican-5 by E14.5 (Fig. 6B). More caudally, expression was also seen in cells of the isthmus, or midbrain/hindbrain junction (Fig. 6B). In the spinal cord, glypican-5 hybridization was seen as early as E12 within two bilaterally symmetric pairs of continuous columns of cells extending rostrocaudally within the ventral gray matter (not shown), a pattern that could still be detected at E16.5 (Fig. 6C). Strong hybridization within the medulla was also evident at E16.5. On lateral sections of E16.5 ani-

mals (Fig. 6C), little glypican-5 hybridization was seen in midbrain and forebrain structures, with the exception of anterior ventral forebrain; however, on more medial sections of the same animal (Fig. 6D), glypican-5 mRNA was found in several additional structures. For example, in the diencephalon, glypican-5 was detected in subsets of thalamic nuclei and in much of the ventral and lateral hypothalamus. The striatal primordium, or ganglionic eminence, showed little glypican-5 hybridization, except along its ventricular border (see Discussion). Hybridization was also seen in the midbrain tectum, in the deep nuclei of the cerebellum, and in parts of the pons. Other tissues expressing glypican-5 at E16.5 included the trigeminal ganglion, the pituitary, and the cochlea of the inner ear (Fig. 6C). Interestingly, the latter is derived specifically from the ventral wall of the otic vesicle (Martin and Swanson, 1993), where glypican-5 hybridization had been detected at E10.5 (Fig. 7).

Because of its interesting patterns of expression within the nervous system, as well as its relatively greater abundance in that organ system in adult tissues (cf. Figs. 4 and 5), *in situ* hybridization analysis of glypican-5 expression at later developmental stages was limited to the central nervous system. In comparison to the E16.5 central nervous system, P0 brain demonstrated widespread expression of glypican-5 mRNA throughout the forebrain (Fig. 7A). In very anterior sections (not shown), glypican-5 mRNA was detected within the subventricular zone and mitral cell layer of the olfactory bulb. Slightly more posteriorly, glypican-5 was detected in structures of the ventral forebrain, including the preoptic nuclei and the olfactory tubercle. As at E16.5, the striatum was generally negative for glypican-5. Still more posteriorly, glypican-5 mRNA was detected in both the hippocampus as well as the cerebral cortex. In the cerebral cortex, transcript appeared most abundant within the deepest layers of the developing cortical plate. In the diencephalon, mRNA expression was detected within some, but not all thalamic nuclei. In particular, hybridization was detected in the lateral geniculate, centromedian, and laterodorsal nuclei of the thalamus. In the epithalamus strong hybridization was seen primarily in the lateral, but not the medial habenular nuclei, while in the hypothala-

mus, hybridization was strongest in lateral and ventral regions (Fig. 7A). In the more posterior regions of the midbrain, glypican-5 hybridization was widely observed within the deeper nuclei of the cerebellum, tectum, and pons. No hybrdization to any CNS structures was seen with a control sense-strand probe (Fig. 7B).

In the adult brain, glypican-5 mRNA was expressed even more widely, although at levels that appeared quite low (as judged by lower signal intensity after similar autoradiographic exposure times). Figure 7 presents the pattern of expression of glypican-5 in the forebrain and contrasts it with that of glypican-1 (Fig. 7C), a glypican that is also widely expressed in the brain (Litwack *et al.,* 1994). Glypican-5 hybridization was seen at fairly uniform levels throughout the cerebral cortex, hippocampus, and diencephalon, with the exception of hippocampal areas CA1–CA2, in which pyramidal cells showed elevated levels of hybridization (Fig. 7D). In contrast, glypican-1 is most highly expressed in hippocampal areas CA3–CA4 and in thalamus, as well as at somewhat lower levels in the dentate gyrus, hippocampal areas CA1–CA2, and cerebral cortical layers 2/3, 5 and 6 (Litwack *et al.,* 1994).

Chromosomal Mapping of Human Glypican-5

To gain other potential clues about the biological function of glypican-5, we mapped the glypican-5 gene within the human genome using radiation hybrid (RH) mapping, a technique that involves scoring the presence or absence of a genomic marker among independent human/hamster hybrid cell clones bearing human genomic fragments produced by X-irradiation. A pattern of amplification of PCR products is compared with the patterns contained in a database of previously mapped genomic framework markers. Among the advantages of this technique is its ability to order markers spanning millions of base pairs of DNA, at a resolution not easily obtained by other techniques. In addition, the estimated distances obtained are directly proportional to physical distance.

