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Expression of the Heparan Sulfate Proteoglycan Glypican-1 in the Developing Rodent

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ABSTRACT **The glypicans are a family of glycosylphosphatidylinositol (GPI)-anchored proteoglycans that, by virtue of their cell-surface localization and possession of heparan sulfate chains, may regulate the responses of cells to numerous heparin-binding growth factors, cell adhesion molecules, and extracellular matrix components. Mutations in one glypican cause a syndrome of human birth defects, suggesting important roles for these proteoglycans in development. Glypican-1, the first-discovered member of this family, was originally found in cultured fibroblasts, and later shown to be a major proteoglycan of the mature and developing brain. Here we examine the pattern of glypican-1 mRNA and protein expression more widely in the developing rodent, concentrating on late embryonic and early postnatal stages. High levels of glypican-1 expression were found throughout the brain and skeletal system. In the brain, glypican-1 mRNA was widely, and sometimes only transiently, expressed by zones of neurons and neuroepithelia. Glypican-1 protein localized strongly to axons and, in the adult, to synaptic terminal fields as well. In the developing skeletal system, glypican-1 was found in the periosteum and bony trabeculae in a pattern consistent with expression by osteoblasts, as well as in the bone marrow. Glypican-1 was also observed in skeletal and smooth muscle, epidermis, and in the developing tubules and glomeruli of the kidney. Little or no expression was observed in the developing heart, lung, liver, dermis, or vascular endothelium at the stages examined. The tissue-, cell type-, and in some cases stage-specific expression of glypican-1 revealed in this study are likely to provide insight into the functions of this proteoglycan in development.** ***Dev. Dyn. 1998;211:72–87.***

Key words: glypican; heparan sulfate; proteoglycan; nervous system

INTRODUCTION

The responses of cells to many kinds of developmental signals involve cell surface heparan sulfate proteoglycans (HSPGs). Heparan sulfate (HS) binds to cell adhesion molecules, extracellular matrix proteins, proteases and their inhibitors, and growth factors (Jackson et al., 1991; Lander, 1994), and can participate in cell-cell adhesion (Cole et al., 1986; Reyes et al., 1990; Stanley et al., 1995), cell-substratum adhesion (Haugen et al., 1992a; LeBaron et al., 1988; Sanderson et al., 1992), neurite outgrowth (Haugen et al., 1992b; Walz et al., 1997; Wang and Denburg, 1992), and signaling mediated by growth factors, including fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), and factors of the wingless/Wnt family (Kispert et al., 1996a; Rapraeger et al., 1991; Reich-Slotky et al., 1994; Reichsman et al., 1996; Yayon et al., 1991; Zioncheck et al., 1995)

The majority of cell surface HS appears to be provided by two classes of integral membrane HSPGs, which comprise distinct gene families. Members of the syndecan family are transmembrane proteins; most possess both chondroitin sulfate (CS) and HS chains (for review, see Bernfield et al., 1992). The syndecans are composed of an extracellular domain with several glycosaminoglycan (GAG) attachment sites, a hydrophobic transmembrane domain, and a highly conserved cytoplasmic domain. The other family of integral membrane HSPGs is the glycosylphosphatidylinositol (GPI)-anchored glypicans. Currently this family is comprised of glypican-1 (formerly known as glypican; David et al., 1990), glypican-2 (formerly cerebroglycan; Stipp et al., 1994), glypican-3 (formerly OCI-5; Filmus et al., 1988, 1995), glypican-4 (formerly K-glypican; Watanabe et al., 1995), and glypican-5 (Saunders et al., 1997; Veugelers et al., 1997). A glypican-1 homolog has also been identified in chick (Niu et al., 1996), and a glypican family member has been identified in Drosophila (Nakato et al., 1995).

The fact that two distinct, evolutionarily conserved protein families carry cell surface HS suggests that proteoglycan core proteins may play unique roles in the

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functions of cell surface HSPGs. In support of this idea, highly regulated developmental expression of different syndecan family members has been observed, including the switching of cells from expression of one syndecan to expression of another (Bernfield et al., 1992; David et al., 1993). Although the glypican family has been less extensively studied, existing data point to patterns of developmental expression that are also intricate (Karthikeyan et al., 1994; Litwack et al., 1994; Saunders et al., 1997; Stipp et al., 1994; Watanabe et al., 1995). Glypican-2, for example, is found only in the nervous system, where it is transiently expressed by newly postmitotic neurons (Stipp et al., 1994; Ivins et al., 1997).

Little is known about the specific functions of glypicans, nor how their functions differ from those of the syndecans. Several studies indicate that glypicans can regulate signaling by FGFs-2 and -7 in vitro (Bashkin et al., 1992; Bonneh-Barkey et al., 1997; Brunner et al., 1991; Steinfeld et al., 1996). Compelling evidence that glypicans are involved in controlling cell growth and development in vivo has recently come from genetic studies. Mutations at the *dally* locus in Drosophila cause alterations in specific patterns of cell division, and give rise to abnormalities in the development of several structures (Nakato et al., 1995). Null mutations in the human glypican-3 gene are responsible for the Simpson-Golabi-Behmel overgrowth syndrome (Pilia et al., 1996), which is characterized by pre- and postnatal somatic overgrowth affecting multiple organ systems, as well as an increased incidence of certain tumors. It has been proposed that glypican-3 modulates tissue growth by regulating cellular interactions with growth factors such as IGF-2 (Pilia et al., 1996).

To begin to understand the developmental roles of glypicans, it is necessary to establish where and when they are expressed. In the rodent, the expression of glypican-1 in the adult nervous system has been studied in detail: Glypican-1 is the major HSPG of the adult brain and is expressed by many, but not all, classes of projection neurons (Karthikeyan et al., 1994; Litwack et al., 1994). Some observations on the expression of glypican-1 during development have also been made (Asundi et al., 1997; Karthikeyan et al., 1994; Lander, 1993). Beyond these reports, most studies on glypican-1 expression have focused on cultured cells, of which nearly all of the adherent cell lines and primary cells that have been tested have been found to express this proteoglycan (Karthikeyan et al., 1994; Litwack et al., 1994; Lories et al., 1992; Watanabe et al., 1995). In the present study, we wished to determine whether glypican-1 expression in vivo is as widespread as the data from cultured cells suggested, especially during development. We also specifically set out to examine the localization of glypican-1 in the developing nervous system at times when glypican-2 is known to undergo dramatic changes in its pattern of expression. To address these questions, a combination of in situ hybridization and immunohistochemistry was used in the embryonic and perinatal rat and mouse. Some of these data have been presented previously in abstract form (Litwack et al., Mol. Biol. Cell 5:301a, 1994; Ivins et al., Soc. Neurosci. Abstr. 21:795, 1995).

RESULTS

Initial assessment of sites of glypican-1 expression in the developing rat was made by in situ hybridization at embryonic days 14 and 18 (E14 and E18). At E14, substantial hybridization was seen mainly in the developing central nervous system, with little signal in other tissues (Fig. 1A). At E18, hybridization was more widespread (Fig. 1B,C), with glypican-1 mRNA found at high levels not only in the nervous system, but also in developing bone, and at lower levels in a variety of other locations throughout the embryo. At both E14 and E18, patterns of hybridization using two different glypican-1 RNA probes were identical (data not shown), and detectable hybridization with a sense strand (control) probe was not observed (Fig. 1D; also, see below). In addition, the pattern of glypican-1 expression in E16 mouse (Fig. 1E) was essentially identical to that observed in E18 rat, a comparable developmental stage. Since the highest levels of glypican-1 expression in rodent embryos appeared to be in the nervous and skeletal systems, we focused many of our subsequent observations on these organ systems, as described below.

Glypican-1 Expression in the Developing Nervous System

In E14 rats, glypican-1 mRNA was detected in the forebrain, midbrain, and hindbrain, where regions of hybridization were closely apposed to the the ventricles (Fig. 1A). A closer examination of the telencephalon revealed that glypican-1 mRNA is limited to the ventricular zone (Fig. 2A,B), a region containing the proliferating progenitors of both neurons and glia. Consistent with an association with proliferating neural precursors, high levels of glypican-1 hybridization were also observed in the ganglionic eminence—the primordium of the corpus striatum—and the developing sensory epithelia of the inner ear and nasal cavity (Fig. 1A), all sites of active neural precursor proliferation.