We utilized for these experiments the GeneBridge 4 RH panel (Walter *et al.,* 1994) and two pairs of glypican-5-spe-

FIG. 6. Expression of glypican-5 mRNA in Embryonic Day 14.5 (A, B) and 16.5 (C–F) mouse. Sections were hybridized with ^{35}S-labeled murine glypican-5 antisense probe, subjected to autoradiography, and viewed with dark-field illumination (A–E) or after counterstaining with toluidine blue (F). (A) Parasagittal section through distal hindlimb (lower magnification view, inset). Hybridization is confined to the condensing mesenchyme at the distal tips of the developing digits. (B) Parasagittal section through head. Arrowhead indicates hybridization in the isthmus, or midbrain/hindbrain junction. (C) Parasagittal section through an entire embryo, and (D) a more medial section through the head of the same embryo. Arrowhead indicates hybridization in the neuroepithelium of the ganglionic eminence. (E and F) High magnification view of the kidney. Asterisks indicate hybridization to regions of condensed, undifferentiated mesenchyme; arrowheads, hybridization to differentiated tubular epithelial structures; arrows, nonhybridizing well differentiated tubular epithelial and glomerular structures. A, B, and D were exposed for 3 weeks; C and E were exposed for 5 weeks. Abbreviations: (c) cochlea, (cb) deep cerebellar nuclei, (hb) hindbrain, (ht) hypothalamus, (k) kidney, (m) medulla, (mb) midbrain, (ob) olfactory bulb, (p) pons, (pi) pituitary, (s) spinal cord, (te) telencephalon, (tec) midbrain tectum, (tg) trigeminal ganglion, (th) thalamus. Bar: A, 300 $\mu M;$ B, 1 mm; C, 2 mm; D, 1 mm; E, 300 μm. Apparent hybridization to the dorsal aorta and to some skeletal elements in the section shown in C was considered to be nonspecific because similar patterns were seen using control probes. In addition, consistent hybridization to these elements was not seen in all experiments.
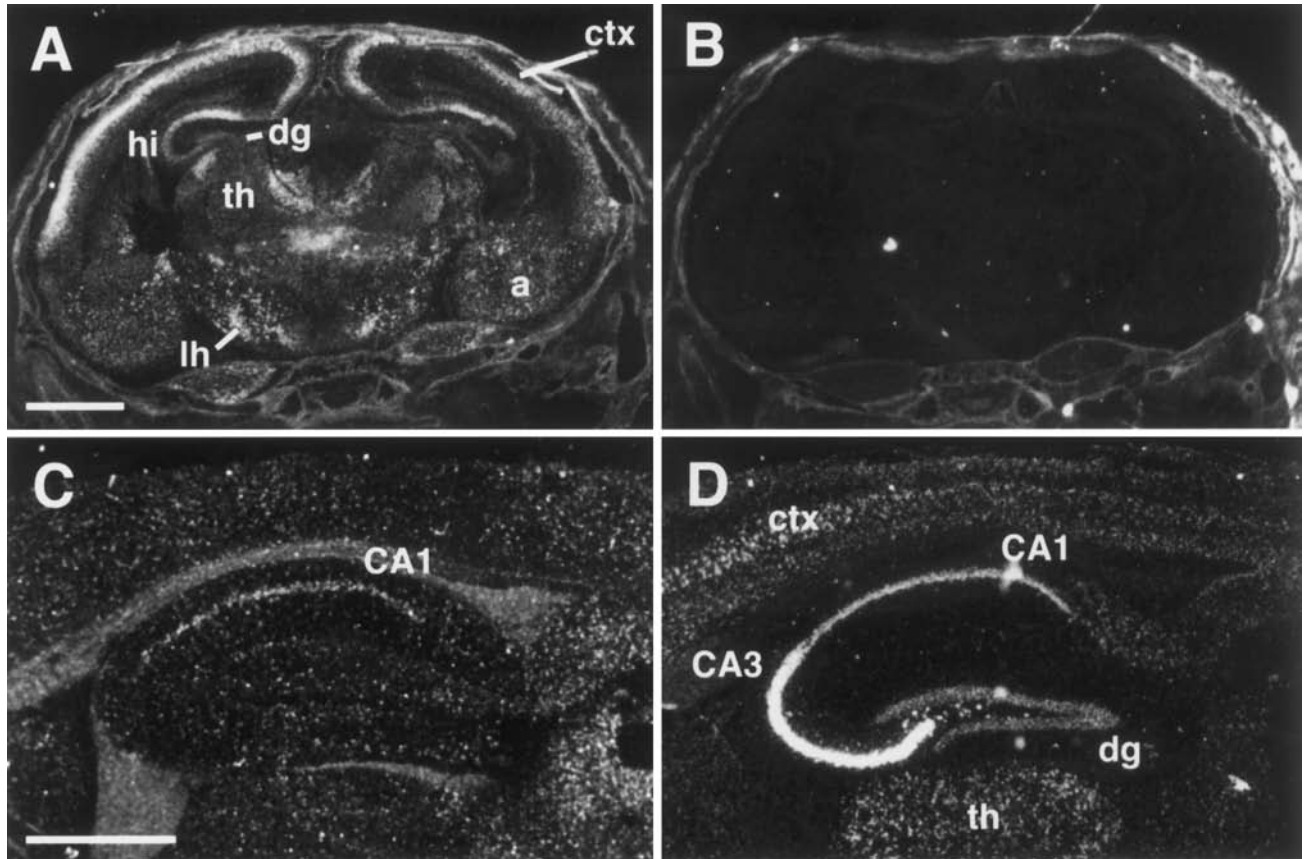
FIG. 7. Expression of glypican-5 mRNA in neonatal and adult central nervous system. Coronal sections of neonatal mice (A,B) and parasagittal sections of adult mouse brains (C, D) were hybridized with ^{35}S-labeled murine glypican-5 antisense probe (A,C), sense control probe (B), or an antisense probe for glypican-1 (D). A and B were exposed for 6 weeks; C and D were exposed for 5 weeks. Abbreviations: (a) amygdala, (CA1 and CA3) pyramidal cell layers of hippocampal cortex, (ctx) cerebral cortex, (dg) dentate gyrus, (hi) hippocampal cortex, (lh) lateral hypothalamus, (th) dorsal thalamus. Bar, 1 mm.

cific PCR primers. One pair spanned the C-terminus to 3′ untranslated region and the other spanned a splice donor site located following nucleotide residue 1235 (GenBank Accession No. AF001462) that was identified serendipitously by 3′ RACE of an unspliced glypican-5 mRNA (data not shown). These primer pairs were preselected and evaluated for specificity (ability to amplify a single band from human, but not hamster, genomic DNA). Data were submitted for analysis via computer to the Whitehead–MIT Center for Genome Research which is creating an integrated map of the human genome based on the GeneBridge RH panel (Hudson, 1995). The results localized the glypican-5 genomic markers to human chromosome 13. Specifically, the marker spanning the exon–intron boundary at nucleotide residue 1235 of the human cDNA mapped 1.51 cR telomeric to the framework marker CHLC.GATA73H01, while the genomic marker from the C-terminus and 3′UTR mapped 1.61 cR telomeric to framework marker WI-9265

(Fig. 8). As seen in Fig. 8, the genomic framework markers surrounding this region of the glypican-5 gene correspond to the region characterized cytogenetically as bands q31-32, consistent with the localization of the glypican-5 gene to 13q32 via fluorescence *in situ* hybridization (Veugelers *et al.,* 1997). While no genetic syndrome has been mapped specifically to this discrete locus, as discussed below, a number of human patients have been characterized with chromosomal deletions, of various sizes that would include the glypican-5 gene. These patients display a phenotype referred to as 13q− syndrome which includes a number of developmental abnormalities.