In contrast, glypican-1 was not expressed in the outer layer (preplate) of the telencephalon (Fig. 2A,B), which consists of early post-mitotic neurons. In this regard, the pattern of glypican-1 expression is quite different from that seen in the adult (Litwack et al., 1994) and appears to be complementary to that of glypican-2 (cerebroglycan; Fig. 2E,F), which is expressed only in post-mitotic neurons (Ivins et al., 1997; Stipp et al., 1994). In many other parts of the nervous system, however, glypican-1 was readily detected in zones of post-mitotic neurons, albeit at lower levels than those seen in ventricular zones. Examples in Figure 1A

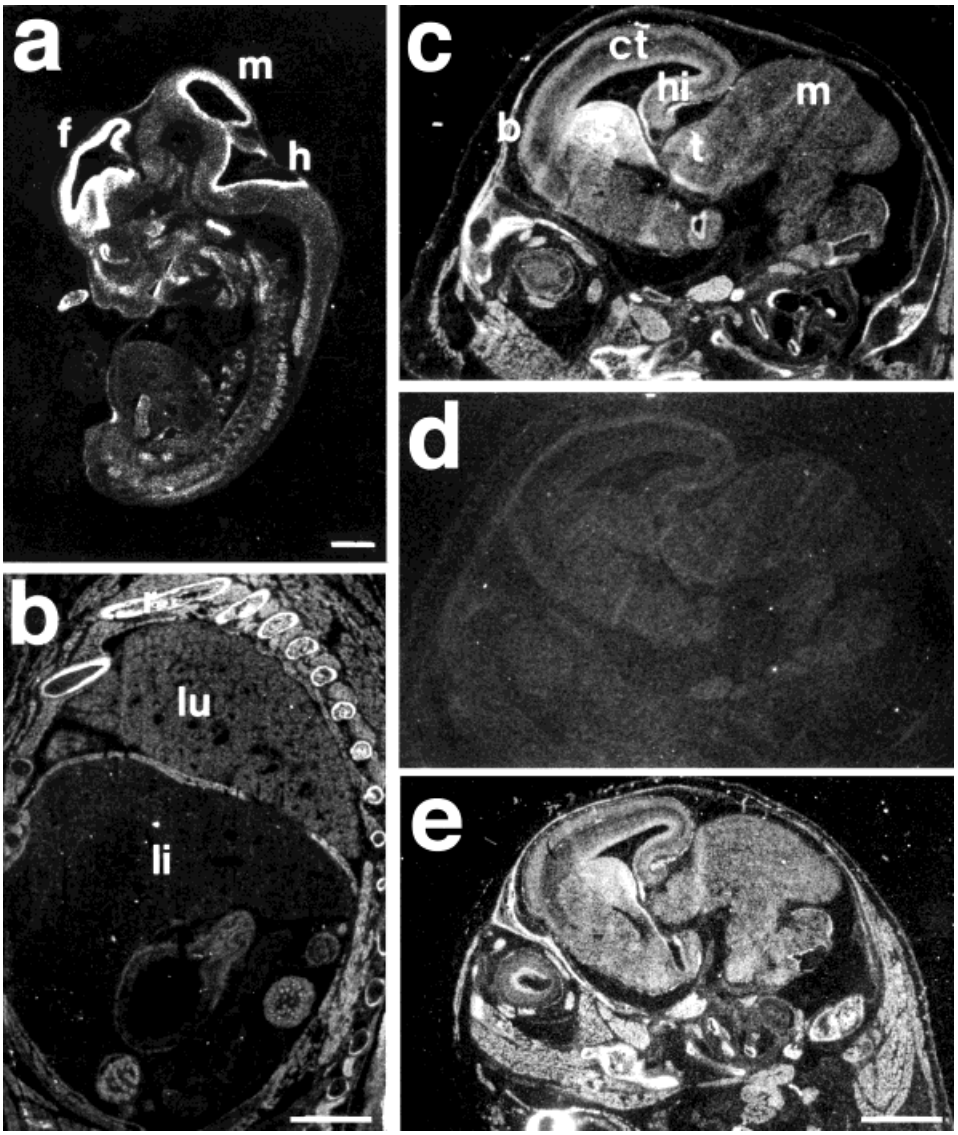

Fig. 1. In situ hybridization of glypican-1 in the embryonic rat and mouse. Darkfield views of sagittal sections through (**A**) E14 rat, (**B**) E18 rat torso, (**C,D**) E18 rat head, and (**E**) E16 mouse head probed with ^{35}S-labeled antisense (A,B,C,E) and sense (D) glypican-1 RNA probes and developed by autoradiography. The glypican-1 antisense probes used were 4X1 (A,E) and 4P2 (B,C). Sections hybridized with sense probes were processed identically to sections hybridized with antisense probes. Abbreviations: b, bone; ct, cortex; f, forebrain; h, hindbrain; hi, hippocampus; li, liver; lu, lung; m, midbrain; t, thalamus. Scale bars: A,B, 1 mm; E, 0.8 mm. The magnification in C and D is the same as in B.

include the dorsal root ganglia and the ventrolateral mantle layer of the ventral spinal cord (site of the developing motoneurons of the lateral motor column). Dorsal root ganglion neurons and spinal motoneurons continue to express glypican-1 in the adult (Litwack et al., 1994).

As development proceeds, glypican-1 continues to be expressed in zones containing neural precursors, but expression in regions of post-mitotic neurons also becomes widespread. Both at E18 and postnatal day 0 (P0), hybridization was observed in all regions of the nervous system (Figs. 1C, 3A,B; data not shown). In the forebrain, signal was seen in the cerebral cortex, the thalamus, all regions of the hippocampus, and the corpus striatum. Likewise, hybridization was observed througout the midbrain and hindbrain (Fig. 1C and data not shown). In the cerebral cortex, glypican-1 mRNA was observed both in the ventricular zone and in the cortical plate, the latter the site of differentiating neurons (Fig. 3A,B). In the hippocampus, glypican-1 mRNA was observed both in the hilar region, which contains granular precursor cells, and in the dentate gyrus and pyramidal cell layers, both of which contain differentiated neurons (Fig. 3A,B). In the spinal cord, expression in the the lateral motor columns similar to that observed at E14 persisted (data not shown), and

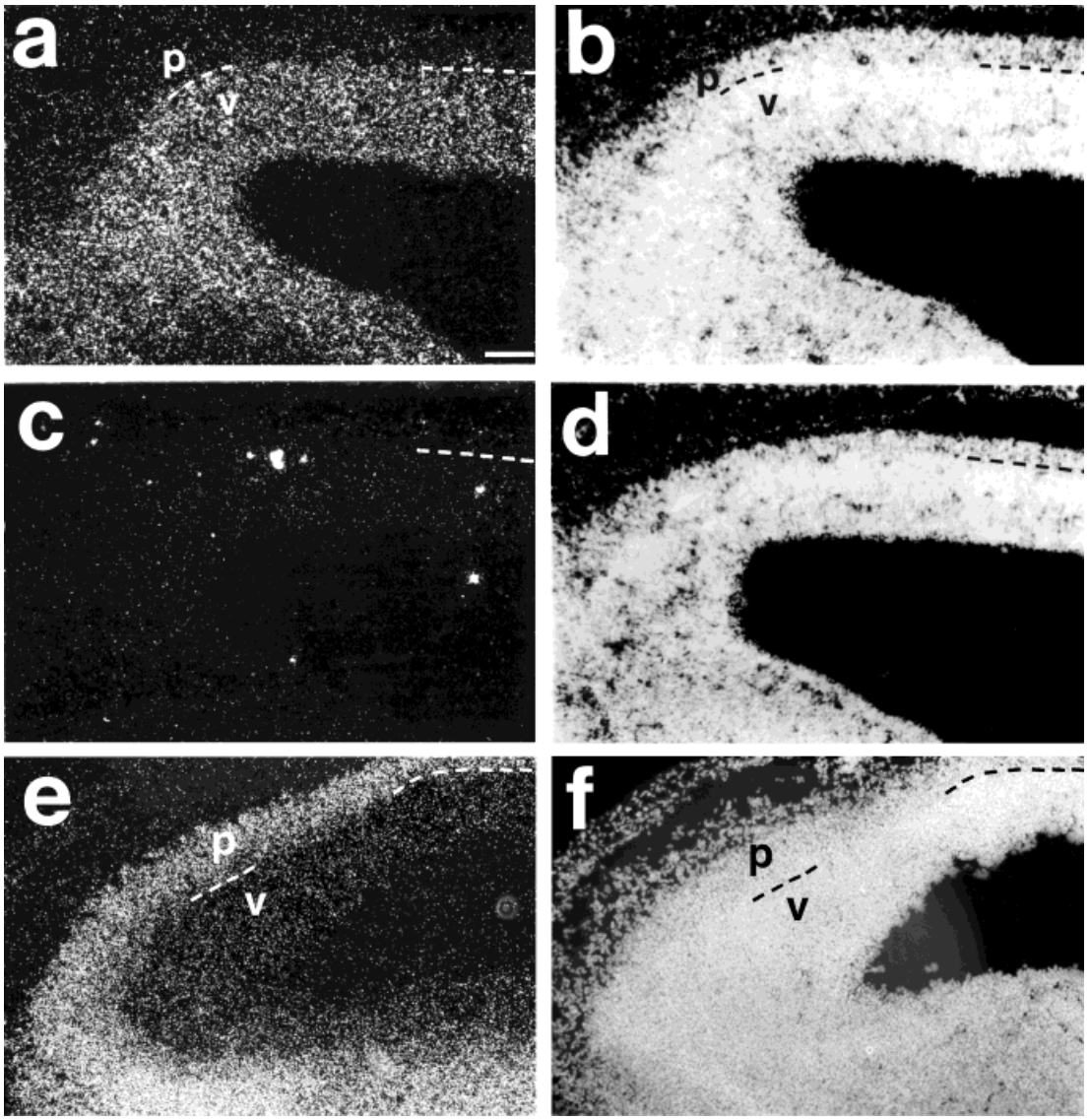

Fig. 2. Glypican-1 expression in the ventricular zone of E14 rat telencephalon. Sagittal sections of E14 telencephalon were hybridized with the glypican-1 antisense RNA probe 4X1 (**A,B**), sense probe (**C,D**), or with an antisense glypican-2 RNA probe (**E,F**). Darkfield views are in A,C,E; sections were also counterstained with bisbenzimide and viewed by fluorescence (B,D,F). Dashed lines show the approximate boundary between the ventricular zone and the preplate. Abbreviations: p, preplate; v, ventricular zone. Scale bar: 100 μm.

substantial hybridization was seen in dorsal root ganglia (see Fig. 7C,D, below).