DISCUSSION

The experiments described above characterize the structure and expression of a fifth member of the glypican family
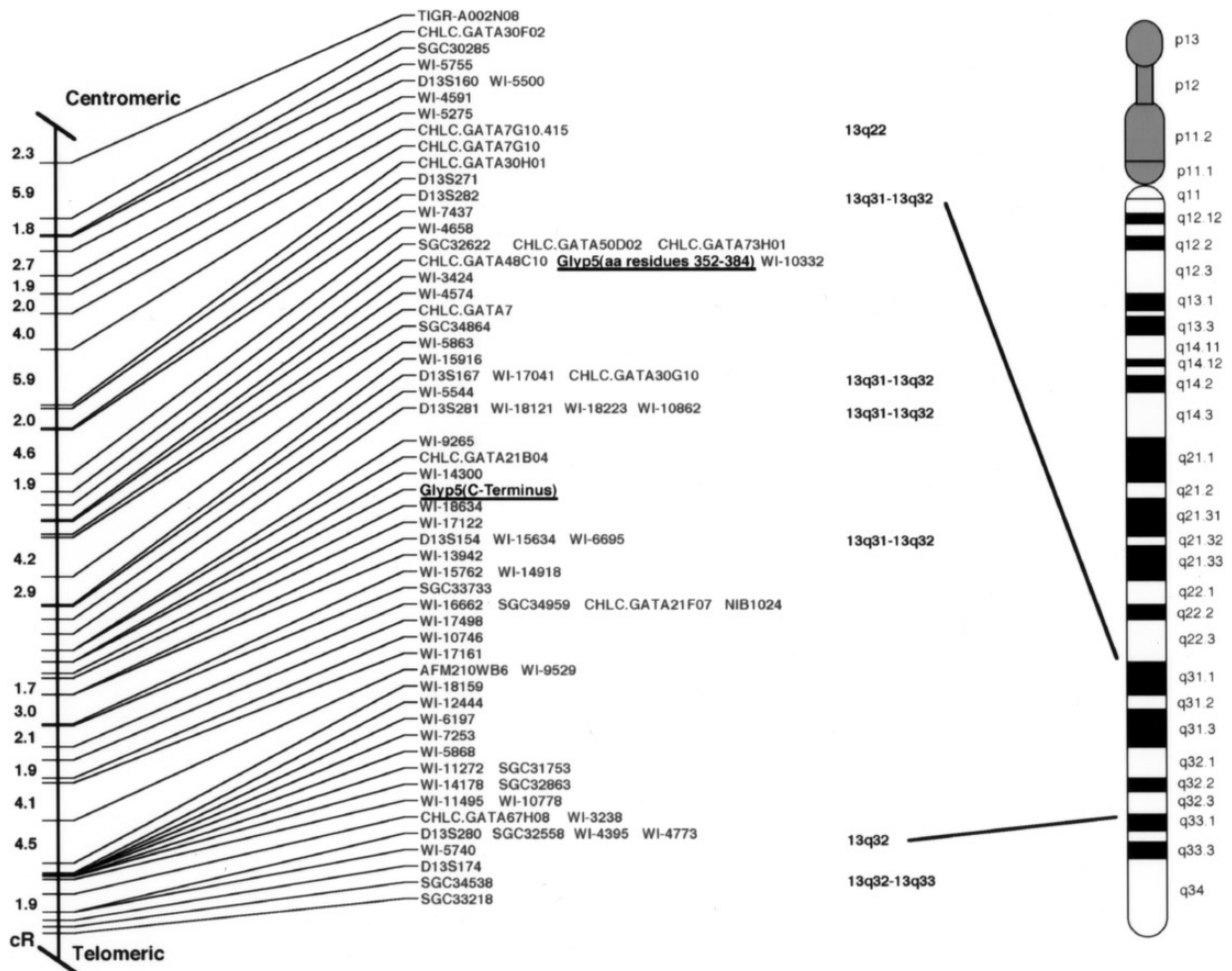
FIG. 8. Localization of the human glypican-5 gene to the chromosome 13 radiation hybrid and cytogenetic maps. On the left the chromosome 13 radiation hybrid map positions of two glypican-5 exons, which include amino acid residues 352–384, and the glypican-5 C-terminus, respectively, are shown. On the right the correspondence of this region of the radiation hybrid map to cytogenetic bands q31–32 is shown.

of cell surface proteoglycans. Glypican-5 has a structure typical of other glypicans (Fig. 1), is modified with glycosaminoglycan side chains, at least when expressed *in vitro* (Fig. 2), and, like other glypicans, is expressed in a highly tissue-specific manner during embryogenesis (Figs. 3–7).