At P7, glypican-1 expression continues to be found in all regions of the brain (Fig. 3C–G), including the olfactory bulb (Fig. 3F), corpus striatum (Fig. 3C), and the internal and external granule cell layers of the cerebellum (Fig. 3E). Both the pyramidal cell layer and dentate gyrus of the hippocampus express glypican-1. However, the levels of expression have become distinctly higher in the pyramidal cell layer (Fig. 3D), a pattern that persists in the adult (Litwack et al., 1994). In addition, a laminar pattern of expression has started to become apparent in the lateroventral regions of the cerebral cortex (Fig. 3G). It is likely that this pattern reflects the loss of glypican-1 expression by layer IV, the cortical layer that does not express glypican-1 in the adult (Litwack et al., 1994). As cortical development proceeds in a lateral-to-medial gradient, the gradient of glypican expression observed here may parallel a maturational gradient of layer IV.

By P14, glypican-1 expression in the brain has come to resemble that in the adult relatively closely. In the hippocampus the highest levels of expression continue to be seen in the pyramidal cell layer, with less expression in the dentate gyrus. However, by this stage, differences in intensity of hybridization within the

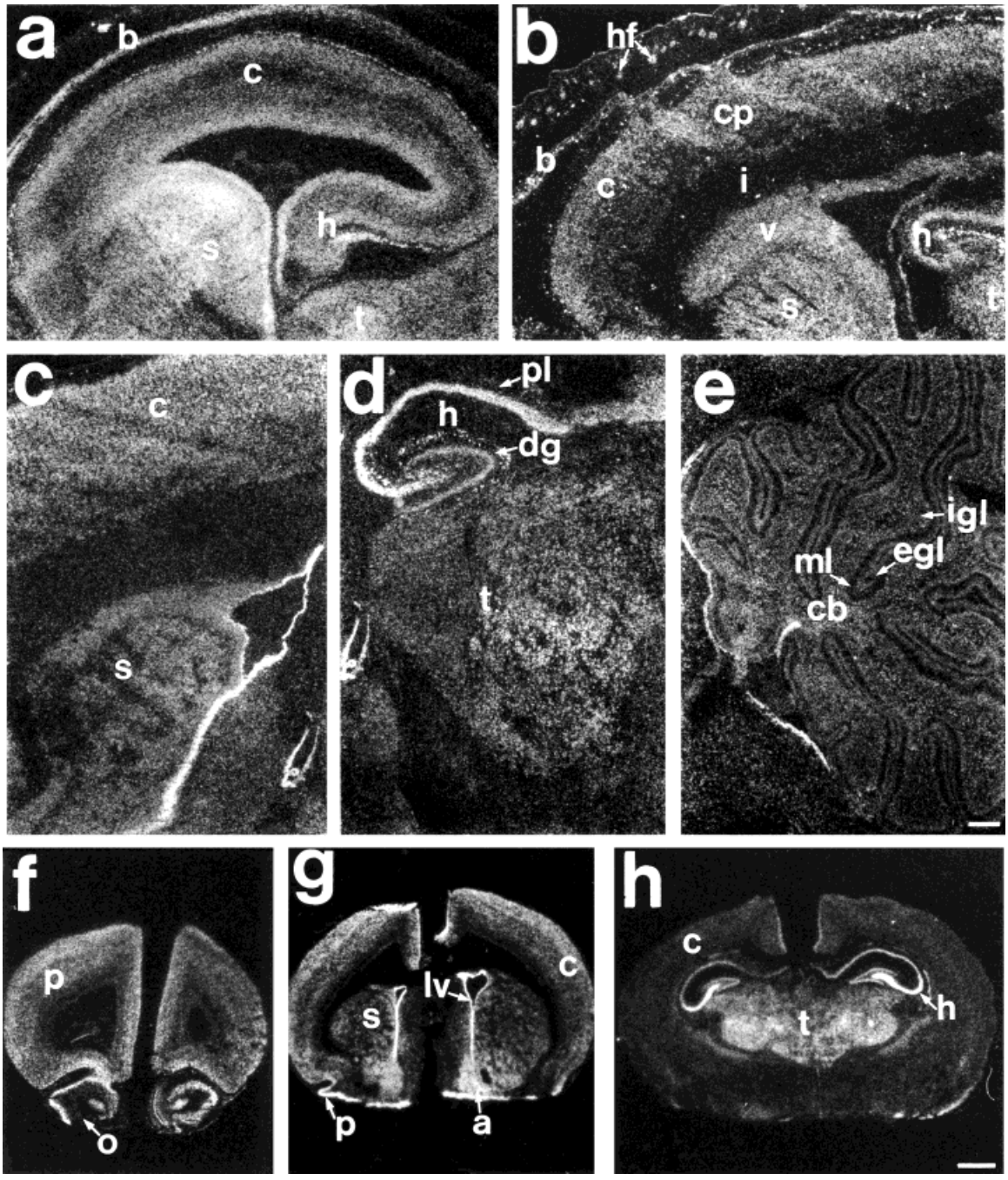

Fig. 3. Glypican-1 mRNA expression in the E18-P7 rat nervous system. Sagittal (A–E) or coronal (F–H) sections through (**A**) E18, (**B**) P0, (**C–G**) P7, and (**H**) P14 rat nervous system were hybridized with glypican antisense RNA probes 4P2 (A,B,F,H), 4EX (C-E) or 4X1 and 4P2 simultaneously (G), and viewed in darkfield. In A-E, anterior is to the left, dorsal is up. Abbreviations: a, nucleus accumbens; b, bone; c, cerebral cortex; cb, cerebellum; cp, cortical plate; dg, dentate gyrus; egl, external granule cell layer; h, hippocampus; hf, hair follicles; i, intermediate zone; igl, internal granule cell layer; lv, lateral ventricle; o, olfactory bulb; ml, molecular layer; p, piriform cortex; pl, pyramidal cell layer; s, striatum; t, thalamus; v, ventricular zone. The magnification is identical in A-E (scale bar in E is 250 μm), and in F–H (scale bar in H is 1.5 mm).

pyramidal layer have also emerged, with the CA3 region expressing the highest levels of glypican-1 (Fig. 3H). At this age, the dorsal thalamus highly expresses glypican-1, whereas levels of cortical expression have become relatively low (Fig. 3H). This overall pattern—high expression in CA3 and the dorsal thalamus, and low levels in the cortex—is similar to what is observed in adult (Litwack et al., 1994). Unlike in the adult,

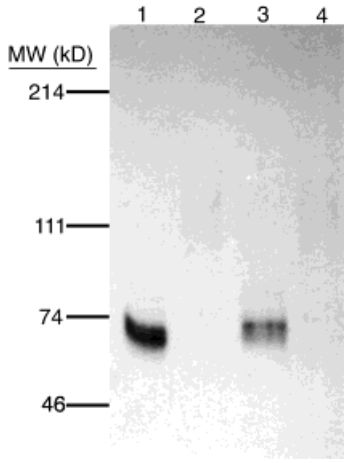

Fig. 4. Detection of glypican-1 protein in rat brain by western blotting. A P0 rat brain membrane fraction was treated with heparitinase and/or chondroitinase ABC, separated by SDS-PAGE, transferred to nitrocellulose, stained with 343-1, and developed using a peroxidase-conjugated second antibody, and enhanced chemiluminescence. Eighteen µg of membrane protein was used in each lane. Lane 1, heparitinase- and chondroitinase-treated; lane 2, chondroitinase-treated; lane 3, heparitinase-treated; lane 4, untreated. Numbers on the left refer to the size (kD) of molecular weight markers.

however, some hybridization to the ventral thalamus is observed (Fig. 3H).

Expression of Glypican-1 Protein in the Nervous System

To verify that sites of glypican-1 mRNA expression in the nervous system correspond to sites of glypican-1 protein expression, an antiserum, designated 343-1, was raised to a glypican-1-derived peptide and affinity purified (see Materials and Methods). 343-1 recognizes a single band of about 66 kD (the apparent size of the glypican core protein) on Western blots of heparitinase-treated P0 rat brain membrane extracts (Fig. 4). In lanes containing P0 brain membranes not treated with heparitinase, a faint smear (consistent with intact glypican PG) can be seen around 110 kD (Fig. 4). The low signal in untreated lanes is likely due to the poor ability of intact PGs to bind to nitrocellulose (Rapraeger et al., 1985). In fact, 343-1 is able to recognize intact glypican-1, as it readily immunoprecipitated glypican-1 from rat brain growth cone particles (Ivins et al., 1997) and synaptosomes (data not shown). 343-1 also recognizes intact glypican-1 on cell surfaces, as it readily and specifically stained live myeloma cells (which do not normally express glypican-1) following transfection of those cells with the glypican-1 cDNA (Litwack and Lander, unpublished observations).