Glycosaminoglycan Modification of Glypican-5

Five serine–glycine dipeptides, representing potential glycosaminoglycan attachment sites, are present in the deduced amino acid sequence of glypican-5. As in other glypicans, these dipeptides are clustered near the carboxy terminus. Three of them, Ser^{486}, Ser^{507}, and Ser^{509}, occur as SGSG or SGXG sequences in close proximity to acidic residues, features particularly characteristic of glycosaminoglycan at-

tachment sites (Bourdon *et al.,* 1987), including those that are specifically modified with heparan sulfate (Zhang *et al.,* 1995). The remaining serine–glycine pairs, at Ser^{441} and in particular Ser^{495}, also have neighboring acidic residues within 6 amino acids and might represent glycosaminoglycan attachment sites as well (Zhang *et al.,* 1995).

Results of transient expression in COS-7 cells (Fig. 2) support the notion that at least two sites in glypican-5 are substituted with glycosaminoglycans, since both heparitinase and chondroitinase ABC were required to degrade the intact proteoglycan to its core protein. Although cell surface proteoglycans of the syndecan family frequently contain both heparan sulfate (HS) and chondroitin sulfate (CS), members of the glypican family have been reported to bear only HS (Steinfeld *et al.,* 1996), even when isolated from

cells whose syndecans are clearly hybrid (bearing both CS and HS) (Shworak *et al.,* 1994a). Although glypican-5 may differ from other glypicans in this respect, it is also possible that the pattern of glycanation seen in the present study was a consequence of overexpressing this molecule in COS-7 cells, by transient expression, and not indicative of the pattern normally found *in vivo.* Interestingly, when Veuglers *et al.* (1997) expressed glypican-5 in K5652 cells by stable transfection, only heparan sulfate chains were found.

The apparent molecular weight of COS-7 cell-synthesized glypican-5, following removal of glycosaminoglycan chains, was approximately 65 kDa when analyzed by SDS–PAGE under reducing conditions. This is larger than the 58.5-kDa calculated mass of the core protein (with signal peptides removed), and suggests possible posttranslational modification at the three potential N-linked glycosylation sites predicted from the cDNA sequence.

Relationship of Glypican-5 to Other Cell Surface Proteoglycans

Comparison of the amino acid sequences of the known vertebrate glypicans suggests greater relatedness between some family members than others. Using the alignment of glypican-1 through -5 shown in Fig. 1A (with N- and C-terminal signal peptides removed), and the Distances software package from Genetics Computer Group, we find 46–52% similarity among human glypican-1, rat glypican-2, and mouse glypican-4, and 50% similarity between human glypican-5 and -3. In contrast, comparisons between members of these two groups reveal similarities of only 25–29%, suggesting these groups form distinct subfamilies (Fig. 1C).

Close inspection of the sequences of glypican family members suggests that these proteins may be divisible into two domains: About 85% of the amino acids in each glypican comprise a large N-terminal domain in which all 14 of the invariant cysteines reside, but in which, to the extent tested so far, no glycosaminoglycan chains are found (Mertens *et al.,* 1996, and unpublished observations). Following that is a C-terminal domain containing glycosaminoglycan attachment sites (clustered serine–glycine dipeptides flanked by acidic residues), a single pair of cysteine residues in some, but not all, glypicans, and finally a short region, lacking cysteines but relatively enriched in serine, threonine, proline, and/or basic amino acids, that provides a linkage to the GPI anchor. Interestingly, most of the strong sequence conservation among glypicans is confined to the N-terminal domain. For example, within this region, glypican-1 and -4 are 54% similar, and glypican-3 and -5 are also 54% similar. Within the C-terminal domain, sequence conservation is mainly confined to the immediate vicinity of the serine–glycine dipeptides, with very little similarity seen elsewhere. For example, within this region, glypican-1 and -4 are 38% similar, whereas glypican-3 and -5 are only 25% similar. Comparisons between members of these two

groups, within this C-terminal region, reveal similarities of only 8–18%.