Immunohistochemistry using 343-1 was carried out on sections of rat and mouse embryos of various ages. At levels of secondary antibody required to produce strong specific staining with 343-1, unacceptable background staining (staining with non-immune rabbit immunoglobulin at the same concentrations as 343-1) was often

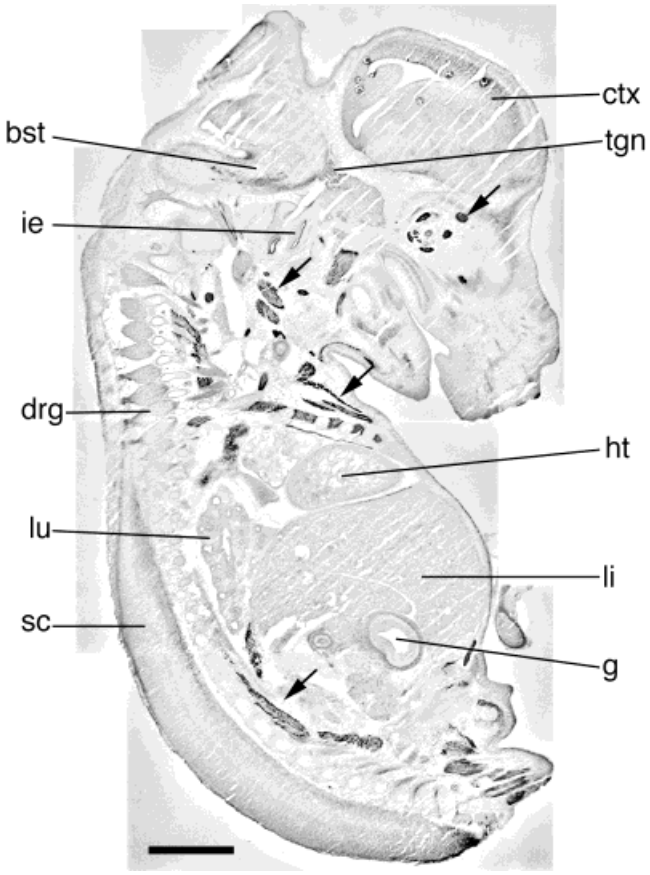

Fig. 5. Expression of glypican-1 protein in the embryonic mouse. E13 mouse sections were stained with 343-1 as described in Materials and Methods. Arrows indicate examples of skeletal muscle stained with 343-1. Abbreviations: bst, brainstem; ctx, cerebral cortex; drg, dorsal root ganglion; g, gut; ht, heart; ie, inner ear; li, liver; lu, lung; sc, spinal cord; tgn, trigeminal nerve, central root. Scale bar: 1 mm.

seen in rat, but not mouse, embryos (data not shown). As the patterns of glypican-1 mRNA expression in embryonic mouse and rat were indistiguishable (Fig. 1 and data not shown), mice were used for immunohistochemical experiments involving embryonic ages. As with rat, 343-1 specifically recognizes a band of the appropriate molecular weight on Western blots of heparitinase-treated protein purified from mouse brain (Williamson et al., 1996).

In the E13 mouse, a stage similar to the E15 rat, glypican-1 staining was seen in the nervous system in a pattern consistent with neuroepithelial and neuronal expression (Fig. 5). Significant staining was observed surrounding ventricles in the forebrain and hindbrain, and appeared to outline neuroepithelial cells of the ventricular zone (Fig. 6B). The highest levels of staining were found along the surface of the ventricle, suggesting that neuroepithelial cells polarize glypican-1 to their apical surface. Glypican-1 immunoreactivity was also observed on central axon tracts and peripheral nerves, including the intermediate zone of

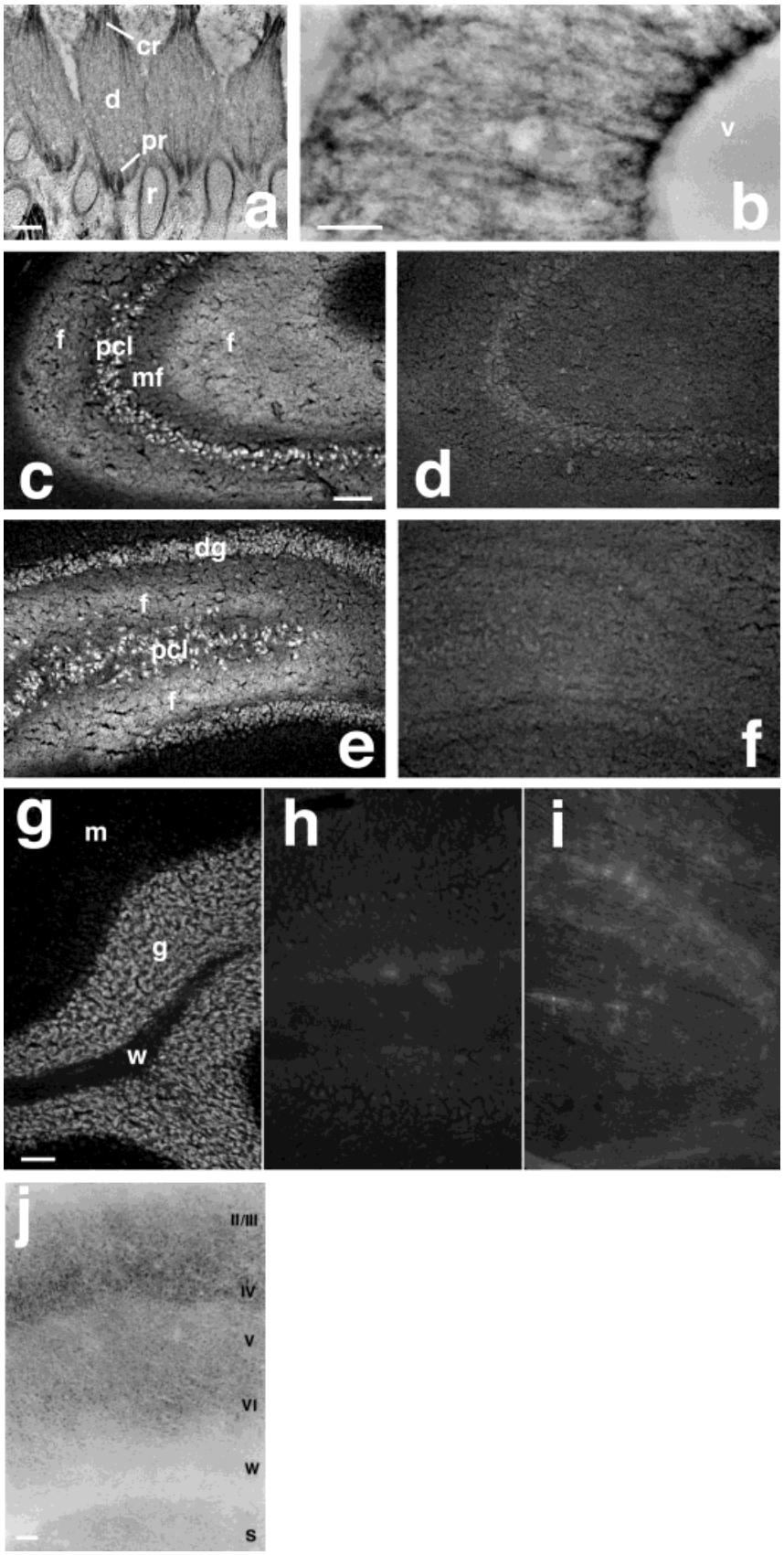

Fig. 6. Glypican-1 immunostaining in the nervous system. **A**: Sagittal section of E13 mouse dorsal root ganglia stained with 343-1. Muscle and the periosteum of developing ribs are also stained with 343-1. **B**: Sagittal section through E13 mouse neuroepithelium, along the posterior margin of the lateral ventricle, stained with 343-1. **C–F**: Sagittal sections through adult rat hippocampus stained with (C,E) 343-1 or (D,F) antibodies to KLH. **G–I**: Sagittal sections through adult rat cerebellum stained with (G) 343-1, (H) antibodies to KLH, or (I) 343-1 in the presence of a 10-fold molar excess of the peptide antigen. **J**: Coronal section through adult rat cerebral cortex stained with 343-1. Abbreviations: cr, central root; d, dorsal root ganglion; dg, dentate gyrus; f, associational and commissural fibers of hippocampal pyramidal cells; g, cerebellar granule cell layer; m, molecular layer; mf, hippocampal mossy fiber layer; pcl, pyramidal cell layer; pr, peripheral root; r, rib; s, striatum; v, ventricle; w, white matter; II-VI, cortical layers II-VI. Scale bars: A, G, J, 100 µm; B, 25 µm; C, 50 µm. The magnification is identical in C-F, and in G-I.

the telencephalon, which contains cortical and thalamic fibers (Fig. 5), fiber tracts in the brainstem and in the spinal cord (Fig. 5), the trigeminal nerve (Fig. 5), and both the peripheral and central roots of dorsal root ganglia (Fig. 6A). Much less staining was associated with the cell bodies of dorsal root ganglion neurons, suggesting that glypican-1 may be polarized to axons, as has previously been seen for glypican-2 (Ivins et al., 1997). While we cannot rule out that some glypican-1 staining in the peripheral nervous system reflects expression on Schwann cells (cf., Carey et al., 1993), rather than axons, we note that in the E14 and E18 rat very little if any glypican-1 mRNA was detected in the peripheral roots of dorsal root ganglia (Figs. 1A, 7C,D and data not shown), where Schwann cells, but not neurons, are located.