It is interesting to contrast this organization with that described for the syndecans, the other major family of cell surface proteoglycans. Although syndecans are transmembrane proteins, their extracellular domains are notable for a high content of serine, threonine and proline, an absence of cysteine, and extremely poor sequence conservation except in the immediate vicinity of glycosaminoglycan attachment sites (Bernfield *et al.,* 1992). Clearly, the syndecans have much in common with the short C-terminal domain of the glypicans, but little in common with the N-terminal domain. Yet it is the N-terminal domain of glypicans that has been most highly conserved by evolution, raising the strong possibility that this domain mediates some function unique to the glypicans. It is tempting to speculate that this domain is involved in the direct interaction of glypicans with as yet unknown extracellular ligands. Indeed, several cases have been described where proteoglycans are known to specifically bind other molecules via protein–protein, rather than glycosaminoglycan–protein, interactions (Cheifetz and Massague, 1989; Krusius and Ruoslahti, 1986; Vogel *et al.,* 1984).

Developmental Regulation of Glypican-5 mRNA Expression in the Mouse Embryo

In the E10.5 embryo, the earliest stage examined, glypican-5 mRNA was found abundantly throughout the relatively undifferentiated, proliferative mesenchyme of the developing limb bud. As these cells begin to differentiate into skeletal and connective tissue elements, glypican-5 mRNA becomes restricted to mesenchymal cells at the distal tip of the limb. For example, by E12.5 glypican-5 mRNA expression is restricted to the prechondrogenic mesenchyme at the distal tips of the forming digits. Thus, in both cases, glypican-5 is localized to highly proliferative cells that are in the process of generating differentiated structures.

Glypican-5 mRNA was also detected in E10.5 mesonephros, in association with the caudal nephric duct. The nephric duct gives rise to the bilateral ureteric buds which, in turn, induce proliferation and condensation of metanephric mesenchyme into pretubular aggregates. At later stages, clear association of glypican-5 mRNA with these pretubular aggregates, as well as with the differentiating tubular epithelial cells recently derived from these aggregates, could readily be seen. In contrast, more highly differentiated tubules and glomeruli failed to express glypican-5 mRNA. This pattern of expression contrasts with that of both glypican-4 (K-glypican) and glypican-1. Glypican-4 mRNA is expressed relatively late in kidney development where it is detected in some pretubular aggregates, but is found mainly in the most differentiated tubules. Expression in tubules persists at later times, but little expression is found in glomeruli (Watanabe *et al.,* 1995). By comparison, glypican-1 mRNA appears to be expressed strongly in newly formed

glomerular structures (S. Paine-Saunders and A. D. Lander, unpublished data).

Kidney development is a complex process that is thought to require multiple soluble factors to drive both induction of tubules and branching of the ureteric bud (Hammerman, 1995; Hammerman *et al.,* 1992). Interestingly, both *in vitro* and *in vivo* experiments have implicated heparan sulfate-dependent growth factors, including FGFs and Wnts, in these events (Herzlinger *et al.,* 1994; Perantoni *et al.,* 1995; Stark *et al.,* 1994). A recent *in vitro* study has also demonstrated a requirement for heparan sulfate itself in branching morphogenesis of the ureteric bud, and in controlling the expression of wnt-11 (Kispert *et al.,* 1996). The highly restricted and dynamic patterns of expression of different glypicans raise the possibility that these proteoglycans may play as yet undefined roles in coordinating the many growth factor-driven morphogenetic events that underlie kidney development.

Although expression of glypican-5 mRNA in both the developing kidney and limb correlates with the locations of highly proliferative cells, the pattern of glypican-5 expression in the nervous system clearly points to functions unrelated to proliferation. Most tellingly, the cell layers of the central nervous system in which most neural proliferation is confined (the early neuroepithelium and later ventricular zones) are conspicuously lacking in glypican-5 expression throughout the brain, with the sole exception of the striatal primordium (Fig. 6D). In contrast, ventricular zones show strong hybridization for glypican-1 throughout the brain (Lander, 1993; and E. D. Litwack and A. D. Lander, unpublished data) and for glypican-4 in parts of the telencephalon (Watanabe *et al.,* 1995).