As glypican-1 is a product of neurons in the adult (Litwack et al., 1994), we stained adult rat brain to determine if glypican-1 is also associated with axons in the mature nervous system. Consistent with this, 343-1 stained the hippocampal associational and commissural fiber layers, which consist of axons arising from glypican-1 mRNA-expressing hippocampal pyramidal cells (Fig. 6C,E). Cell bodies of pyramidal cells were also positive for glypican-1 immunoreactivity. The level of glypican-1 immunuoreactivity in the dentate gyrus—especially in the mossy fiber layer, which contains axons arising from neurons of the dentate gyrus—was lower than the level of immunoreactivity associated with the cell bodies and axons of the pyramidal cell layer. This is consistent with the relative levels of glypican-1 mRNA expression in the adult hippocampus as assessed by in situ hybridization (Litwack et al., 1994). Furthermore, this staining was specific for glypican-1, as control antibodies to KLH purifed from the same antiserum as was 343-1 (see Materials and Methods), did not stain hippocampal fibers and only weakly stained hippocampal and granular cell bodies (Fig. 6D,F).

Glypican-1 protein expression was also strongly associated with synaptic terminal fields in adult cerellum and cerebral cortex. A high level of glypican-1 immunoreactivity was observed in the granule cell layer of the adult cerebellum (Fig. 6G). Since adult cerebellar granule cells do not express glypican-1 mRNA (Litwack et al., 1994), glypican-1 is likely being supplied by afferents that project into the granule layer (i.e., mossy fibers) from glypican-1-positive cells that lie outside the cerebellum. Control antibodies did not stain the cerebellar granule cell layer; furthermore, glypican-1 staining was abolished by absorption of 343-1 with an excess of peptide antigen (Fig. 6H,I). In the cerebral cortex glypican-1 staining was associated primarily with layer IV (Fig. 6J). Layer IV neurons express the least amount of glypican-1 mRNA relative to the other cortical layers; however, the major afferent input to layer IV comes from the dorsal thalamus, which expresses exceptionally high amounts of glypican-1 mRNA (Litwack et al., 1994). Thus, the glypican-1 staining in layer IV most likely derives from thalamic axons. The association of glypican-1 with synaptic fields is supported by the observation that glypican-1 is a major HSPG of synaptosomal preparations (Ivins and Lander, unpublished observations).

Skeletal Expression of Glypican-1

Together with the nervous system, the skeletal system is one of the sites of strongest glypican-1 in situ hybridization in the embryo (Fig 1B,C). At all ages examined, strong in situ hybridization was associated with developing bones. For example, in the E18 rat, glypican-1 mRNA was observed in the periosteum and bony trabeculae of the humerus (Fig. 7A,B), vertebral bodies, and ribs (Fig. 1B, 7C,D). In the P7 limb, glypican-1 mRNA was detected in cell bodies near bony spicules in the epiphyseal plates of long bones (Fig. 7E,F). Apparent hybridization to bony spicules themselves was seen with both with antisense (Fig 7E) and sense (Fig. 7G) probes, and was therefore judged to be artifactual. Hybridization to distinct clusters of cells in adjoining bone marrow (Fig. 7I,J), however, was judged to be specific, as it was not seen with sense probes (data not shown). In no cases was glypican-1 mRNA detected in zones containing resting, proliferating, or hypertrophic chondrocytes (Fig. 7A–F).

In addition to bones undergoing endochondral ossification, high levels of glypican-1 mRNA were also observed in bones undergoing intramembranous ossification. For example, evidence of specific hybridization to the periosteum and trabeculae of bones of the calvarium can be seen in Figures 1C, and 3A,B.

Overall, the pattern of hybridization in bone suggests that glypican-1 is expressed by developing and mature osteoblasts, which are localized to the periosteum and trabeculae during both intramembranous and endochondral ossification. Consistent with this view are variations in the pattern of glypican-1 mRNA expression in bones of the rib cage at different rostrocaudal levels, which correlate with developmental stage. For example, in more caudal (less well developed) ribs of the E18 rat, expression is seen in the periosteum. In more rostral ribs, glypican-1 expression becomes extensive in the periosteum and also appears in trabeculae (Fig. 1B).

Immunostaining of both mouse and rat skeletal tissue with 343-1 gave results similar to those seen by in situ hybridization. In both E13 mouse ribs (Fig. 6A) and in P7 rat talus (Fig. 8C), the periosteum was stained with 343-1, while chondrocytes were unstained. Expression of glypican-1 protein in the epiphyseal plates of P7 limb could not be evaluated due to high non-specific binding of secondary antibody to that region (data not shown).

Expression of Glypican-1 in Other Tissues

Glypican-1 mRNA and protein expression was observed in the skin of developing mouse and rat (Figs. 3B, 5). In P7 rat skin, significant glypican-1 protein staining was associated with all layers of the epidermis

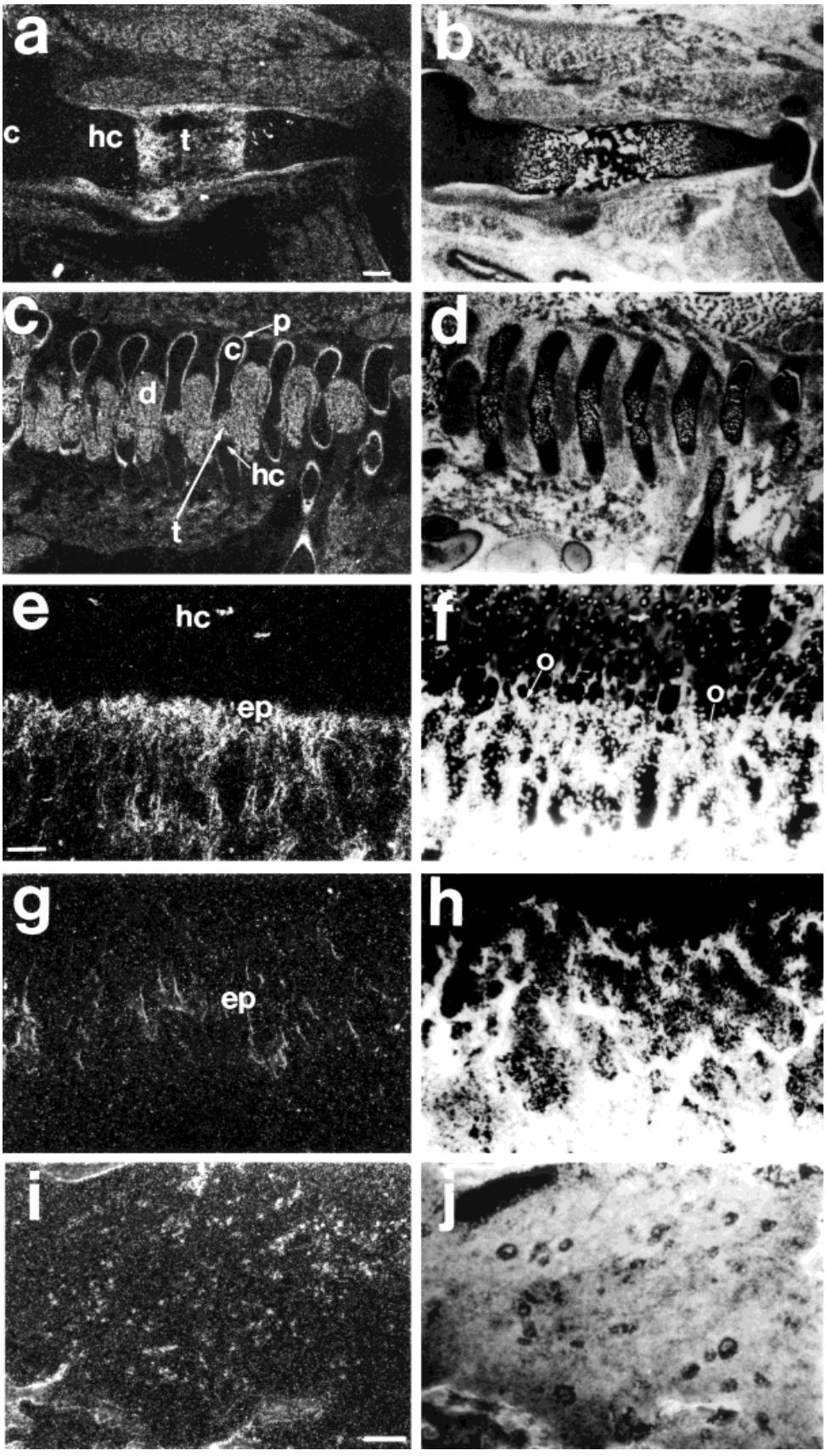

Fig. 7.

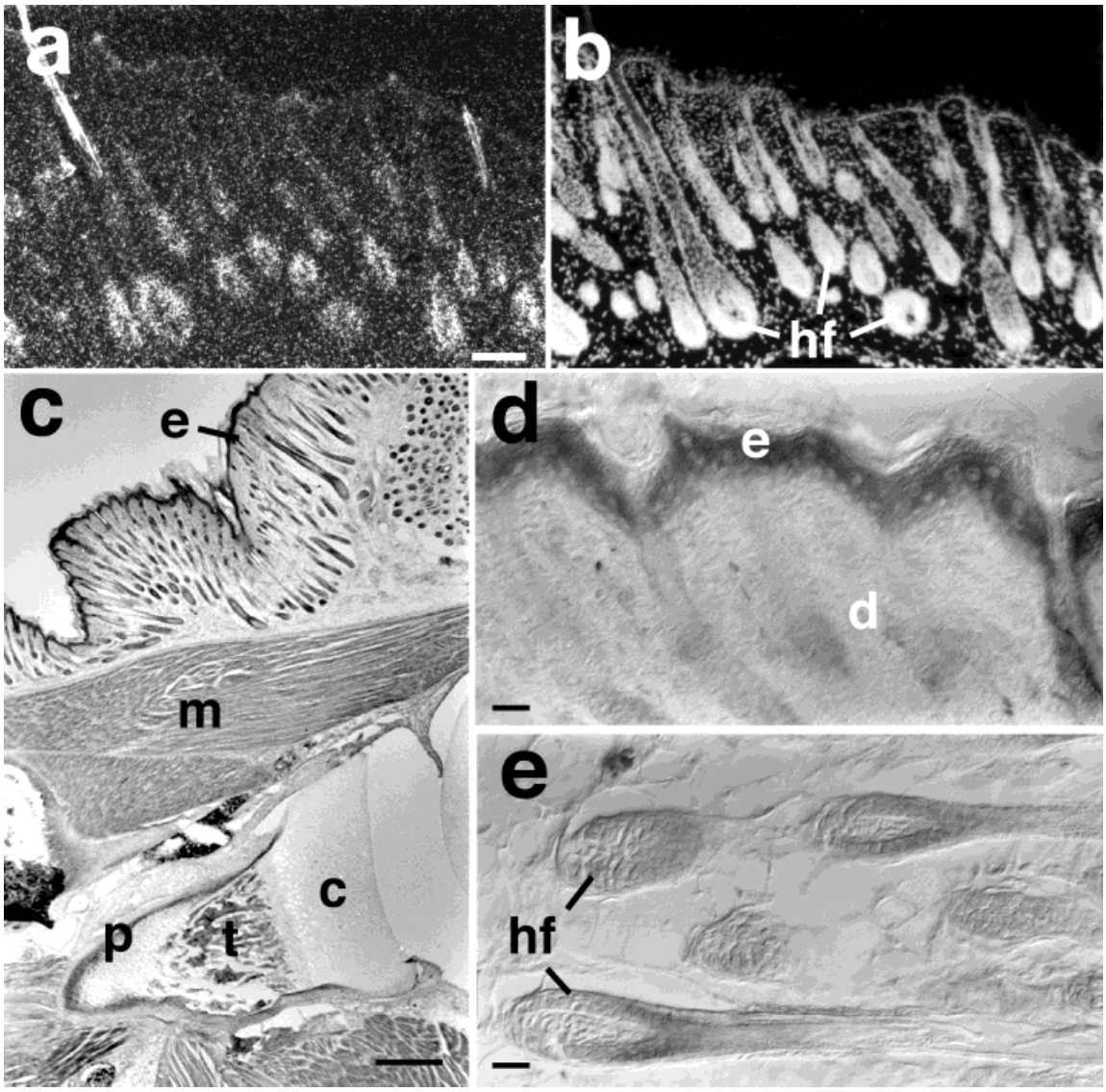

Fig. 8. Expression of glypican-1 in skin and hair follicles. **A**: Hair follicles in P7 rat limb, probed with a glypican antisense RNA probe; darkfield view. **B**: Same section as in (A), stained with bisbenzimide and viewed by fluorescence. **C**: Section through P7 rat limb, stained with 343-1. The staining in bone trabeculae observed in this section is due to nonspecific binding of the secondary antibody, and does not reflect 343-1 staining. **D,E**: High magnification views of skin and hair follicles from P7 rat limb, stained with 343-1. Abbreviations, c, cartilage; d, dermis; e, epidermis; hf, hair follicles; m, skeletal muscle; p, periosteum; t, trabeculae. Scale bars: A,B, 100 µm; C, 500 µm; D,E, 20 µm.

Fig. 7. Glypican-1 mRNA expression in the skeletal system. **A**: Darkfield view of section through an E18 rat humerus, after in situ hybridization for glypican-1. **B**: Adjacent section to that in (A), stained with hematoxylin and eosin. **C**: In situ hybridization to E18 rat vertebral column, darkfield view. **D**: Section 40 µm from that in (C), stained with hematoxylin and eosin. **E,G,I**: In situ hybridization showing epiphyseal plate (E,G) and bone marrow (I) from P7 rat limb; darkfield view. **F,H,J**: Same sections as in (E), (G), and (I) respectively, stained with bisbenzimide and viewed by fluorescence. Sections were hybridized with antisense glypican-1 RNA probes 4X1 (A) or 4P2 (C,E,I), or with a sense glypican-1 RNA probe (G). In (C,D), rostral is to the left, and dorsal is to the top. Abbreviations: c, chondrocytes; d, dorsal root ganglia; ep, epiphyseal plate; hc, hypertrophic chondrocytes; o, osteoblast layer; p, periosteum; t, trabeculae. The magnification is identical in A-D (scale bar in A is 250 µm), E-H (scale bar in E is 100 µm), and I-J (scale bar in I is 100 µm).

with the exception of the stratum corneum; little protein was observed in the dermis (Fig. 8C,D). This pattern is also preserved in hair follicles, whether examined by in situ hybridzation or immunohistochemistry (Figs. 3B, 8). A close examination of hair follicles in P7 limb (Fig. 8E) reveals that glypican-1 expression is associated with the epithelial cells of the matrix and/or the inner root sheath, but not the dermal papilla.

Skeletal muscle was observed to express glypican-1 mRNA by in situ hybridization (Figs. 1, 7A), and was very strongly stained with 343-1 (Figs. 5, 6A, 8C). Smooth muscle (the muscularis externa of the developing gut) also exhibited moderate glypican-1 staining