In general, glypican-5 expression in the early nervous system marks distinct subsets of newly generated postmitotic neurons, such as dorsal root ganglia and columns of presumptive primary motoneurons in the ventral spinal cord (Figs. 5 and 6). In some cases, glypican-5 expression also demarcates morphological boundaries, such as the midbrain–hindbrain junction, or isthmus, and the dorsal and ventral surfaces of the evaginating olfactory bulb (Fig. 6B). Only in later embryonic and perinatal life does glypican-5 begin to be widely expressed throughout the brain. At these stages, relative positions of cells can sometimes be used as indicators of stage of differentiation. For example, in the developing cerebral cortex, the oldest postmitotic neurons lie in the deep portion of the cortical plate, while the most recently generated are located more superficially. Preferential expression of glypican-5 in the deep cortical plate (Fig. 7A) strongly suggests that glypican-5 is not expressed by neurons as soon as they become postmitotic, but rather it appears somewhat later. Similarly, the relatively low glypican-5 *in situ* hybridization signals that are seen over individual neurons in the adult brain (Figs. 7C and 7D) suggest that, in expressing cells, glypican-5 mRNA levels decline significantly with cell maturation.

These dynamic changes in glypican-5 expression are quite different from, and in some senses complementary to, those seen with other glypicans. Glypican-2 is expressed by developing neurons almost as soon as they become postmitotic, but then rapidly disappears (Ivins *et al.,* 1997; Stipp *et al.,* 1994). Glypican-1 mRNA seems to appear in post-mitotic neurons slightly later than glypican-2 (at least in the cerebral cortex (E. D. Litwack and A. D. Lander, unpublished data)), but reaches its highest level of expression in the adult brain (Litwack *et al.,* 1994). These data suggest that, in some developing neurons at least, expression of glypicans may progress from predominantly glypican-2, to predominantly glypican-5, to predominantly glypican-1. The significance of such alternation between glypican family members is unclear, and is likely to remain so until the relevant ligands of individual glypicans in the nervous system are better understood. To this end, it is interesting to note that many of the heparin-binding growth factors that are candidates for interacting with glypicans in regions such as the developing limb and kidney (e.g., fibroblast growth factors, wnts, transforming growth factor-β, bone morphogenetic proteins) are also believed to regulate the differentiation and behavior of postmitotic neurons (Chalazonitis *et al.,* 1995; Fradkin *et al.,* 1995; Iwasaki *et al.,* 1996; McFarlane *et al.,* 1995; Unsicker *et al.,* 1996).

Deletion of the Glypican-5 Gene May Be Associated with Some Phenotypic Features of the Human 13q− Syndrome

Using radiation hybrid mapping we localized the human glypican-5 gene to a region of the long arm of chromosome 13 defined cytogenetically as 13q31-32; these data are consistent with the mapping of human glypican-5 to 13q32 by fluorescence *in situ* hybridization (Veugelers *et al.,* 1997). Interstitial deletions involving this region of the human genome are associated with a constellation of developmental abnormalities known collectively as 13q− syndrome. The phenotype of individual patients is variable, owing likely to the variable size of the specific interstitial deletions involved as well as other possible genetic modifiers. However, phenotypic features associated with interstitial deletions involving 13q31-32 include severe intrauterine growth retardation; functional as well as structural abnormalities of the nervous system including mental retardation, hypotonia, microcephaly, encephalocele, holoprosencephaly, partial anencephaly, agenesis of the corpus callosum, and deafness; developmental abnormalities of other organ systems including hypoplastic kidneys, hypoplastic ureters, hypoplastic adrenals, ambiguous genitalia, imperforate anus; and skeletal abnormalities primarily including absent or hypoplastic thumbs and metacarpal/metatarsal bones of the extremities (Brown *et al.,* 1993, 1995). Brown and colleagues have identified a correlation between the major phenotypic features of this disorder and interstitial deletions encompassing portions of 13q32, suggesting the location of gene(s) in this region responsible for 13q− syndrome (Brown *et al.,* 1993, 1995).

While even the smallest interstitial deletions involved in 13q− syndrome are large enough to include multiple genes, the localization of glypican-5 to this region of the genome, as well as the significant overlap in the developmental expression pattern of glypican-5 and those tissues affected in 13q− syndrome (e.g., brain, kidney, digits) suggests that glypican-5 is a candidate gene for at least some of the phenotypic features of this syndrome. Interestingly, an association between 13q− syndrome and glypican-5 deficiency would suggest that glypican-5, like glypican-3, has a normal physiological role in regulating cell proliferation and differentiation. However, the nature of the respective phenotypes might suggest opposite activities in regulating these processes.

ACKNOWLEDGMENTS

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