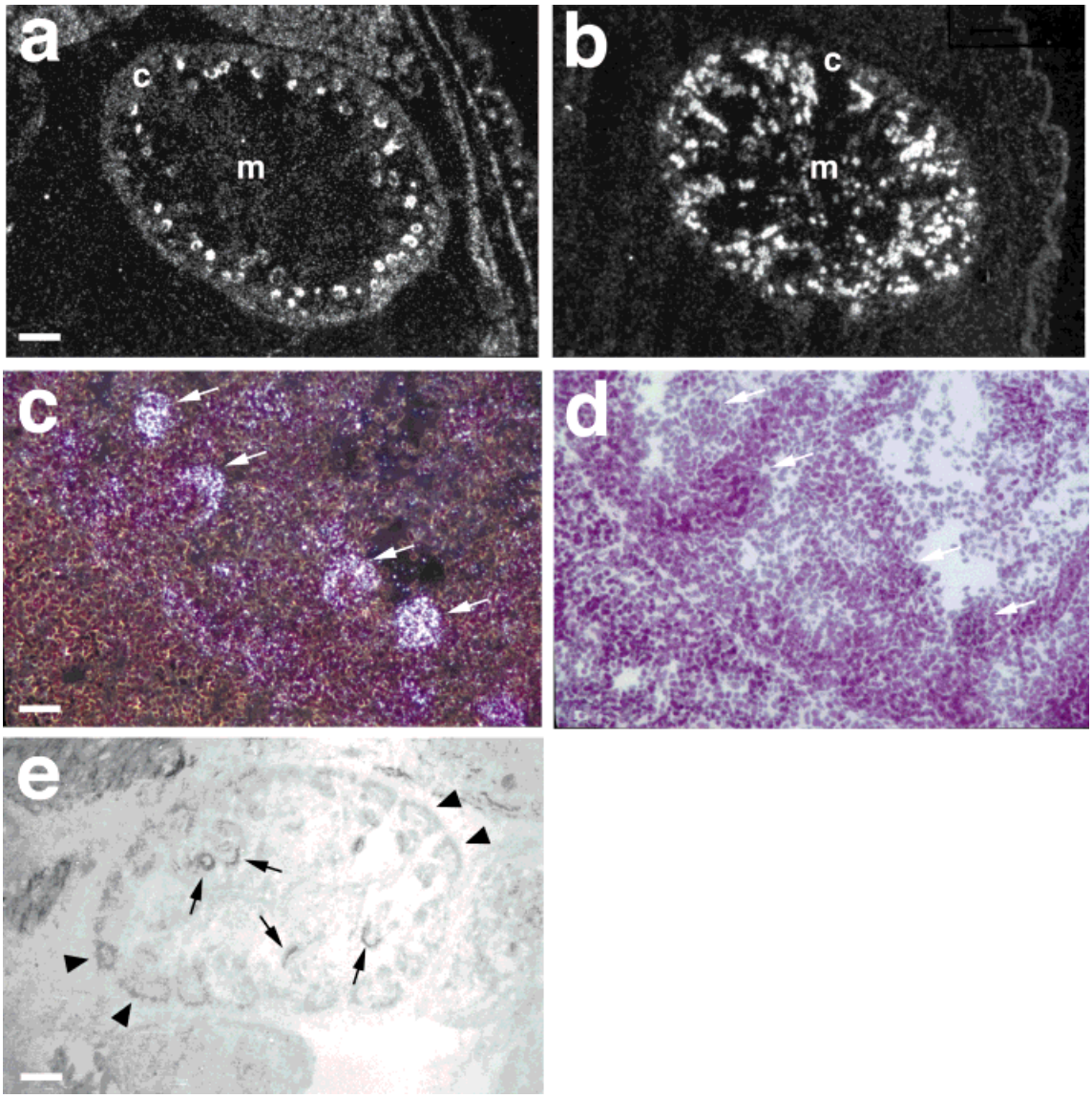

Fig. 9. Glypican-1 expression in developing kidney. **A,C**: Sections through E16 mouse embryos were hybridized with the glypican-1 antisense RNA probe 4X1, or (**B**) a glypican-5 antisense RNA probe. **D**: The same section as in (C), stained with hematoxylin and eosin, and viewed in bright field. **E**: E13 mouse kidney stained with the 343-1 antibody (skeletal muscle stained with 343-1 is also present in the upper left of the photograph). Arrows in (C,D) show glomeruli positive for glypican-1; arrowheads in (E) show developing glomerular structures, while arrows point to pretubular epithelial aggregates. Abbreviations: c, cortex; m, medulla. Scale bars: A,B, 250 µm; C,D, 50 µm; E, 100 µm.

(Fig. 5). There was, however, only weak staining of cardiac muscle in mouse (Fig. 5).

In developing metanephric kidney, glypican-1 protein was localized to epithelial aggregates, some of which showed the characteristic C-shape of condensing nephrons; little glypican-1 expression was observed in uninduced mesenchyme (Fig. 9E). Glypican-1 mRNA expression was also observed in the glomeruli of E18 rat kidney (Fig. 1B) and E16 mouse kidney (Fig. 9A,C,D), but not in tubular epithelial cells (Fig. 9A,C,D), indicating that glypican-1 continues to be expressed in glomeruli after mesenchymal condensation. Interestingly, the pattern of glypican-1 expression in the kidney constrasts with that of glypican-5, which is strongly expressed in developing tubular structures (Fig. 9B; see Discussion). Some glypican-1 expression was also seen in the E18 rat testis (Fig 1B).

In addition to dermis and heart, other tissues that showed little or no glypican-1 expression either by in situ hybridization or immunohistochemistry were developing liver, lung, and blood vessels (Figs. 1, 5, and data not shown).

DISCUSSION

In the present study, in situ hybridization and immunohistochemical staining were used to identify the

major sites of glypican-1 expression during mouse and rat embryogenesis. Glypican-1 was found to be specifically expressed by particular tissues and cell types during development. Especially high levels of glypican-1 mRNA were seen in neural and skeletal tissues. Other glypican-1-expressing tissues included kidney, epidermis, skeletal muscle, and visceral smooth muscle.

Previous studies on cultured cells lines have suggested that glypican-1 is widely expressed by most types of epithelial and fibroblastic cells (e.g., Lories et al., 1992). The results presented here suggest that this is not the case in vivo. For example, connective tissue appeared generally negative for glypican-1. This was true even in lung, despite that fact that cultured lung fibroblasts served as the original source for the biochemical purification of glypican-1 (David et al., 1990). It may be that induction of glypican-1 expression is a response of many cell types to growth under in vitro conditions.

Some of the locations in which glypican-1 was found in this study have been noted by others. For example, David et al. (1992) reported glypican-1 immunoreactivity in human epidermis. Karthikeyan et al. (1994) observed glypican-1 hybridization and immunostaining in embryonic and postnatal rats in a subset of the neural structures described here (see below). Asundi et al. (1997) reported that glypican-1 is not present in the embryonic rat heart—in agreement with the findings reported here—but, interestingly, also found that glypican-1 appears at high levels in the heart after birth.

Glypican-1 Expression in the Nervous System

Glypican-1 is the major HSPG of the adult brain (Herndon and Lander, 1990; Karthikeyan et al., 1992; Litwack et al., 1994), where it is expressed by many, but not all, populations of projection neurons (Litwack et al., 1994). Here we have shown that, prior to appearing in neurons, glypican-1 is also highly expressed in zones containing proliferating neural precursors throughout the nervous system. In addition, during embryonic development, glypican-1 expression is associated with many more populations of neurons than are glypican-1-expressing in the adult. Structures that transiently express glypican-1 include the corpus striatum, ventral thalamus, layer IV of cerebral cortex, and cerebellum. Indeed, it is possible that, during development, all neurons and neural precursors express glypican-1.

This view differs from that of Karthikeyan et al. (1994), who saw very little glypican in the rat brain prior to E19, and who therefore suggested that neurons first express glypican-2 (which appears as soon as neurons become post-mitotic; Stipp et al., 1994, and later switch to expressing glypican-1. Instead, it appears that glypican-1 is expressed before glypican-2 (in neural precursors) and then is likely to be co-expressed with glypican-2 in many neurons (with a possible exception being the preplate of the telencephalon; see Fig. 2A,B). Differences in the amount of expression seen by Karthikeyan et al. (1994) and in the present study most likely reflect differences in the sensitivity of the staining methods used.

Both the regions of the nervous system in which glypican-1 is expressed, and the appearance of strong glypican-1 immunoreactivity on fiber tracts, suggest that glypican-1 expression is primarily associated with neurons rather than glial cells. It has been reported that Schwann cells (Carey et al., 1993), the glial cells of the peripheral nervous system, and oligodendroglia (Bansal et al., 1996) express glypican-1, however these results have come from the analysis of cells in culture and, as already mentioned, many cell types that express glypican-1 in culture appear not to do so in vivo. Indeed, the absence of glypican-1 in situ hybridization to regions of white matter (the territory populated by oligodendrocytes) in adult (Litwack et al., 1994) and developing (Fig. 3C–H) brain strongly suggests that these glial cells do not express glypican-1 in vivo. Similarly, our failure to detect hybridization over peripheral nerves and nerve roots (Figs. 1A, 7C,D and data not shown), suggests that Schwann cells express, at most, low levels of glypican-1 mRNA.

Differential Expression of Cell-Surface HSPGs During Development

It is becoming increasingly clear that the glypican family, like the syndecan family, is widely expressed throughout development and adulthood. Individual family members, however, show dramatic tissue-specific and stage-specific patterns of expression, with the result that switches in expression between family members can mark specific developmental stages or lineages (see Table 1).

For example, in the nervous system, neural precursor cells generally appear to possess glypican-1, but not glypicans -2, -4, or -5, except in the ventricular zone of the cerebral wall, where they express glypican-4 (Watanabe et al., 1995), and parts of the striatal primordium, where they express glypican-5 (Saunders et al., 1997). Early neurons express glypicans-1 and -2, but a few populations also express glypican-5 (Ivins et al., 1997; Saunders et al., 1997; Stipp et al., 1994). Later, glypican-2 rapidly disappears from all neurons (Ivins et al., 1997; Stipp et al., 1994), glypican-1 disappears from some neurons (see above), and glypican-5 appears, but at low levels, in many neurons (Saunders et al., 1997). In addition, there is widespread transient neuronal expression of syndecan-3 in late embryonic and early postnatal rodents (Carey et al., 1997).

In kidney development, at least three glypicans are associated with the formation of nephrons: Glypican-5 mRNA is expressed by pre-tubular aggregates and early tubular structures, but is down-regulated before complete glomerular structures are formed (Saunders et al., 1997). Glypican-1 is also expressed by early epithelial aggregates, but persists in their glomerular derivatives (Fig. 9). Glypican-4 is associated with mature tubular, but not glomerular, derivatives (Watanabe et al., 1995). Early epithelial aggregates also ex-

TABLE 1. Patterns of Expression of Glypicans*

	Glypican-1[a,b]	Glypican-2[a,c]	Glypican-3[d,e,f]	Glypican-4[f]	Glypican-5[g]
brain					
neuroepithelium	+	−	−[i]	+	−
postmitotic neurons	+	+		−	+
mature neurons	+	−		−	+
kidney					
mesenchyme	−	−	+	−	−
epithelial aggregates	+			+/−	+
tubules	−			+	+
glomeruli	+			−	−
adrenal gland	nd	−	nd	+	nd
lung	−	−	+	+	+[j]
liver	−	−	+[i]	+	+[j]
heart	+[h]	−	nd	−	−
intestine	−	−	+	−	−
testis	+	−	nd	nd	+
blood vessels	−	−	nd	−	−
muscle					
skeletal	+	−	nd	nd	−
smooth	+	−	nd	+	−
bone					
periosteum/osteoblasts	+	−	nd	nd	−
chondroytes	−	−			−
bone marrow	+	−			−
skin					
epidermis	+	−	nd	nd	−
dermis	−	−			−
hair follicles	+	−			−

*Published data on expression patterns of mammalian glypicans are summarized. For glypicans 1–2, data on both mRNA and protein are included; for glypicans 3–5, only mRNA expression data are available. Brackets indicate results that have only been obtained for an organ as a whole, e.g. by Northern blotting. nd, not determined.

[a]This study.

[b]Asundi et al., 1997; Roskams et al., 1995; Karthikeyan et al., 1994; and David et al., 1992.

[c]Stipp et al., 1994, Ivins et al., 1997 and unpublished observations.

[d]Pilia et al., 1996.

[e]Filmus et al., 1988.

[f]Watanabe et al., 1995.

[g]Saunders et al., 1997 and Veugelers et al., 1997.

[h]Expression seen in postnatal but not embryonic animals.

[i]Findings are those of Pilia et al. (1996) using human tissue; Watanabe et al. (1995), studying the mouse, reported that glypican-3 mRNA is found in brain and not in liver.

[j]Expression observed in embryonic but not adult animals.

press syndecan-1 (Vainio et al., 1989), and the surrounding mesenchyme that induces them expresses syndecan-2 (David et al., 1993).

Less is known about the expression of HSPGs in skeletal, epidermal, or muscle development, however it is interesting that the pattern of glypican-1 expression we have observed in developing bone is very similar to that seen for syndecan-3 (Gould et al., 1995). Syndecan-2 is also found in the periosteum of developing bone (David et al., 1993), but unlike glypican-1 and syndecan-3, is found in immature chondrocytes as well.

Possible Functions of Glypican-1

As described earlier, several lines of evidence support the idea that cell-surface HSPGs regulate cellular responses to heparin-binding growth factors (Rapraeger et al., 1991; Reich-Slotky et al., 1994; Reichsman et al., 1996; Yayon et al., 1991). It is clear that exogenously supplied or expressed glypicans can participate in such events (Bonneh-Barkey et al., 1997; Steinfeld et al., 1996), and that at least some growth factors can be isolated from cell surfaces as complexes with GPI-anchored HSPGs (Bashkin et al., 1992; Brunner et al., 1991). Furthermore, mutations in glypican-3 (Pilia et al., 1996) and in the *Drosophila* glypican, *dally* (Nakato et al., 1995), clearly interfere with tissue growth control.

Consistent with an involvement of glypicans in the responses of cells to growth factors, patterns of glypican-1 expression that are reported here frequently (although not always) correspond to locations of high mitotic activity, where HS-dependent and heparin-binding growth factors are known to play important developmental roles. FGFs, for instance, have effects on both neural precursor proliferation and neuronal survival (DeHamer et al., 1994; Hughes et al., 1993; Murphy et al., 1990). Furthermore, given that glypican-1 is expressed on axons, it is relevant that both

FGFs and endogenous HS have been implicated in controlling axon targeting during neural development (McFarlane et al., 1996; McFarlane et al., 1995; Walz et al., 1997; Wang and Denburg, 1992).

In developing bone, numerous heparin-binding growth factors are present and are known to act as mitogens for osteoblasts; these include both FGFs and BMPs (bone morphogenetic proteins; Canalis et al., 1991; Hauschka et al., 1986; Reddi, 1992). In the bone marrow, where glypican-1 is also expressed, HS is known to regulate the presentation and availability of heparin-binding growth factors such as GM-CSF and IL-3 (Coombe, 1996; Gordon et al., 1987; Roberts et al., 1988). That glypicans are involved in such interactions is supported by the finding that phospholipase C treatment of bone marrow cultures results in the release of an FGF-2/HSPG complex (Brunner et al., 1991).

HSPGs are known to play an essential role in kidney morphogenesis (Kispert et al., 1996b), possibly reflecting the HS-dependence of growth factors of the Wnt family (Reichsman et al., 1996). In skeletal muscle, HSPGs have been implicated in morphogenesis of the neuromuscular junction, which may reflect interactions with the heparin binding protein agrin (Ferns et al., 1993; Gordon et al., 1993; Hirano and Kidokoro, 1989; Wallace, 1990).

Although it is tempting to speculate that glypican-1 acts as an important source of cell surface HS for these and other cellular interactions with HS-binding molecules, it only begs the question of why multiple glypican species exist, and why they undergo such dramatic changes in expression over the course of development. Nor is it yet clear why, in many cells, cell-surface HS should need to be carried by members of both the glypican and syndecan families. These unresolved issues raise the possibility that the core proteins of cell-surface HSPGs have unique functional roles. Whether core proteins act indirectly, by influencing the type of HS chains that are synthesized on them, or directly, by binding to cell-surface or extracellular ligands, is an important question that will need to be explored in the future.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

RNA probes from rat glypican-1 clones 4X1 and 4P2 were synthesized as previously described (Litwack et al., 1994). Probes for glypican-2 and glypican-5 were as described by Stipp et al. (1994) and Saunders et al. (1997), respectively. Embryos were dissected from timed pregnant Sprague-Dawley rats and CD-1 mice, with the date of sperm-positivity (for rats) or observation of a vaginal plug (for mice) considered embryonic day 0 (E0). E14 rat embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C, and then equilibrated sequentially in 5%, and then 15%, sucrose in PBS. All other animals were fresh frozen in isopentane chilled on dry ice. Twenty µm cryostat sections were collected on Probe-On Plus microscope slides (Fisher Biotech, Orangeburg, NY). In situ hybridization experiments were performed as previously described (Litwack et al., 1994; Saunders et al., 1997).

Anti-Peptide Antibodies

The peptide CGNPKVNPHGSHPEEKRR was synthesized and purified by reverse-phase HPLC (Biopolymers Lab, M.I.T., Cambridge, MA). This peptide corresponds to amino acids 343-360 of rat glypican-1 (Litwack et al., 1994). Twenty mg of keyhole limpet hemocyanin (KLH; Pierce) was reacted with 2 mg sulfo-SMCC (Pierce) at room temperature with stirring for 30 minutes. Activated KLH was purified over a Sephadex G-25 column (Pharmacia, Gaitherburg, MD) in 0.1 M sodium phosphate (pH6). 22 mg of this peptide was dissolved in the same buffer, and reacted overnight at room temperature with the activated KLH. KLH-343 complexes were purified over a Sephadex G-25 column. Rabbits were injected intradermally with 2.5 mg KLH-343 in complete Freund's adjuvant and boosted four times intramuscularly with 2.5 mg KLH-343 in incomplete Freund's adjuvant (Pine Acres Rabbitry and Farms, Norton, MA). Antibodies were collected and purified over 343 peptide coupled to Sulfo-Link (Pierce). This antiserum was designated 343-1. Antibodies to KLH were prepared by affinity purification on a KLH column (as described above) from the same serum used to prepare 343-1.

GAG lyase digestions and Western blots were performed as previously described (Litwack et al., 1994).

Immunohistochemistry

E13 CD-1 mouse embryos were dissected and fixed by overnight immersion in 4% paraformaldehyde in PBS at 4°C. Tissue was equilibrated sequentially in 5% and 15% sucrose in PBS. All other animals were quick-frozen by immersion in isopentane chilled on dry ice. Twnety µm cryostat sections of all samples were collected on Probe-On Plus slides (Fisher) and stored at -80°C until further use. For immunohistochemistry, sections were fixed in 4% paraformaldehyde in PBS, washed, and then incubated in blocking solution (2% BSA, 100 mM Tris [pH 8 at 4°C], 150 mM NaCl, 0.3% Triton X-100). Sections were washed in TBS (100 mM Tris [pH 8 at 4°C], 150 mM NaCl) and, when sections were to be developed with horseradish peroxidase, treated twice for 30 min each in 0.3% H_2O_2, and washed again in TBS. Affinity purified 343-1 was applied at 2.5–5 µg/ml in blocking solution. For immunofluorescence, a 1:100 dilution of Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) was used. Sections were coverslipped using GelMount (Biomeda Corp., Foster City, CA). For horseradish peroxidase, sections were incubated in biotin-conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted 1:300 in block as a secondary antibody. Sections were washed in TBS and then incubated in avidin-horseradish peroxidase (ABC reagent, Vector) diluted in block.

Sections were washed again in TBS and developed in 50 μg/ml diaminobenzidine/0.02% H_2O_2/50 mM Tris. In some cases (Fig. 6J), staining was enhanced with the Metal Enhanced Substrate Kit from Pierce. Sections were washed in H_2O, dehydrated, cleared in xylenes, and coverslipped using Permount.

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